Dual functions of the Retinal Determination Gene Network member EYES ABSENT as a transcription factor and protein phosphatase

by

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B.A. Biology
B.A. English and American Literature
New York University, 1999

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

[September 2004]

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submitted to the Biology Department on June 10, 2004 in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in Biology

Proper development of cell types and tissues requires the integration of extracellular signals to provide context specific information that insures appropriate differentiation. The Drosophila eye is an excellent model for the study of this signal integration, as its development is orchestrated by the interactions of common signal transduction pathways in conjunction with organ specific gene expression. Signaling through these pathways sets the stage for appropriate deployment of the Retinal Determination (RD) gene network members to direct formation of the eye and other organs.

Our studies have focused on the RD gene network member EYES ABSENT as a point of signal integration necessary for the formation of the *Drosophila* eye. We have examined two functions for EYA, the first as a transcriptional co-activator, and the second, more novel function as a protein tyrosine phosphatase.

Previous work suggested that EYA functions as a transcriptional co-activator, particularly in a complex with the DNA binding domain containing RD network member SINE OCULIS (SO). In order to better understand RD network regulation, we performed a structure-function analysis of the EYA protein, which defined the P/S/T rich region of EYA as crucial for EYA transactivation potential. This region is also necessary for EYA mediated ectopic eye induction and rescue of the eya^2 mutant phenotype. We showed that RAS/MAPK signaling potentiates EYA transactivation, providing a mechanism for previously described *in vivo* activation of EYA by MAPK. We have also demonstrated roles for GROUCHO and DACHSHUND in negative and positive regulation of the EYA-SO transcription factor, respectively.

Recently we have begun to study a novel function of EYA suggested by the homology of the highly conserved EYA domain (ED) to the Haloacid dehalogenase (HAD) family. Using the substrate analog para-nitrophenyl phosphate (pNPP), we showed that recombinant ED possesses phosphatase activity, which is affected by tyrosine phosphatase inhibitors but not serine/threonine phosphatase inhibitors. To determine whether this activity is important for EYA function in vivo, mutants that reduce or abrogate phosphatase activity, as shown by lower specific activity or higher K_m in pNPP assays, were tested for their ability to induce ectopic eyes or rescue the EYA mutant phenotype. These mutants, which we refer to collectively as EYA^{HAD}, are unable to induce ectopic eyes or rescue the eya^2 phenotype to the degree of wildtype EYA. As the EYA^{HAD} mutants are all within the ED, which is known to bind to SO, we tested whether these mutants are competent transcriptional coactivators with SO, and found that they retain this activity. Thus the phosphatase and transactivation functions of EYA may represent two distinct essential functions of EYA.

As EYA represents one of the first transcription factors found to possess phosphatase activity, and modulation of phosphorylation state represents a common mode of transcriptional regulation, it will be of particular interest to elucidate the role of EYA phosphatase function *in vivo*, studies which will require identification of transcriptional targets and phosphatase substrates.

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Acknowledgements

I was first introduced to the powerful and beautiful genetic tool that is *Drosophila* by my undergraduate advisor at NYU, Chris Rushlow. When I joined her lab, Chris introduced me to fly crosses, *in situ* hybridizations, and the world of developmental biology, all with her infectious love of science. She was an amazing first mentor, and I feel privileged to have had her friendship and guidance.

I think I knew I would join Ilaria's lab almost as soon as she finished her talk during IAP, and after spending the past four years in her lab, I am still sure that I made the right choice. Ilaria's passion for science is contagious; when labwork seems dismal, a pep talk from her is sure to encourage students to return to the bench full of new fire. In Ilaria's lab I have learned molecular biology, genetics, and even biochemistry, but most importantly I have learned to trust my instincts and to be unafraid of new challenges (or at least not to be paralyzed by my fear). She is a wonderful graduate advisor and I am so happy to have been part of her lab.

I would like to thank Matthew Voas and Andrina Williams Zink for all of their help and guidance when I first joined the lab. Everyone in our lab has made it a wonderful place to be a graduate student, but I would like to particularly thank Mousumi Mutsuddi, David Doroquez, and Jennifer Jemc for their supportive and insightful discussions over the past few years. I have also been lucky enough have the help of a very talented undergraduate for the past few summers, Laura Doyon. My perspective outside of the world of flies was greatly enriched by discussions with my classmates Maria Courel and Jessica Tytell. I would also like to thank my committee members, Paul Garrity Hazel Sive, and Terry Orr-Weaver, for their helpful advice on many occasions.

I would especially like to thank my classmate, labmate, and very good friend, Tina Tootle. We joined the lab together and worked on very separate projects for three years. Over that time we developed an amazing friendship and support system for each other, a relationship that was strengthened rather than tested by working together for the past year on the EYA phosphatase project. I know that I will always be able to count on her for sound scientific advice as well as a good laugh.

Of course, none of this would be possible without the love and encouragement of my family: first of all, my husband Adam, who has been by my side through the ups and downs of graduate school, keeping me smiling and sane. I would also like to thank my fantastic support network in New York; my sister Jaki, and my parents, Mark & Shelley Silver.

Chapter 1

The Retinal Determination Gene Network: A Point of Signal Integration for Specification and Proliferation

Abstract

Proper development of cell types and tissues requires the integration of extracellular signals to provide context specific information that ensures appropriate differentiation. The Drosophila eye is an excellent model for the study of this signal integration, as its development is orchestrated by the interactions of common signal transduction pathways in conjunction with organ specific gene expression. Signaling through the NOTCH, DECAPENTAPLEGIC/ TRANSFORMING GROWTH FACTOR-β, WINGLESS, HEDGEHOG, and EPIDERMAL GROWTH FACTOR RECEPTOR pathways sets the stage for appropriate deployment of the Retinal Determination (RD) gene network members to direct formation of the eye and other organs.

The RD gene network encodes a group of evolutionarily conserved transcription factors and co-factors that are crucial for the formation of many organs including the eye. These nuclear factors, which include proteins in the PAX6, EYA, SIX, and DACH families, are regulated by their interactions with each other and by effectors of the signaling pathways mentioned above. The mechanistic links between the RD gene network and signaling pathways are just beginning to be understood, particularly at the level of phosphorylation and regulation of transcriptional targets. One crucial role for the crosstalk between signaling pathways and this network of transcription factors is to coordinate the processes of cell proliferation and cell differentiation so that appropriate organ size and structure can be achieved.

A Conserved Regulatory Network for Eye Development

Eye development in different organisms produces strikingly different structures: the primitive eye of planaria, the compound eye of insects, and the camera-like eye of vertebrates. While these visual organs are morphologically distinct, the molecular mechanisms that lead to these different eyes are remarkably conserved. Specification of the eye field in these diverse organisms requires expression of homologous members of the Retinal Determination (RD) gene network, a group of transcription factors and co-factors crucial for the development of the eye as well as other organs. Much has been learned about these transcription factors, which include members of the PAX6, EYA, SIX, and DACH families (Figure 1), from studies in *Drosophila* as well as vertebrates.

A Master Regulator? - EYELESS/PAX6

The first RD gene network member to be identified molecularly was *Drosophila eyeless* (*ey*), the homolog of vertebrate *Pax6* (Quiring et al., 1994). The PAX6 family is a subgroup of the large family of PAX proteins, each containing two DNA binding motifs; a PAIRED box DNA binding domain, and a HOMEOBOX type DNA binding motif (Figure 1; Quiring et al., 1994).

ey was first named due to eye specific alleles which result in loss of eye tissue (Quiring et al., 1994). Subsequent cloning of this gene and isolation of null alleles, which are homozygous lethal, revealed a broader role for EY in the embryo and brain (Kammermeier et al., 2001). This pattern of discovery, where isolation of eye specific alleles is followed by recognition of broader roles in development, would be repeated for many of the RD gene network members.

Heterozygosity at the *Pax6* locus is associated with aniridia and Peters' anomaly in humans and the small eye phenotype in mouse (Hanson et al., 1994; Quiring et al., 1994), indicating conservation of PAX6 family roles in eye and head development in addition to the striking sequence conservation. Consistent with studies in *Drosophila*, homozygous mutant vertebrates, including humans, do not survive due to broader requirements for PAX6 during development (Hanson, 2001).

In addition to *ey*, the *Drosophila melanogaster* genome contains a second closely linked PAX6 homolog, *twin-of-eyeless* (*toy*), thought to be the result of a duplication during insect evolution, as two PAX6 homologs are also found in the closely related *Drosophila virilis* and the more distantly related silkmoth *Bombyx mori*, but not in the grasshopper *Schistocerca nitans* nor a primitive insect, the springtail *Folsomia candida* (Czerny et al., 1999). TOY and EY are independently required for proper eye development (Kronhamn et al., 2002; Quiring et al., 1994) and display distinct as well as overlapping embryonic and larval expression patterns (Kammermeier et al., 2001; Kronhamn et al., 2002). Therefore these homologous genes are not merely redundant, and insects have taken advantage of the presence of two PAX6 genes by adapting them for different uses.

Both TOY and EY are highly homologous to human PAX6, with over 90% identity in the PAIRED domain and 90% identity in the HOMEOBOX domain. TOY is more similar to PAX6 in length and in sequence outside of the two DNA binding domains, and also shows more similar DNA binding specificity (Czerny et al., 1999). This distinction may play a role in the binding specificities of TOY and EY *in vivo*, as the PAIRED domain of TOY binds to five sites in the eye specific enhancer of their target gene *sine oculis*, while the PAIRED domain of EY binds only to a subset of those sites (Niimi et al., 1999; Punzo et al., 2002).

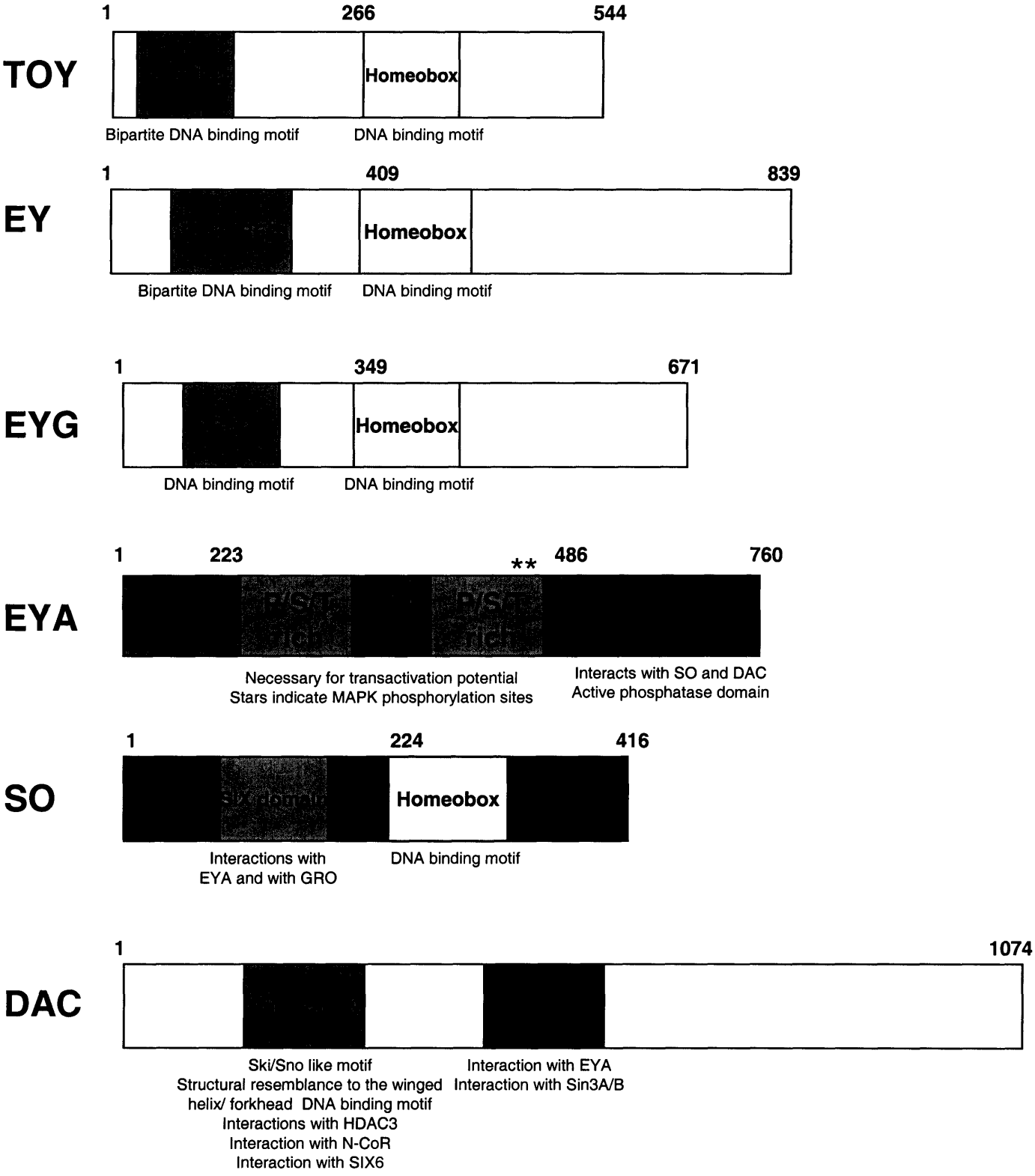


Figure 1: Domain Structures of the RD gene network members

In the eye, TOY and EY are expressed in a regulatory hierarchy, whereby TOY acts upstream to activate *ey* through binding to PAX6 consensus motifs in the *ey* intron (Czerny et al., 1999; Hauck et al., 1999), and both are required for proper development of the head and eyes (Kronhamn et al., 2002). Perhaps one of the most striking aspects of PAX6 family members is their ability to act as "master regulators" of eye formation, by directing the formation of ectopic eyes upon overexpression (Halder et al., 1998). Expression of either TOY or EY is sufficient to induce ectopic eyes (Czerny et al., 1999; Halder et al., 1995), and even human PAX6 can induce ectopic eyes when expressed in flies (Halder et al., 1995).

Although PAX6 proteins contain two DNA binding motifs, studies of *Drosophila* EY suggest they may be used independently. For example, analyses of truncated EY proteins reveal a requirement for the PAIRED domain but not the HOMEOBOX to rescue the eye specific ey^2 mutant phenotype and also to induce ectopic eyes (Punzo et al., 2001). The PAIRED domain may also be involved in cooperative interactions with other transcription factors, as is observed for the PAIRED domain of a related protein, PAX5, in its interactions with the transcription factor ETS-1 (Garvie et al., 2001). In contrast, the HOMEOBOX is required to downregulate genes important for the development of appendages, such as *distalless*, suggesting a role for this DNA binding domain in repression (Punzo et al., 2001). It is interesting to note that these two DNA binding domains appear to mediate opposite effects, activation versus repression, and function in the development of discrete organs. It is likely that co-factor interactions, such as those observed between PAX5 and ETS-1, may favor the use of one or the other DNA binding domain, co-recruiting it to target sequences and giving PAX6-DNA complexes context specificity.

TOY and EY function at the top of a transcriptional hierarchy, where they are required for the expression of downstream members of the RD gene network (Figure 2; Halder et al., 1998). Like TOY and EY, members of the RD gene network have largely been characterized as transcription factors, and include the PAX6-like gene *eyegone* (*eyg*), which is thought to act in parallel to *ey*, and downstream components *eyes absent* (*eya*), *sine oculis* (*so*), and *dachschund* (*dac*). EYA, SO, and DAC are all the founding members of families of proteins important for organ development in both invertebrates and vertebrates, and are homologous to members of the EYA, SIX, and DACH families, respectively. SO contains a HOMEOBOX DNA binding domain (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), while EYA and DAC are novel nuclear proteins (Bonini et al., 1993; Mardon et al., 1994). However, the transcriptional hierarchy observed in the eye is not linear, and EYA, SO, and DAC contribute to positive feedback loops that ensure continued expression of EY (Figure 2; Halder et al., 1998). Another SIX family member, *optix*, also appears to act in parallel to TOY and EY to direct eye growth (Seimiya et al, 2000).

Despite study of the function of the PAX6 family in many organisms, few direct targets of these transcription factors have been identified. Recently, microarray analysis of ectopic eyes induced by *eyeless* was performed in order to understand the developmental program initiated upon eye formation, identifying 371 genes induced by EY (Michaut et al., 2003). Of these, many are already known to be involved in eye formation, others have been previously characterized for roles in other developmental processes, while over half remain uncharacterized, including 100 novel genes (Michaut et al., 2003). It still must be determined which of these

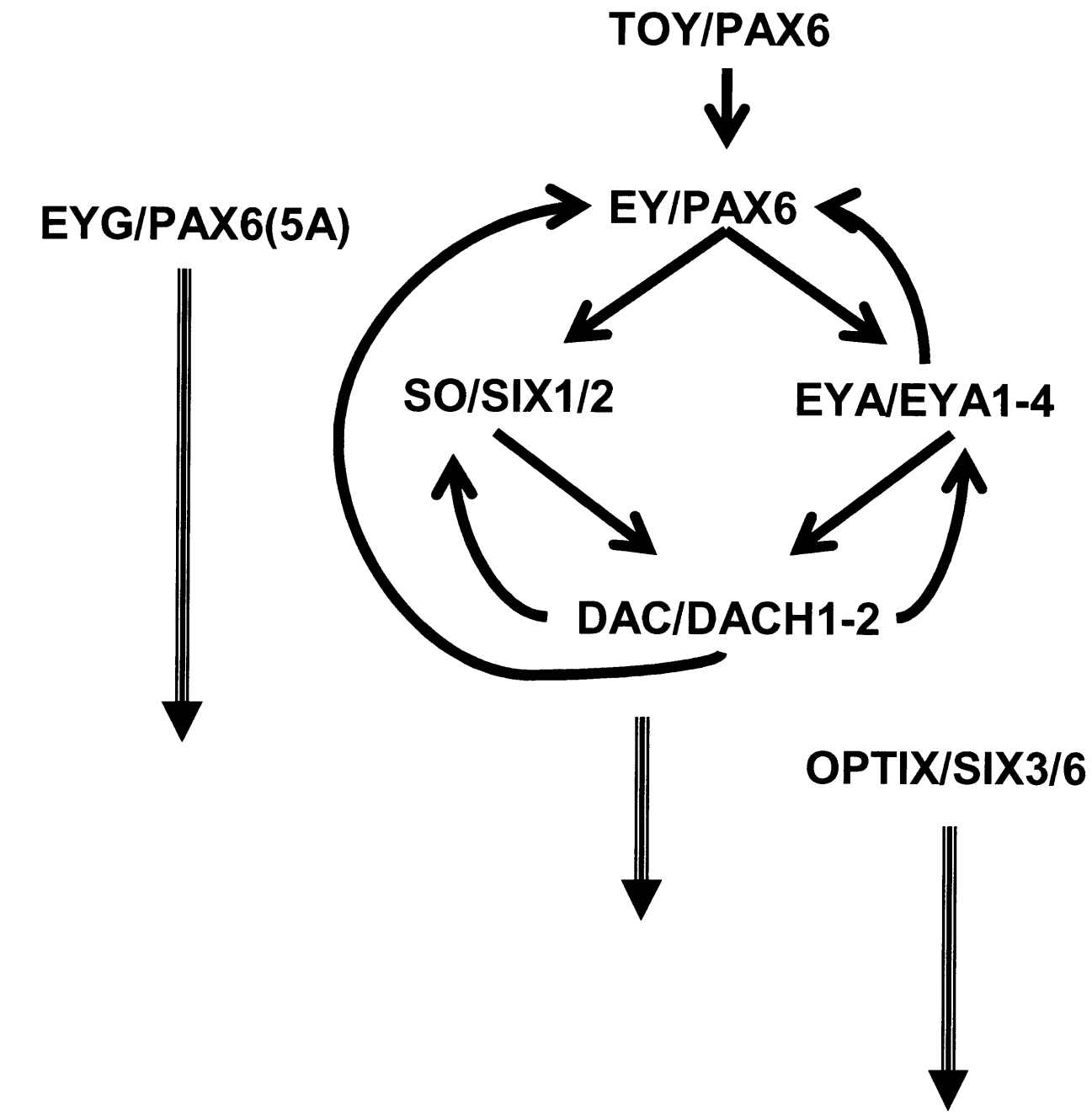


Figure 2 - Retinal Determination Gene Network
The RD gene network is expressed in a transcriptional hierarch whereby TOY leads to EY expression leading to SO, EYA, and DAC expression. Lower tier members of the network such as EYA and DAC operate in reiterative feedback loops to turn on EY. Other RD members EYG and OPTIX are required independently for proper eye development.

genes are direct targets of EY and which play roles further downstream in eye formation. Performing similar microarray analysis of tissue expressing the closely related TOY might reveal overlapping and distinct targets for these two PAX6 proteins.

It is of particular note that greater than one-fourth of the total enriched gene class encode novel proteins, especially in light of two novel but now well studied members of the RD gene network, EYA and DAC. EYA and DAC when cloned could not be categorized immediately as transcription factors, enzymes, or DNA binding proteins based on amino acid sequence, but indeed each perform some of these functions (Ikeda et al., 2002; Ohto et al., 1999; Silver et al., 2003; Tootle et al., 2003). It will require more detailed study to the identify functions of these 100 novel proteins, through analysis of localization, mutant phenotype, structure, and biochemistry. The study of the novel genes identified by this microarray screen may reveal new paradigms for functional motifs as well as the mechanistic roles of individual genes in organ development.

EYEGONE, a PAX6-like gene in *Drosophila*

In addition to the PAX6 orthologs TOY and EY, there are two *Pax6*-like genes in *Drosphila*, *eyegone* (*eyg*), and its related gene, *twin-of-eyegone* (*toe*). Like most RD gene network members, loss of function alleles of *eyg* result in loss of eye tissue, while overexpression of EYG promotes ectopic eye formation (Jang et al., 2003). *eyg* and *toe* encode Pax proteins which contain a truncated PAIRED domain and a complete HOMEOBOX (Figure 1; Jang et al., 2003; Jun et al., 1998). The PAIRED DNA binding domain contains two separable motifs, PAI and RED, which can each bind DNA independently (Jun and Desplan, 1996); *eyg* and *toe* contain only the RED motif (Jun et al., 1998). *eyg* expression does not

require the presence of *ey*, nor does *ey* expression require *eyg* function (Jang et al., 2003). Furthermore, ectopic expression of neither *ey* nor *eyg* can induce the other, indicating that they may act in parallel during eye formation (Jang et al., 2003).

While there are no homologs of *eyg* in mammals, it has been suggested that the functional ortholog of this gene is the 5a splice isoform of *Pax6* (*Pax6(5a)*) (Dominguez et al., 2004; Jun et al., 1998), which also contains only the RED motif portion of the PD. In support of this theory, the RED domain of EYG and the RED domain of PAX6(5A) can bind to similar sequences (Jun et al., 1998), and overexpression of PAX6(5A) in *Drosophila* produces the same overgrowth effects as overexpression of EYG (Dominguez et al., 2004). Therefore the functions of EYG are likely to represent conserved processes in vertebrates.

In *Drosophila*, EY and EYG can function synergistically to induce larger ectopic eyes, for which a mechanism has recently been suggested. Specifically, EY and EYG may play discrete roles in eye development, where EY is important for eye specification while EYG is crucial for eye growth (Dominguez et al., 2004). It is crucial to the development of a functional organ that these two processes be coordinated during tissue formation, and EY and EYG may be important in this coordination through their parallel roles in differentiation and growth.

In the animal, the discrete roles of EY and EYG can be observed by analysis of marker gene expression in developing eye tissue mutant for either *ey* or *eyg*. *ey* mutant tissue, which can be examined in eye specific *ey* alleles, develops into the larval eye primordia, the eye disc, but lacks expression of retinal determination markers such as EYA and SO (Halder et al., 1998). Due to the absence of differentiation, this tissue then undergoes programmed cell death, leading to the "eyeless" phenotype (Halder et al., 1998).

In contrast, growth defects in *eyg* mutant tissue can be observed using the FLP-FRT system (Xu and Rubin, 1993) to induce specific, marked clonal patches of homozygous mutant tissue wherever FLP is expressed. When this is done in the eye disc, *eyg* mutant clones display clear growth disadvantages compared to wildtype tissue, but express the normal complement of retinal determination markers such as ELAV and EYA (Dominguez et al., 2004). Thus the "eyegone" phenotype is due to growth defects in the developing eye.

Thus two distinct proteins, encoded by different genes in *Drosophila* but a single gene in vertebrates, separate the control of two essential processes for the development of an organ; growth and differentiation. These processes must be coordinated precisely to generate appropriate adult structures, coordination that relies upon members of the RD gene network to communicate between multiple signaling pathways.

EYES ABSENT: a novel protein with two conserved functions

eyes absent (*eya*) was first identified in *Drosophila* as a gene important for cell survival and differentiation in the *Drosophila* eye (Bonini et al., 1993). Further studies of *Drosophila* EYA and of vertebrate homologs EYA1-4 have revealed a wider role for EYA in organogenesis (Bonini et al., 1998; Xu et al., 1999). In *Drosophila*, strong alleles of *eya* are lethal and affect cell proliferation and apoptosis, most clearly resulting in defects in head morphology, but also defects in gonad formation and body wall musculature (Bonini et al., 1998; Boyle et al., 1997). Weaker alleles survive to adulthood but display defects in eye and ocelli as well as male and female sterility (Bonini et al., 1998; Leiserson et al., 1998).

In humans, heterozygosity for mutations in the *Eya1* gene are associated with Branchio-oto-renal (BOR) syndrome, as well as Branchio-oto (BO) syndrome and Ocular Defects (OD)

(Abdelhak et al., 1997; Ozaki et al., 2002), while homozygotes display more severe defects and do not survive. *Eya1* heterozygous mice display similar defects in ears and kidneys, as well as more pleiotropic defects in organogenesis in homozogyotes (Xu et al., 1999; Xu et al., 2002). The four vertebrate EYA homologs have both discrete and overlapping expression patterns, suggesting that their functions may not be wholly redundant (Xu et al., 1997a; Zimmerman et al., 1997). While knockout mice of *Eya2*, *Eya3,* and *Eya4* have yet to be reported, in humans mutations in the *Eya4* locus are associated with familial hearing impairment (Pfister et al., 2002; Wayne et al., 2001).

The EYA family is characterized by a highly conserved ~250 amino acid C-terminal domain called the EYA DOMAIN (ED), while the N-terminus of different EYA orthologs shows little conservation aside from the tyrosine rich EYA DOMAIN2 (ED2; Figure 1; Xu et al., 1997b; Zimmerman et al., 1997). The amount of EYA must be carefully titrated to maintain viability, as broad overexpression in *Drosophila* is lethal (Hsiao et al., 2001), while in cell culture overexpression of EYA triggers the programmed cell death pathway (Clark et al., 2002).

The ED was initially characterized as a protein-protein interaction domain, a point of contact between EYA and other RD gene members SO (Pignoni et al., 1997), and DAC (Chen et al., 1997), an observation that has been extended to interactions between vertebrate EYA and SIX and DACH families (Ohto et al., 1999; Heanue et al., 1999).

EYA has been best characterized as a transcriptional co-activator which is recruited to the DNA of target genes via interaction with the SO/SIX family. SIX proteins bind DNA through their HOMEOBOX domain, recruiting the transactivation potential of EYA to the promoter of target genes (Ohto et al., 1999). This transcriptional co-activator function of EYA requires the

Proline/Serine/ Threonine (PST) rich region of EYA's N-terminus including the ED2, which is also required for EYA function *in vivo* (Figure 1; Silver et al., 2003).

Recently a second function has been described for EYA, through the identification of the ED as a catalytic motif belonging to the Haloacid dehalogenase family of enzymes (Tootle et al., 2003). Recombinant EYA has been shown to dephosphorylate tyrosyl phosphorylated peptides (Rayapureddi et al., 2003; Tootle et al., 2003) and serine/threonine phosphorylated peptides (Li et al., 2003) suggesting it may be a dual-specificity protein phosphatase. Thus far there are only two suggested substrates, both of which can be dephosphorylated by EYA *in vitro*: RNA polymerase II (Li et al., 2003), and EYA itself (Tootle et al., 2003). The phosphatase function of EYA is required for rescue of the eye specific eya^2 allele (Tootle et al., 2003), indicating that this role is utilized *in vivo* during eye development.

EYA is the first identified transcription factor to posses intrinsic phosphatase function. As phosphorylation plays a key role in modulation of transcription factor activity, and EYA is a nuclear protein associated with DNA, it is likely that the targets of the EYA phosphatase may play important roles in transcriptional regulation. The identification of *in vivo* substrates of the EYA phosphatase, and targets of EYA transcriptional regulation, will help determine how the phosphatase and transcription factor functions of EYA are coordinated to establish the appropriate developmental program.

More insight as to EYA function may come from identification of co-factors other than SO and DAC. Heterotrimeric G proteins are important components of cellular communication, responding to extracellular cues through activation of the Gα subunit. They are known to regulate the function of numerous transcription factors indirectly through second messengers, but in the case of EYA may directly affect transcription factor function by competition for co-

factors. Activated $G\alpha_z$ and $G\alpha_{i2}$ proteins have been shown to interact directly with the ED using Yeast and Mammalian-two hybrid analysis as well as glutathione S-transferase (GST) fusion protein pull-down assays, and may compete with SIX family members for EYA, thus preventing target gene activation (Fan et al., 2000). This represents a novel mode of transcriptional regulation through Gα subunits, and it is intriguing to ask if this interaction might serve to direct EYA's phosphatase activity towards particular substrates, allowing EYA to regulate other transcription factors or perhaps a different aspect of the cellular machinery.

The ED also contains a potential interaction motif for the GROUCHO (GRO) family of corepressors, although GRO-EYA interactions are not observed by co-immunoprecipitation in *Drosophila* cell culture (Silver et al., 2003). Recent genome wide yeast-two-hybrid screens of *Drosophila* and *C. elegans* proteins have identified other potential EYA binding partners, 8 in *Drosophila* and 51 in *C. elegans*, whose relevance remains to be determined through more detailed experimental analysis (Giot et al., 2003; Li et al., 2004). Unfortunately, other than the isolation of SO and its *C. elegans* homologs *ceh-33* and *ceh-35*, there is no overlap between the two screens, and more analysis will be needed to identify true binding partners.

Although it is best known for its role in eye development, EYA is found in organisms which do not have eyes, including as mentioned above the nematode *C. elegans* and perhaps more strikingly, plants such as *Arabidopsis thaliana*. Despite the absence of eyes, *C. elegans* have many sensory neurons which interact with the environment to sense modalities such as temperature and odor (for review see Mori and Ohshima, 1997), and homologs of RD gene network members EY, SO, and DAC are expressed in the worm head in discrete and overlapping domains (Chisholm and Horvitz, 1995; Dozier et al., 2001; Duncan et al., 1997; Zhang and

Emmons, 1995; A. Brown, personal communication), while the EYA expression pattern has yet to be reported. The presence of the RD gene network in these animals underscores their use in early development of sensory organs, and ultimately the adaptation of this network to organize multiple tissue and organ types in vertebrates (Wawersik and Maas, 2000).

EYA is thus far the only member of the RD gene network to be found in plants, in both monocots and dicots (Takeda et al., 1999). EYA homologs in *Oryza sativa* (rice) and *Arabidopsis* consist solely of an EYA DOMAIN preceded by a short 18 amino acid peptide, and *Oryza* EYA is highly expressed during embryogenesis (Takeda et al., 1999). As the *Arabidopsis* genome is now complete and annotated, and searches do not reveal homologs of EY, SO, or DAC, analysis of EYA function in plants may reveal independent and evolutionarily older functions for this family of proteins.

Absence of transcriptional cofactors in addition to the absence of the N-terminal transactivation domain suggests that EYA may not function as a transcription factor in plants. However plant EYA contains all conserved residues necessary for HAD family phosphatase activity, and indeed is the most active EYA of all orthologs tested (Rayapureddi et al., 2003). Thus plant EYA may represent EYA's original role as a protein phosphatase which was later in evolution brought into transcriptional regulation through interactions with "new" proteins such as SO and DAC. This would then bring EYA's phosphatase activity to promoter complexes, where it might act in the regulation of transcription factors through control of phosphorylation state, a common mechanism of transcriptional regulation. It will be interesting to ask whether plant EYA is localized to the nucleus, as constructs of the *Drosophila* EYA domain alone are both nuclear and cytoplasmic (T. Tootle, personal communication), and whether plant and animal EYA phosphatases will display similar substrate preferences.

SO/SIX family members; crucial mediators between EYA, DAC, & DNA

The SIX family can be grouped into three subgroups, one member each in *Drosophila* and two members each in vertebrates thought to result from the duplication of a "SIX cluster" (Kawakami et al., 2000; Seo et al., 1999). All family members are marked by the presence of two conserved domains, the SIX domain, which mediates protein-protein interactions, and a HOMEOBOX DNA binding domain (Figure 1; Pignoni et al., 1997). s*ine oculis* (*so*), the founding member of this family, is crucial for proper proliferation and patterning during eye development (Pignoni et al., 1997), and also plays roles in brain (Daniel et al., 1999) and gonad development (Fabrizio et al., 2003). Strikingly, SO is the only RD gene network member that cannot induce ectopic eyes when expressed alone, although it can synergize with EYA to increase the frequency of ectopic eyes (Pignoni et al., 1997).

so falls into a SIX family subgroup with vertebrate SIX1 and SIX2. SIX1 mutant mice display defects in ear, kidney, thymus, skeletal muscle and nose (Ozaki et al., 2004; Xu et al., 2003; Zheng et al., 2003). Recently SIX1 was shown to be upregulated in a mouse model of metastatic skeletal muscle cancer (Yu et al., 2004), and increased levels of SIX1 were associated with greater ability to form metastases (Yu et al., 2004), perhaps by overcoming mitotic checkpoints in G2 (Ford et al., 1998).

Another subgroup of the SIX family contains *Drosophila d-SIX4*, which plays an important role in muscle and gonad formation (Kirby et al., 2001), and vertebrate homologs *Six4* and *Six5*. *Six4* mutant mice have no apparent defects (Ozaki et al., 2001), despite its strong expression throughout the nervous system and in many sensory organs, and *Six5* knockout mice appear normal except for a higher incidence of cataracts (Klesert et al., 2000; Sarkar et al.,

2000). The effect of simultaneous knockout of these closely related genes with overlapping expression patterns remains to be examined.

In humans, the *Six5* locus is associated with myotonic dystrophy (DM1), and patients with that disease show lower levels of *Six5* expression (Wansink and Wieringa, 2003). Although the loss of *Six5* in mice does not mimic the entire phenotype of patients with DM1, it is thought that loss of *Six5* may contribute to the cataracts observed in these patients (Wansink and Wieringa, 2003), while downregulation of the associated gene DM protein kinase (DMPK) is responsible for the myopathy phenotype (Sarkar et al., 2000).

As described earlier, the members of the SIX subfamilies SIX1/2 and SIX4/5 are thought to generally function in a complex with EYA proteins as bipartite transcription factors. The SIX Domain interacts with the EYA domain, forming a transcription factor with the SIX HOMEOBOX as the DNA binding domain and the N-terminus of EYA as the transactivation domain that together direct transcription of target genes (Kawakami et al., 2000; Silver et al., 2003). This also brings the phosphatase activity of EYA to the promoter region, where it may play a role in regulation of itself or other factors. In another example of independent functions of members of the RD gene network, vertebrate SIX1/2 and SIX4/5 genes have some activation ability independent of EYA (Kawakami et al., 2000).

The most divergent branch of the SIX family includes *Drosophila optix* and the vertebrate genes *Six3* and *Six6* (Kawakami et al., 2000). All members of this subfamily show strong expression in the developing eye (Kawakami et al., 1996c; Seimiya and Gehring, 2000), and are necessary for proper eye formation (Carl et al., 2002; Li et al., 2002; Seimiya and Gehring, 2000). Similar to its vertebrate counterparts, which when overexpressed induce enlarged forebrains (Kobayashi et al., 1998), overexpression of *optix* leads to ectopic eyes

(Seimiya and Gehring, 2000). OPTIX, like EYG, does not require EY for the induction of ectopic eyes, and may act in a parallel pathway to direct eye formation (Figure 2; Seimiya and Gehring, 2000). However the lack of mutants that affect *optix* makes it difficult to place this protein more definitively within the RD gene network hierarchy.

Mutations in the human *Six3* gene are associated with holoprosencephaly (Pasquier et al., 2000; Wallis et al., 1999), stemming from defects in neural plate formation. Loss of the other human member of that subfamily, *Six6*, is associated with bilateral anophthalmia, a condition where babies are born with no eyes, as well as pituitary defects (Gallardo et al., 1999). Similar to the human phenotype, mice homozygous for knockout of *Six6* survive but have retinal and pituitary hypoplasia (Li et al., 2002). Overexpression of SIX3/6 causes overproliferation, and mutations in these genes cause phenotypes associated with proliferation defects, suggesting that they may play a direct role in cell cycle regulation. Recent work suggests that the SIX3/6 subfamily performs this role through transcriptional regulation and protein-protein interactions.

Unlike the other SIX family members, which are largely thought to coordinate transcriptional activation through interactions with EYA, the SIX3/6 subfamily do not interact with EYA family members (Kawakami et al., 2000). Instead, they are proposed to act as transcriptional repressors, one mechanism for which may be their interactions with the GROUCHO (GRO) family of corepressors, called GRG in vertebrates, through an engrailed homology 1 (eh1) motif in the SIX domain (Kobayashi et al., 2001; Lopez-Rios et al., 2003; Zhu et al., 2002).

The interaction between SIX3/6 and GRG family members has been demonstrated to be crucial for proper eye and brain formation in zebrafish and medaka (Kobayashi et al., 2001; Lopez-Rios et al., 2003) as well as mice (Zhu et al., 2002). In overexpression studies, the GRG

homolog TLE can act synergistically with SIX3 and SIX6 to expand the eye field, while a dominant negative form has the opposite effect (Lopez-Rios et al., 2003).

At least one direct target of SIX6 mediated repression is the cyclin-dependent kinase inhibitor p27Kip1, which must be repressed to allow early proliferation of the presumptive eye (Li et al., 2002), providing a direct link between SIX and cell cycle regulation. In this way, SIX family members may serve to coordinate growth with other targets important for appropriate differentiation of the organ.

A transcription independent role of SIX3 in control of cell proliferation has recently been identified in vertebrate eye development. SIX3 and SIX6 were shown to interact with the DNA replication inhibitor GEMININ (Del Bene et al., 2004). GEMININ inhibits cell proliferation through sequestration of CDT1, an important component of the replication machinery, such that SIX3 can compete with CDT1 for GEMININ, thus releasing CDT1 and with it cell cycle inhibition (Del Bene et al., 2004). This combined with the more direct transcriptional regulation of cell cycle components, illustrates how cell proliferation and cellular differentiation may be knitted together by deployment of common proteins to interact with distinct elements that insure the appropriate development of a complex organ.

Transcriptional repression may not be limited to the SIX3/6 subfamily, as the GRG interaction motif is found in all SIX proteins within the SIX domain (Kobayashi et al., 2001), indicating interactions with this corepressor family may be a common feature of the entire SIX family. Recently we showed that *Drosophila* GRO can interact with SO, and can repress SO mediated transcription of a reporter gene, likely by competing with EYA for SO binding (Silver et al., 2003). As studies in mice have shown that SIX1 can also have transcriptional repressor function (Li et al., 2003) it is likely that the members of the SIX family may play more elastic

roles in transcriptional regulation dependent upon their specific cofactor or context. As EYA and SO expression patterns are not wholly coincident, this dual function may provide a clear distinction between cells which express genes necessary for differentiation into eye tissue, expressing EYA and SO, and cells where those genes are actively repressed, expressing only SO. This model may be better understood once more targets of the SO/SIX family are identified.

Thus far only a handful of direct transcriptional targets have been identified for the SIX family (Table 3), most of them in mice. The SIX1/2 and SIX4/5 subfamilies have the most similar homeobox domains and are likely to bind the same or similar target sequences (Kawakami et al., 2000). The promoters of the *myogenin* and *aldolase A* genes contain SIX binding sites known as MEF3 (TCAGGTT), which is necessary for the embryonic expression of *myogenin* (Spitz et al., 1998). Another known target from mice is the housekeeping gene *Na^+/K^+ ATPase alpha 1 subunit* which contains a SIX family target sequence in the enhancer element AREC3 (core sequence GGNG**NCNGGTT**GC, includes TCAGGTT in bold; Kawakami et al., 1996b).

SIX1 and SIX4 have been shown to activate transcription via binding to the MEF3 site (Spitz et al., 1998), while SO, SIX2, SIX4 and SIX5 can all bind the AREC3 site and with EYA activate transcription of a reporter gene (Kawakami et al., 1996c; Silver et al., 2003). More recently, microarray analysis of mouse cells expressing dominant active or dominant negative SIX1 suggested *c-Myc* and *Gdnf* as direct targets. This result was confirmed by chromatin IP experiments, which showed SIX1 and EYA localization to the *c-Myc* and *Gdnf* promoters (Li et al., 2003). The single target of this family identified thus far in *Drosophila* is the *lozenge* gene, whose expression is activated by SO binding to an imprecise repeat in the *lozenge* minimal eye enhancer (LMEE) (Yan et al., 2003).

Table 3 – SIX family target genes have been identified in vertebrates and *Drosophila*

Target gene	SIX family member 1st identified to bind	DNA binding site (# found)	Reference
$Na^+/K^+ATPase$ $\alpha 1$ subunit	SIX4	TCAGGTT (1)	(Kawakami et al., 1996a)
pUC119 (plasmid)	SO	GATAC	(Hazbun et al., 1997)
myogenin	SIX1/SIX4	TCAGGTT (1)	(Spitz et al., 1998)
aldolase A	SIX1/SIX4	TCAGGTT (1)	(Spitz et al., 1998)
Pkip27	SIX6	Seq not ID'd	(Li et al., 2002)
lozenge	SO	TGATAT (2)	(Yan et al., 2003)
c-myc	SIX1	ATCCTGA (1)	(Li et al., 2003)
Gdnf	SIX1	ATCCTGA (3)	(Li et al., 2003)

DACH: A novel DNA binding protein

Dacschund (*dac*) in Drosophila, and its vertebrate homologs, *Dach1* and *Dach2*, encode a family of novel nuclear proteins characterized by two conserved domains, the DachBox-N and the DachBox-C (Figure 1; Davis et al., 2001b; Kozmik et al., 1999) . Analysis of the amino acid sequence of DACH family members revealed some similarity to the *ski* proto-oncogene (Hammond et al., 1998). Recent crystallization of the human DachBox-N revealed a striking structural resemblance to the winged helix/forkhead subgroup of the helix-turn-helix family of DNA binding proteins (Kim et al., 2002). While no specific DNA binding sites for DACH have been identified, it has been shown to bind naked DNA (Ikeda et al., 2002). The DachBox-C is thought to be a protein-protein interaction motif, and has been demonstrated to interact with EYA family members via the EYA DOMAIN (Chen et al., 1997). Thus DAC with its DNA binding ability may like SO be a co-factor responsible for bringing the transcriptional activator and phosphatase activity of EYA to the promoter of target genes.

dac homozygous mutants lack eye tissue, and have striking leg and wing defects; those that survive to adulthood die within a few days (Mardon et al., 1994). In mice lacking *Dach1*, which like *Drosophila dac* is expressed in the eye and limbs (Kozmik et al., 1999), there are no gross defects in development but mice die soon after birth (Backman et al., 2003; Davis et al., 2001a). As the expression patterns of *Dach1* and *Dach2* overlap greatly during development, this relatively mild phenotype may be due to partial redundancy of these genes, and/or may reflect a greater postnatal need for *Dach1*.

Like other members of the RD gene network, ectopic expression of DAC leads to induction of EY expression and the formation of ectopic eyes (Chen et al., 1997; Shen and Mardon, 1997). DAC can synergize with EYA to increase both the size and frequency of ectopic

eyes when the two are expressed together (Chen et al., 1997), support for the model that these two proteins act in a complex to direct eye development.

However, there are some discrepancies in the literature regarding the ability of DAC and EYA to interact directly. For example, Heanue et al. has shown that GST-DACH2 can pull down radiolabeled EYA2 (Heanue et al., 1999), while Ikeda et al. found no interaction between GST-DACH1 and EYA1, EYA2, or EYA4 in a similar assay. However, Ikeda and colleagues report that a DACH/EYA complex is formed in the presence of Creb binding protein (CBP) using co-immunoprecipitation assays and that the DACH/EYA complex can activate transcription of a synthetic promoter (Ikeda et al., 2002). These results may reflect differences between the DACH1 and DACH2, or between cofactors available in different cell types.

Directed yeast-two-hybrid analysis has twice suggested interactions between the DachBox-C and the EYA domain (Bui et al., 2000; Chen et al., 1997), but genome wide yeast-two-hybrid did not observe this interaction (Giot et al., 2003). In *Drosophila* cell culture, we found that we could not observe interactions between EYA and DAC, nor did DAC affect EYA-SO mediated transcription of the reporter *ARE-luciferase* (Silver et al., 2003). However, we do observe co-activation properties of DAC with the EYA-SO transcription factor in the context of a more native reporter (Chapter 4). Taken together, these results suggest that interactions between EYA and DAC are likely to be dynamic, highly context-dependent, and may be influenced by extrinsic factors that strengthen or stabilize the complex.

The DACH protein is a novel nuclear factor that has the potential to promote transactivation of targets (Ikeda et al., 2002; Chapter 4) and to repress them (Li et al., 2002). The DachBoxN from mouse DACH1 has been shown to interact with co-repressors such as Histone Deacetylase 3 (HDAC3) and nuclear receptor corepressor (N-CoR), and this repressor

complex can be recruited to DNA through DACH1 interactions with SIX6 (Li et al., 2002). One target for repression via this complex is the p27Kip1 promoter, which must be repressed to allow mitotic cell division (Li et al., 2002). A similar repressor complex between SIX1 and DACH has been described, and it has recently been suggested that this complex can switch from repressor to activator through the function of the EYA protein phosphatase (Li et al., 2003). Our work has shown a role for DAC in co-activation of EYA-SO mediated regulation of the *lozenge* eye enhancer independent of EYA phosphatase function (Chapter 4). It is interesting that we do not observe DAC co-activation in the same cells when using a reporter gene of seven tandem SO binding sites, *ARE-luciferase* (Silver et al., 2003). All of the reported experiments which observe a DAC/DACH dependent effect on transcription use endogenous promoter sequences ranging in size from 250 bp- 2.2 kb, raising the intriguing possibility that the DachBox-N must bind directly to the DNA to play its role in co-regulation of target genes.

EYE DEVELOPMENT REQUIRES THE INTEGRATION OF SIGNALING PATHWAYS WITH RD GENE NETWORK COMPONENTS

The RD gene network is a group of nuclear transcription factors and co-factors that are required for eye development and can even induce ectopic eyes when overexpressed in the fly. Study of these genes and proteins has revealed new paradigms for transcriptional regulation and a valuable model for organ formation. However these nuclear factors do not act alone, but are employed coordinately by and with components of conserved signaling pathways to achieve the specificity necessary to result in appropriate developmental cues.

Specification of Eye and Head primordia in the Embryo

The beautifully ordered *Drosophila* adult eye is the end result of many coordinated signals and processes that begin with the specification of the initial eye primordium in the embryo. The eye primordium is contained within a dorsal region termed the anterior brain/eye anlage, where it can be identified by early expression of the RD gene network member SO (Cheyette et al., 1994; Daniel et al., 1999). This single domain expands as the cells divide until in Stage 11 when it is split bilaterally and two presumptive eye fields are observed. This split requires that RD gene network expression is repressed in the dorsomedial cells to insure the formation of two discrete eyes, and requires high levels of DPP/TGFβ signaling (Chang et al., 2001).

DPP is a secreted molecule that is thought to act in a gradient, with the highest levels of protein close to where it is expressed and lower levels as the signal travels across cells. Though high levels of DPP signaling specify non-eye, medium levels of DPP signal are required for eye formation and induce expression of EYA and SO (Chang et al., 2001). In strong or null *dpp* alleles, the head and eye are not formed, while in weak alleles, only the most dorsal structures, including the head cuticle, are affected, resulting in a cyclopic phenotype (Chang et al., 2001). Thus two discrete levels of DPP signaling are required for appropriate eye and head formation, similar to the high and low levels of DPP signaling required in the wing (Lecuit and Cohen, 1998). Through this graded interpretation of the DPP signal, cells can determine their precise physical location and develop into the appropriate structure, a system that will be redeployed later in eye development.

Early eye formation also requires signal through the HEDGEHOG (HH) pathway, as *hh* is found in a transverse stripe along the posterior of the eye field, and is necessary for expression

of the RD gene network member *ey*, but not *eya* or *so* (Chang et al., 2001; Suzuki and Saigo, 2000). Consequently, loss of HH signaling is associated with small head structures and absence of visual structures (Chang et al., 2001; Suzuki and Saigo, 2000), and conversely, ectopic HH induces cyclopia (Chang et al., 2001). Thus, independent activation of both DPP and HH signaling must occur together to achieve appropriate expression of multiple RD gene network members to ensure formation of the eye; EYA and SO induction by DPP, and EY induction by HH.

Differentiation of the EYE-ANTENNAL disc

After embryogenesis, *Drosophila melanogaster* embryos hatch into a larval stage, which is broken up into three instars and lasts approximately six days, after which the larva forms a pupal case and undergoes metamorphosis before eclosing as an adult fly. During the larval stages, tissues that will give rise to the adult grow and differentiate in epidermal sacs known as imaginal discs. One of these discs is derived from the eye anlage described above and is known as the eye-antennal disc.

Little is known about the steps taken by the eye-antennal discs between embryogenesis and second instar larval stage beyond cell proliferation. However during the third instar larval stage, the larval eye-antennal disc begins its final differentiation program. Proper formation of the adult eye requires a delicate balance of signaling and communication in order to ensure appropriate size, shape, and place.

One of the earliest decisions to be made in the eye-antennal disc is the separation of this single epithelium into two discrete primordia; the eye region that will give rise to the eye and the ocelli, and the antennal region, which gives rise to the antenna and the palpus. Additionally,

both eye and antennal primordia contribute to formation of specific regions of head cuticle. Initially, *eyeless* is expressed throughout the eye-antennal disc; as cell fates are restricted, these primordia are associated with specific expression of EY in the eye and the marker CUT in the antenna (Kenyon et al., 2003).

One proposed mechanism for this restriction is a balance of antagonism between EGFR and NOTCH signaling, where EGFR induces antennal fate by repression of *ey*, and NOTCH induces eye fate by repression of the antennal gene *dll* and activation of *ey* (Kumar and Moses, 2001). However these studies were performed using eye specific overexpression of dominant active or negative regulators of the NOTCH and EGFR pathways. Dependence on overexpression analysis to determine epistatic relationships can be misleading, as high levels of protein may behave differently than endogenous levels, and appropriate timing may be crucial for endogenous function.

More recent work using clonal analysis of mutant tissue, as well as temperature sensitive mutations, reveals that NOTCH is not required for expression of RD gene network members; in NOTCH mutant tissue *ey*, *eya*, and *dac* expression are unchanged, and the antennal marker *dll* is not ectopically expressed (Kenyon et al., 2003). Thus loss of NOTCH signaling does not result in a fate change from eye to antennal primordia, and it remains to be understood how these primordia truly are restricted. It remains unclear whether EGFR actually plays a homeotic role in antennal specification, however it is certain that in both the eye and antennal primordia EGFR signaling is crucial for cell survival and differentiation, as described below.

Although NOTCH signaling is not required to specify eye fate, it is linked to the maintenance of that fate. If NOTCH signaling is blocked through expression of dominant negative ligands, eye tissue is specified and can express EYA normally (Kenyon et al., 2003).

However this fate is not maintained and EYA expression is soon lost. Tissue without NOTCH signaling displays clear defects in cell proliferation, leading Kenyon et al. to investigate whether the loss of EYA expression might be a secondary defect. They found that EYA expression is tied to cell proliferation, as it can be restored independently of NOTCH signaling via an increase in cell division (Kenyon et al., 2003). At this stage, a link between EYA expression to cell proliferation may be a mechanism for checks and balances system between cell number and cell differentiation.

Thus NOTCH signal is required for maintenance of eye fate through control of proliferation, and one means by which it signals to the cell proliferation machinery may be activation of the PAX6-like protein EYG. Evidence for the relationship between NOTCH and EYG comes from genetic interactions between *eyg* and members of the NOTCH signaling pathway *fng, Dl,* and NOTCH itself, which suggest a role for *eyg* as a positive transducer of NOTCH signaling in the eye (Dominguez et al., 2004). In support of this model, *eyg* expression is induced in regions with high levels of NOTCH signaling, and absent in cells that cannot transduce the NOTCH signal (Dominguez et al., 2004). As discussed earlier, high levels of EYG can induce cell proliferation (Dominguez et al., 2004), and though it is still unclear exactly how EYG performs this function, it is likely to act through transcriptional activation or repression of key cell-cycle regulators.

DPP and HH regulate morphogenetic furrow progression

The eye develops in a wave of differentiation that moves from posterior to anterior and can be visualized by progression of the morphogenetic furrow. For a review of the differentiation events following furrow progression see Voas and Rebay, 2004. Our focus will

be on the establishment and regulation of the furrow itself, which is formed initially at the most posterior part of the eye disc, and requires the expression of *dpp* and *hh* (Figure 3; Curtiss and Mlodzik, 2000).

It has been suggested that DPP and HH signaling act somewhat redundantly to initiate and drive morphogenetic furrow progression, as cells that cannot signal through either pathway never differentiate into photoreceptors, while cells defective for only one signal have defects which are less penetrant (Curtiss and Mlodzik, 2000). DPP and HH are both secreted factors, and thus their signal is thought to be received by cells outside the boundaries of their expression pattern in addition to the cells indicated in Figures 3 and 4, and DPP signaling in particular is important for establishment of the preproneural region (PPN; Bessa et al., 2002). One mechanism for both DPP and HH effects on eye specification is through their regulation of RD gene expression.

dpp transheterozygotes lose expression of RD gene network members *eya*, *so*, and *dac* in the eye disc (Chen et al., 1999b), although they do not affect *eyeless* expression (Kenyon et al., 2003), suggesting that DPP acts downstream of or in parallel to EY to activate expression of RD genes. More detailed work using somatic clonal analysis revealed that DPP signaling is required for EYA, SO, and DAC expression only prior to morphogenetic furrow initiation (Curtiss and Mlodzik, 2000), and that once they are turned on by DPP in the furrow, their expression can be maintained independently of DPP signaling. The expression patterns of the RD gene network members in the eye disc is not wholly coincident (Figure 4), suggesting that even those which can physically interact also play independent roles. EY is expressed in tissue prior to the furrow (Figure 4D; Bessa et al., 2002)), and EYA is expressed just anterior to the morphogenetic furrow and in all eye tissue posterior to the furrow (Figure 4A,D), while DAC is expressed in a

wide stripe centered on the morphogenetic furrow (Figure 4C,D). SO is expressed in all cells in the eye disc (Figure 4B,D), while another SIX family member, OPTIX, is only expressed in undifferentiated tissue anterior to the furrow (Figure 4D; Seimiya and Gehring, 2000).

HH signaling plays a key role in formation of the *Drosophila* eye, and high levels of HH are expressed just posterior to the DPP signal in the morphogenetic furrow (Figure 4), and are required for proper furrow progression (Pappu et al., 2003), which occurs through mutual activation of HH and DPP. Unlike in the embryo, in the eye disc HH is crucial for EYA expression (Pappu et al., 2003), through what seems to be a permissive mechanism. HH signaling inside the cell is effected by changes in the transcription factor CUBITUS INTERRUPTUS (CI); in the absence of signal, CI is cleaved to a shorter repressor form (CI^r), which enters the nucleus and downregulates target genes, while in the presence of signal, phosphorylation of CI is blocked, preventing cleavage thus allowing it to remain an activator and direct transcription of target genes (Chen et al., 1999a). HH's role in regulation of EYA expression is through elimination of a block to transcription, rather than activation; removal of CI^r is sufficient to promote EYA expression, while CI^{act} is not necessary for eye formation (Pappu et al., 2003).

The eye field undergoes a wave of differentiation directed by the expression of DPP and HH at the morphogenetic furrow, which together activate or allow transcription of the second tier of the RD gene network, EYA, SO, and DAC. These three genes are coincident only at, and just adjacent to, the morphogenetic furrow (Figure 4), where they and the DPP signal come together to direct terminal differentiation in the eye. The discrete domains of the eye disc which express each complement of RD gene network members (Figure 4) undergo quite different developmental processes; where SO and OPTIX overlap, cells have yet to differentiate, while

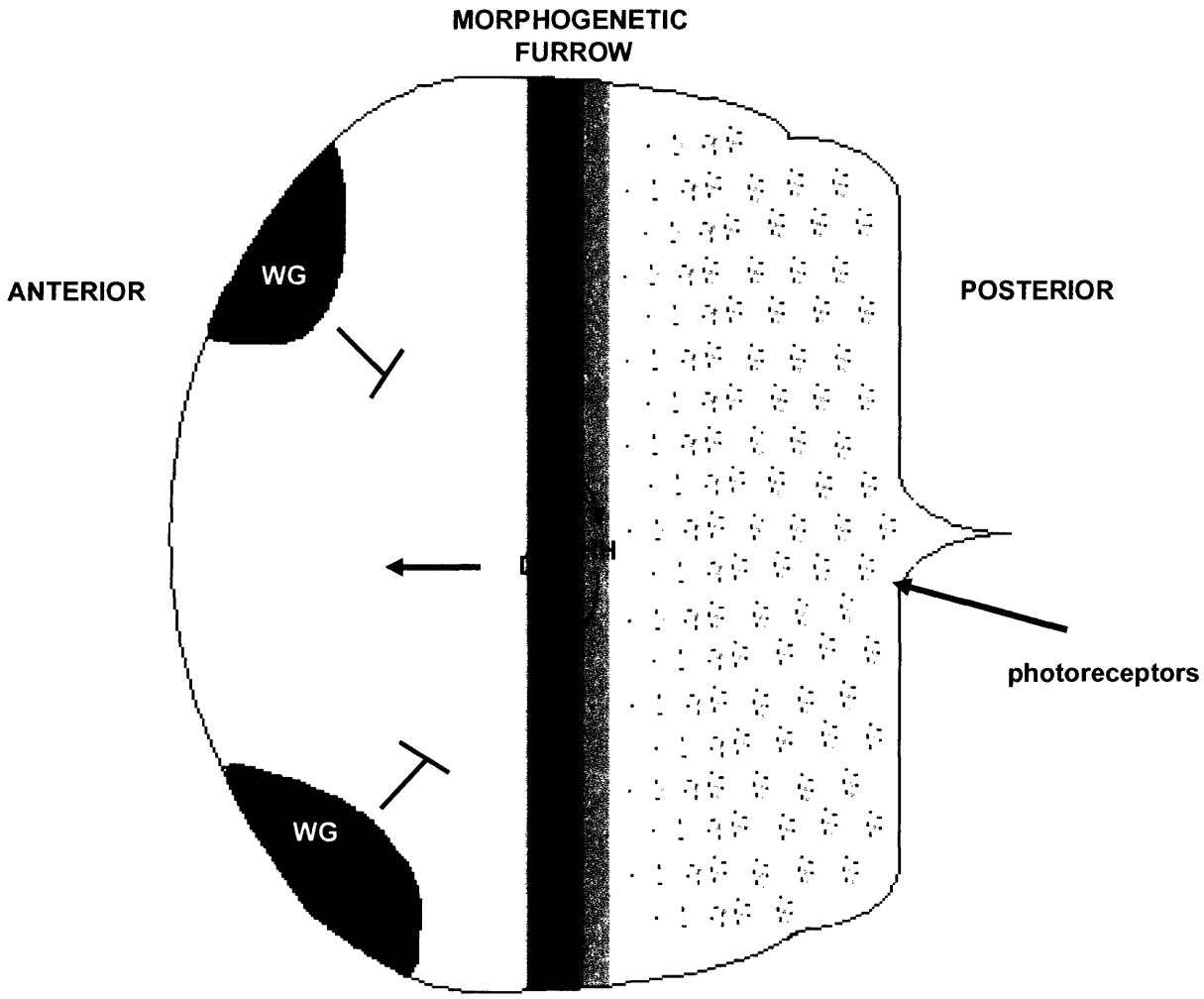
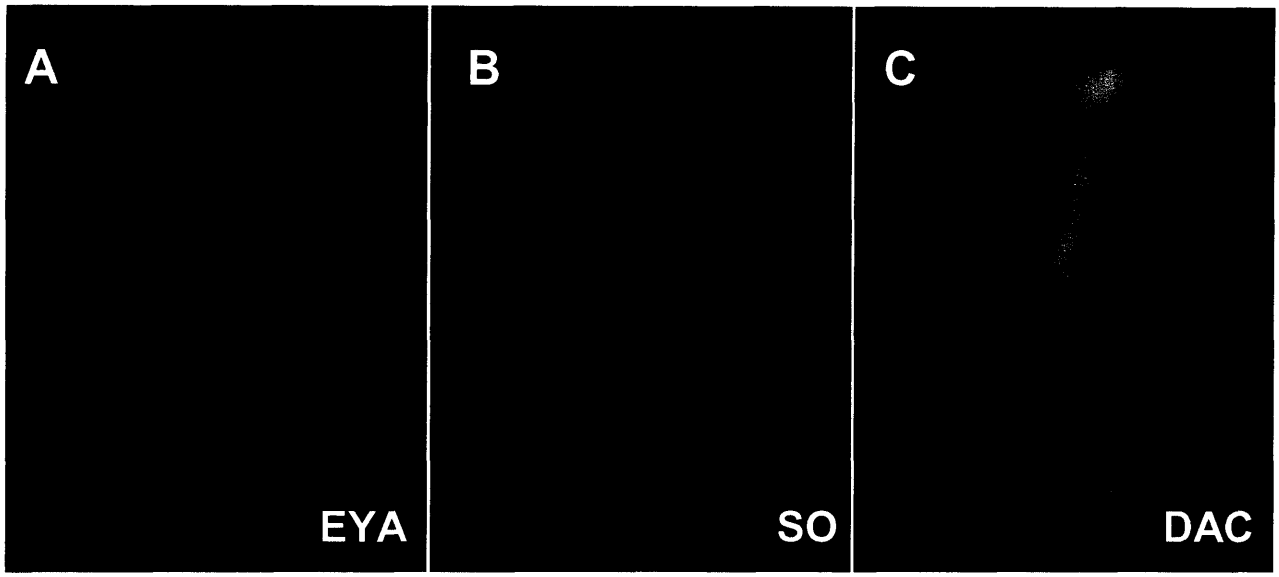


Figure 3: Morphogenetic furrow progression is driven by DPP and HH signals
The eye disc undergoes waves of differentiation as the morphogenetic furrow, driven by cooperative action of the HH and DPP signaling pathways, moves from the posterior to the anterior of the eye disc. The most posterior cells have differentiated into the photoreceptor cells, while anterior cells still proliferate. WG expression in the dorsal and ventral most anterior regions of the disc prevents eye tissue formation in that region leading to head cuticle formation.



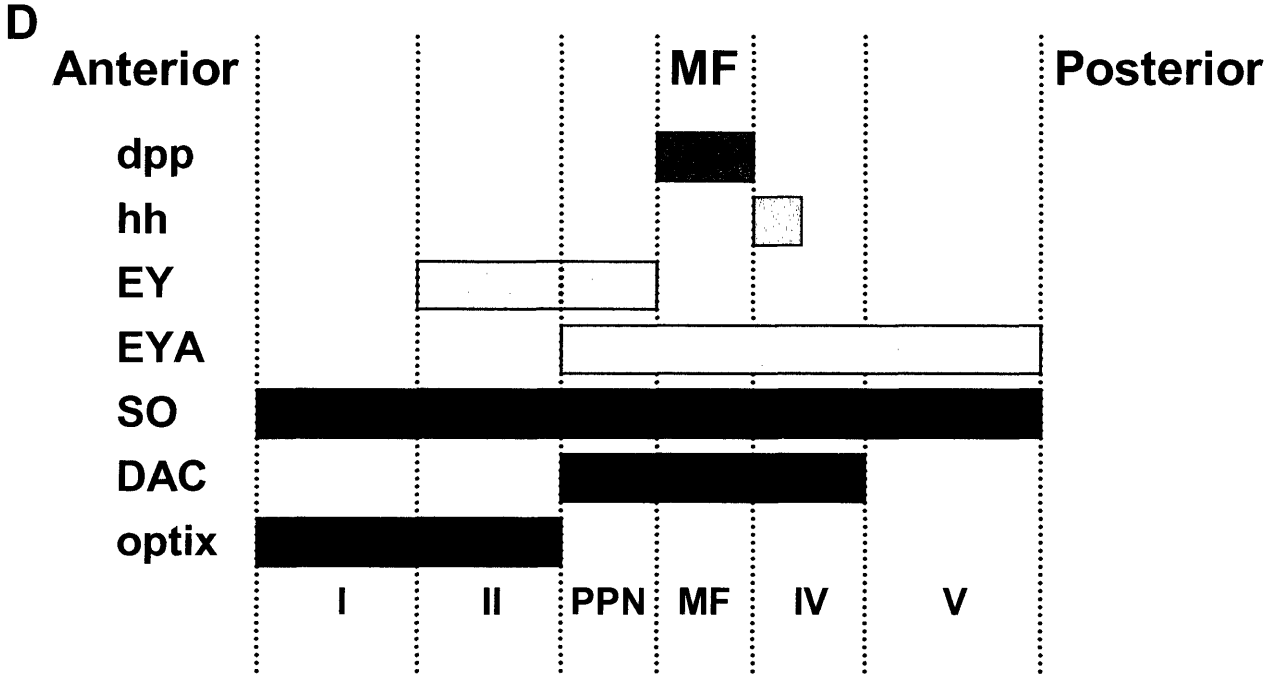


Figure 4: RD gene network members are expressed in overlapping and distinct domains in the eye disc

EYA is expressed just before the furrow in the preproneural (PPN) region and in all differentiated cells after the morphogenetic furrow (MF; A), while SO is expressed in all cells anterior to the furrow and differentiated cells posterior to the furrow (B). DAC is expressed in a broad stripe encompassing the furrow and cells on each side (C). The expression pattern of RD gene network members is depicted schematically in (D), where the eye disc is broken up into six regions based on the level of differentiation. Lowercase indicates RNA expression, while uppercase indicates protein localization. EYG is not depicted on this schematic as it is expressed in a stripe along the Dorsal/Ventral boundary.

where EY, SO, EYA, and DAC overlap, the neural fate of photoreceptors begins to be specified, along with other accessory cells. Finally just EYA and SO remain expressed in the differentiated cells, perhaps playing a role in their survival or function. Further studies of EYA, SO, and DAC, alone and in combination, should reveal their targets both independently and as a group, yielding insight as to how this precise program of eye development is orchestrated.

EGFR signaling: Cell survival and differentiation

The EGFR/RAS/MAPK pathway is an important growth and differentiation cue, which also plays a key role in control of cell survival (Bergmann et al., 2002). In the *Drosophila* eye, EGFR signaling is required at a low level in all cells to prevent apoptosis (Bergmann et al., 2002), but is also used selectively in the process of cellular differentiation. EGFR is required for morphogenetic furrow initiation but not its propagation (Kumar and Moses, 2001), and is used reiteratively to specify the fate of every eye cell in waves of differentiation following the recruitment of the first photoreceptor, R8 (Voas and Rebay, 2004).

Signaling through EGFR leads to activation of the small GTPase RAS, which then activates the MAPK cascade, which regulates downstream transcription factors and thus effects transcriptional change in response to receptor activation (Freeman, 1998). The EGFR pathway provides one of the few direct links between a signaling pathway and regulation of the RD gene network, where at least some part of the mechanism linking EGFR signaling and the network is understood. The first hint that EGFR signaling might directly affect RD gene function was the isolation of mutant alleles of *eya* in a screen for modifiers of the phenotype produced by eye specific expression of an activated negative regulator of EGFR signaling, yan^{act} (Rebay et al.,

2000). Isolation of *eya* alleles as dominant enhancers of yan^{act} suggested a role for EYA in positive transduction of the EGFR signal.

Further analysis of the interactions of EYA with components of the EGFR signaling pathway revealed that activation of RAS signaling leads to activation of EYA, through phosphorylation of EYA by MAPK (Hsiao et al., 2001). More recent work demonstrates that one effect of MAPK phosphorylation of EYA is to increase the activation potential of the EYA-SO transcription factor (Silver et al., 2003). This demonstrates a role for EGFR signaling in modulation of the EYA-SO transcription factor, and may represent a way for EYA to overcome repression of a target gene or simply to express it to higher levels. The role of MAPK in regulation of EYA phosphatase activity is not known, and it will be important to ascertain whether EGFR signaling also affects this function.

Another RD gene member genetically implicated in EGFR pathway function is *dac*, which was initially isolated in a screen for dominant modifiers of the dominant active EGFR allele *Ellipse* (*Elp*) (Mardon et al., 1994). While the mechanistic link between DAC and EGFR signaling remains unclear, mutations in *dac* suppress the *Elp* phenotype (Mardon et al., 1994), suggesting that DAC plays a positive role in transduction of the EGFR signal in the eye. As DAC is expressed just around the morphogenetic furrow (Figure 4C), perhaps it may function during EGFR mediated photoreceptor recruitment near the furrow.

In addition to genetic data linking RD gene network members to EGFR signaling, molecular data has provided clues that link PAX6 family members to this pathway. Early studies of vertebrate PAX6 indicated that it is phosphorylated on Serine residues (Carriere et al., 1993), consistent with MAPK phosphorylation. More detailed studies of zebrafish PAX6 reveal a conserved MAPK phosphorylation site, Serine 413 (Ser413), which is phosphorylated *in vitro* by

MAPK family members Extracellular-signal Regulated Kinase (ERK) and p38 and *in vivo* upon stimulation of ERK or p38 (Mikkola et al., 1999). Ser413 lies within the transactivation domain of PAX6, and similar to EYA, may be a mechanism to modulate activation levels of PAX6 targets, as mutations mimicking phosphorylation display greater transactivation potential (Mikkola et al., 1999).

Another RD gene network member regulated via phosphorylation is the *so* homolog SIX1 (Ford et al., 2000). In this case phosphorylation is tied directly to the cell cycle, as Casein Kinase II (CKII) phosphorylates SIX1 at the G2/M transition, thereby inhibiting SIX1 DNA binding ability (Ford et al., 2000). It is worth noting that the SIX1 phosphoprotein is less active than non-phosphorylated SIX1, the opposite effect observed upon phosphorylation of EYA or PAX6.

It is possible that a complex interplay of cues from both the cell cycle and extracellular signaling pathways are coordinated through the regulation of distinct members of the RD gene network, which then, like EYA and SO, interact physically to allow a coordinated response to both signals. It remains to be determined whether SO is regulated similarly to SIX1, but it is clearly expressed in cells undergoing coordinated mitosis (Bessa et al., 2002), and is a phosphoprotein (E. Davies, personal communication). Another interesting aspect is the interaction observed in the *C.elegans* interactome between EYA and the regulatory subunit of CKII, CKIIβ (Li et al., 2004), which suggests that EYA might be involved in regulation of SO phosphorylation. The mechanism by which CKII proteins are regulated is not well understood, but they are known to be phosphorylated at both serine and tyrosine residues (Litchfield, 2003), raising the intriguing idea that CKII or CKIIβ might serve as a substrate for EYA phosphatase activity.

Antagonistic signals determine EYE versus CUTICLE

Opposing signals in the eye primordia distinguish tissue destined to become eye from that which will become head cuticle. These signals come through the DPP/TGFβ and WINGLESS (WG) signaling pathways. High levels of DPP at the most posterior of the eye disc repress the WG signal and allow the formation of the morphogenetic furrow, while WG expression is highest at the most dorsal and ventral boundaries of the disc (Figure 3), where it inhibits eye formation (Hazelett et al., 1998). The RD gene network members play important roles in the specification and maintenance of these expression patterns.

The eye-antennal discs give rise to most of the adult head, including the eye and antenna as well as head cuticle. The distinction between cells that will go on to form head cuticle versus those that will contribute to the retina depends upon WG signaling (Royet and Finkelstein, 1997). The WG signaling pathway is active at the most dorsal and ventral parts of the disc, which will give rise to nonneural head tissue (Figure 3). In the absence of WG signaling, ectopic morphogenetic furrows form, indicating that WG is necessary to restrict *dpp* and *hh* expression to the most posterior region of the eye disc (Royet and Finkelstein, 1997). In addition, ectopic expression of RD genes EYA and DAC is observed in the absence of WG signal (Baonza and Freeman, 2002), indicating that WG signaling represses RD gene expression. It is difficult to distinguish from this data whether loss of WG signal directly derepresses EYA and DAC, or if this effect is secondary to the ectopic activity of DPP and HH. However, analysis of overexpression of WG suggests that it may play a more direct role in RD gene regulation.

Ectopic WG signaling in the eye field leads to inappropriate cell proliferation and lack of differentiation of eye tissue, resulting in the formation of ectopic head cuticle (Baonza and

Freeman, 2002; Royet and Finkelstein, 1997) as well as leg or antenna-like structure (Baonza and Freeman, 2002), indicating the loss of eye fate specification. Although eye formation is prevented by ectopic WG signaling, the expression of EYELESS is unchanged (Baonza and Freeman, 2002), suggesting that this block occurs either at the level of EY protein function, or through downstream components necessary for eye formation.

Consistent with either of these mechanisms, the expression of EYA, SO, and DAC are downregulated upon ectopic WG pathway activation (Baonza and Freeman, 2002). Downregulation of DAC is likely to be a key step in formation of head cuticle, as in DAC mutant tissue, head cuticle can be formed rather than eye (Mardon et al., 1994). Arguing against repression of DPP signaling as an indirect mechanism for WG downregulation of RD genes, epistasis analysis shows that WG mediated repression cannot be overcome by activation of DPP/TGFβ signaling (Hazelett et al., 1998).

However, blocks in transcription of RD gene network members are not solely responsible for WG mediated repression of eye formation, as ectopic expression of EYA, which induces SO and DAC, cannot rescue this phenotype (Baonza and Freeman, 2002). Thus in tissue with high levels of WG signal, EYA, SO, and DAC are not sufficient to direct eye formation. This suggests that WG signaling may have posttranslational effects on RD gene network function, or may affect unknown factors that act in parallel to the RD gene network.

The opposing roles of DPP and WG signaling in eye development are redeployed in other tissues during ectopic eye induction. DPP appears to play a permissive role in ectopic eye induction; overexpression of RD gene network members promotes eye specification only in regions where DPP signaling is present (Chen et al., 1999b). Moreover, WG signaling can

restrict ectopic eye induction, limiting the potential of EY to induce eye tissue in the wing disc (Halder et al., 1998).

Eye and head development must be regulated spatially to determine the primordia of these structures, but also must be regulated temporally to allow for the appropriate order of cell differentiation. Initial differentiation of eye tissue is initiated by DPP signaling, but then EGFR signaling is required for photoreceptor recruitment. As discussed above, epistasis analysis shows that WG mediated repression occurs downstream of DPP (Hazelett et al., 1998), but it has also been placed upstream of EGFR, as it can be suppressed by activation of the EGFR/RAS pathway (Hazelett et al., 1998). Therefore one effect of WG signaling may be to block EGFR activation, which as discussed above, may also result in lower posttranslational activity of RD gene network members, particularly EYA, DAC, and EY.

WG repression of RD gene network components explains their absence in WG expression domains, but does not explain how WG expression is itself limited to the most lateral regions of the eye disc. One potential mechanism for this limit is through EYG control of *wg* expression (Hazelett et al., 1998; Jang et al., 2003), as WG expression is upregulated in *eyg* mutants and downregulated upon EYG overexpression. Support for this model comes from epistasis analyses where defects in *eyg* mutant eye discs can be suppressed by inactivation of the WG pathway (Hazelett et al., 1998). Thus EYG activates growth, and represses WG, creating a doubly permissive environment for RD gene expression and function. One way to test whether EYG limits WG mediated repression of eye formation is by using ectopic eye formation assays. Analysis of where ectopic eyes are formed after coexpression of EYG with EYA might reveal a combinatorial code that can direct eye formation contexts where EYA alone is not sufficient.

This antagonistic relationship between RD gene network members and WG signaling may be conserved in vertebrates. Evidence from studies of SIX3 in the brain show that loss of SIX3 is associated with posteriorization of the brain, and further analysis suggests that the role of SIX3 in anterior-posterior specification of the brain is to repress WNT expression (Lagutin et al., 2003). It remains to be explored whether OPTIX behaves in a similar manner in *Drosophila*, or whether PAX6(5A) plays a role in WNT repression in vertebrates.

The RD gene network members are expressed in dynamic temporal and spatial patterns in the eye, where they are likely to interact with each other and with other co-factors to regulate transcription of appropriate target genes. Although the RD genes are required for eye development, and misexpression outside of the normal eye field can induce ectopic eye specification, overexpression in the eye itself results in small eyes (Curtiss and Mlodzik, 2000; Hsiao et al., 2001), which may be due to apoptosis (S.J.S. and I.R., unpublished observation). Thus inappropriate expression can have deleterious effects on the same organ that requires these proteins at endogenous activity levels. Endogenous control of RD genes is maintained through interactions with balanced signal networks at the level of both transcriptional and post-translational regulation, elaborate regulatory networks which are just beginning to be understood and will require much future work to reveal the full complement of regulation.

The RD gene network is deployed in the development of multiple organs

While the RD gene network has been best characterized for its role in eye development, insight into its regulation and function has been gained from study of other organs and appendages. In particular, study of RD gene network function in other organs illustrates the context specificity inherent in the interactions between extracellular signaling pathways and the

RD gene network. There are four major organ systems where this has been well studied; in vertebrates, the RD gene network plays important roles in muscle development (Heanue et al., 1999), and some members were independently identified in that context (Kawakami et al., 2000). In *Drosophila*, DAC plays key roles in formation of the proximodistal axis of the leg (Mardon et al., 1994), while multiple members of the network, including EYA, SO, and DAC play critical and context specific roles in gonad development (Bai and Montell, 2002; Bonini et al., 1998; Fabrizio et al., 2003; Keisman and Baker, 2001).

RD genes can direct muscle specification

In early muscle development, the expression of PAX3, a protein related to PAX6 but not orthologous to EY, overlaps with that of DACH2, a DAC homolog, and their expression is mutually regulated through positive feedback loops (Heanue et al., 1999) similar to those observed between EY and DAC during *Drosophila* eye development (Shen and Mardon, 1997). Slightly later during development, other RD gene network members EYA2 and SIX1 are expressed, so that all four components have overlapping expression in the dorsal somite prior to differentiation of skeletal muscle (Heanue et al., 1999).

Strikingly, when these genes are misexpressed in combination in cell culture, EYA2 and SIX1, as well as EYA2 and DACH2, can synergize to direct the expression of muscle markers indicative of myogenic differentiation, including PAX3 (Heanue et al., 1999). This is similar to the synergism observed between EYA and SO, and EYA and DAC in ectopic eye induction upon overexpression in *Drosophila*, and indicates that the interactions between these proteins may play conserved roles in the development of multiple organ types in addition to the eye.

DAC is negatively regulated by EGFR signaling in the leg

DAC earned its gene name for the severe leg defects observed in homozygotes, giving them "dachshund" like proportions of large body and small legs (Mardon et al., 1994). As might be expected given this phenotype, DAC expression must be carefully regulated in the larval leg disc, which will give rise to the adult leg, to allow proper leg development. DAC expression is activated in the leg disc in response to the combined signals of DPP and WG at distinct spatially graded levels of both signals (Lecuit and Cohen, 1997).

DAC is necessary for the formation of proximal structures in the leg, while a gradient of EGFR signaling is required for and can induce the formation of the most distal structures, the tarsus and claw (Campbell, 2002). Analysis of temperature sensitive alleles of EGFR in addition to clonal analysis, reveals cell autonomous repression of DAC by this signaling pathway, where loss of EGFR results in ectopic DAC expression (Campbell, 2002). The relationships between DAC and these signaling pathways in the leg are distinct from the relationships observed in the eye, where DPP and EGFR both act positively and WG negatively regulates DAC expression (Figure 5). In contrast, in the leg DAC is regulated positively by DPP and WG and negatively by EGFR (Figure 5). It is not clear mechanistically how these signals are interpreted in a context specific manner, but context represents an important aspect of RD gene network regulation that requires more intense study. Another example of these context specific interactions between DAC and WG signaling is observed in the gonad.

Sex-specific regulation of RD gene network members

Many patterning genes are expressed in homologous patterns in both male and female genital discs, including the morphogens DPP and WG. In both males and females, *wg*

expression is found in a stripe along the anterior posterior border, and is flanked by broad stripes of *dpp* expression (Keisman and Baker, 2001). Other genes, including RD member *dac* (Keisman and Baker, 2001) are expressed in a sex-specific manner.

In males, DAC expression is seen overlapping the *dpp* stripes, while in females DAC expression is observed overlapping the central *wg* expression domain (Keisman and Baker, 2001). DAC function is important for the proper development of both male and female genitalia, as males lacking DAC have reduced claspers and females lacking DAC have defects in duct formation (Keisman and Baker, 2001).

WG is required to activate DAC expression in the leg (Lecuit and Cohen, 1997), and similarly, is required to activate DAC in the female genital discs (Figure 5; Keisman and Baker, 2001). Strikingly, the opposite effect is observed in male genital discs, where WG appears to restrict DAC expression (Keisman and Baker, 2001), similar to WG repression of DAC in the eye (Figure 5). The converse is observed for DPP signaling, which activates DAC expression in male genital discs, but represses expression in female genital discs (Keisman and Baker, 2001), while in the eye and leg DPP plays only a positive role in DAC regulation (Figure 5).

This sex-specific regulation of DAC is likely mediated by components of the sex-determination pathway *doublesex* and *transformer* (Keisman and Baker, 2001). However it remains unclear just how the sex of this tissue determines DAC responsiveness to WG and DPP signaling, and it is intriguing to ask whether the specificity observed in sex determined expression uses a similar mechanism to determine appropriate DAC response as does the eye and leg. This provides an example of the importance of context, which is observed again and again to be crucial for the function of RD gene network members, and is outlined in Figure 5.

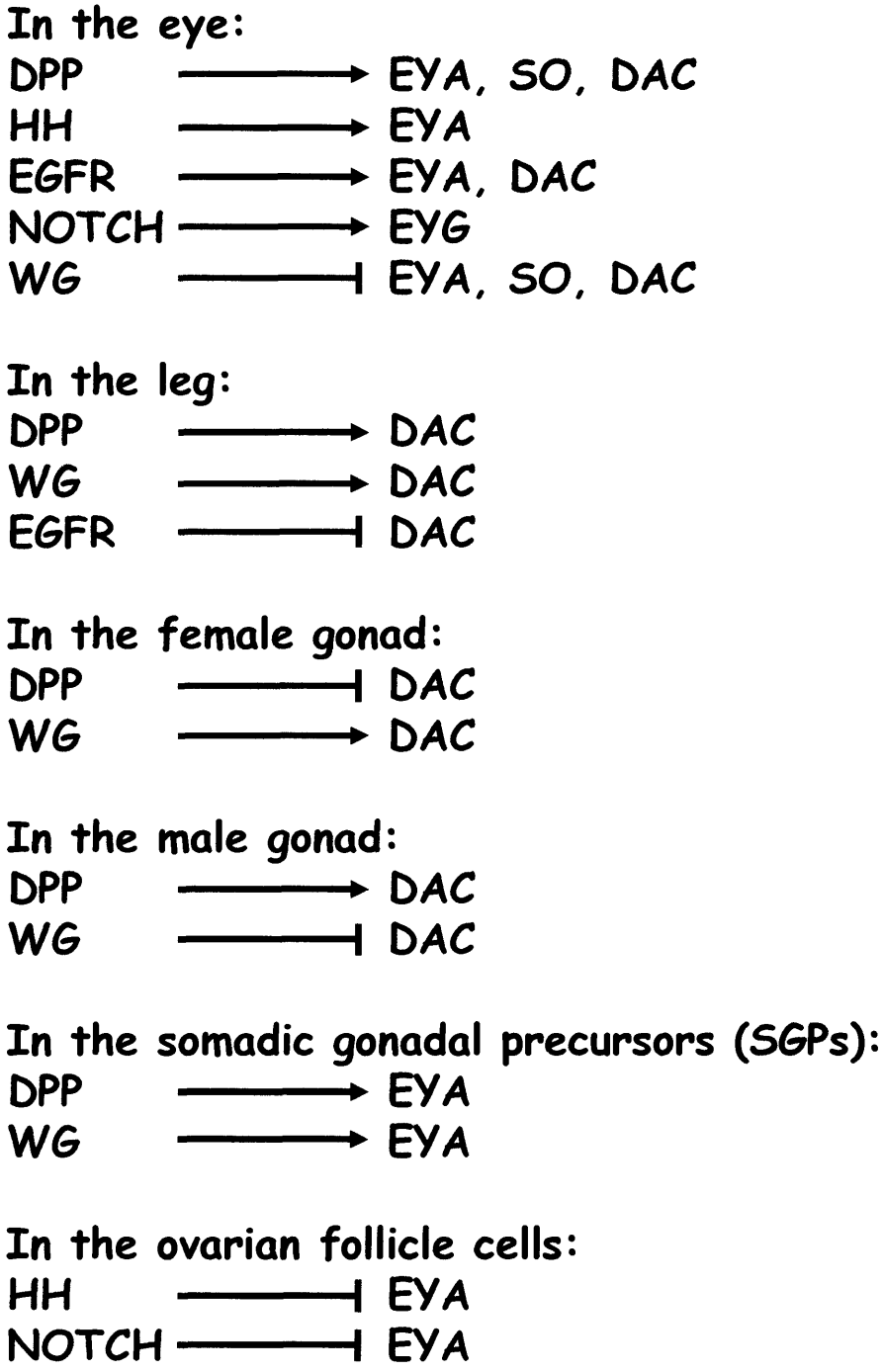


Figure 5: The relationship between signaling pathways and the RD gene network is highly context dependent. Positive interactions shown in green, negative in red.

EYA is a crucial determinant for somatic gonadal precursor cells

EYA also plays a role in both female and male fertility; hypomorphic alleles that survive to adulthood are sterile in addition to defects such as small eyes (Boyle et al., 1997; Fabrizio et al., 2003). EYA is expressed in the somatic gonadal precursor (SGP) cells, cells that associate with the germ cells and insure proper incorporation of the germ cells into the gonad (Boyle et al., 1997). In *eya* mutants, the SGP cells develop but do not maintain their fate and thus do not coalesce around the germ cells (Boyle et al., 1997). EYA may work downstream of the WG signaling pathway, which is also required for SGP formation, as ectopic activation of WG signaling leads to ectopic EYA and the recruitment of extra SGPs (Boyle et al., 1997). Here, WG plays a positive role in EYA regulation, while in the eye WG and EYA are antagonists.

In contrast, DPP and EYA retain their positive relationship from the eye. DPP signaling is crucial for EYA expression and the formation of SGPs through the activation of a secondary regulator, in this case *tinman* (Boyle et al., 1997). Thus context once again determines the direction of interaction between signaling pathways and the RD gene network, where here WG and DPP together activate EYA in contrast with their opposing functions in the eye (Figure 5).

EYA plays a SO independent role in oogenesis

In addition to patterning roles early in gonad formation, EYA function is required during oogenesis, in the somatic tissue crucial for proper germ cell development and function. There are three types of somatic follicle cells that surround the oocyte during oogenesis; polar cells, stalk cells, and main body epithelial cells. Polar cells are found at the anterior of the egg chamber, and recruit border cells to surround them and facilitate movement through the nurse cells to the anterior of the oocyte, where they develop into the micropyle, the organ that allows

sperm to enter the oocyte. In ovaries mutant for EYA, extra polar cells are observed, a phenotype similar to that observed upon ectopic activation of the HH signaling pathway (Bai and Montell, 2002). Conversely ectopic expression of EYA prevents specification of polar cells (Bai and Montell, 2002).

These results suggest an antagonistic link between HH and EYA, which is supported by loss of EYA in follicle cells upon HH pathway activation (Bai and Montell, 2002). As discussed above, the HH signal is transduced by the full length form of CI, CI^{act}, which in the absence of signaling is processed to a shorter repressor form, CI^{R}. In EYA mutant cells, higher levels of CI^{act} are present (Bai and Montell, 2002), suggesting a mutually repressive relationship between HH signaling and EYA in the differentiation of ovarian follicle cells.

Another signaling pathway associated with the proper formation of polar cells is the NOTCH pathway, which like HH is required for polar cell formation. Ectopic NOTCH signaling can induce the formation of ectopic polar cells in a non-cell autonomous manner which includes downregulation of EYA expression (Bai and Montell, 2002), although the mechanisms behind NOTCH and HH mediated repression of EYA remain to be determined. Both NOTCH and HH here function antagonistically to EYA, unlike their positive or at least permissive effects on EYA in the eye (Figure 5), suggesting that there is some key tissue specific difference which changes the way these signals interact with the RD gene network.

Oogenesis also provides an example of independent roles for EYA and SO. SO is not expressed in polar cells, where EYA seems to act alone. Instead, SO plays a role in development of a different type of follicle cell, the stalk cell, and is required for the specification of that cell type without help from EYA (Bai and Montell, 2002).

In contrast, spermocyte development may require the EYA-SO complex, as they are both required in the somatic cyst cells for proper spermocyte development, and genetic interactions between the two are observed in this tissue (Fabrizio et al., 2003). This suggests that in spermatogenesis, the EYA-SO complex may act to direct transcription of target genes, while in the oocytes EYA may complex with different DNA binding proteins or may only function as a protein phosphatase. SO may interact with other co-activators in the stalk cells, or may play its role there through transcriptional repression. It will be interesting to determine how the transcriptional targets of the EYA-SO complex might be different in these distinct contexts.

Concluding Remarks

The RD gene network provides a sensitive model for signal integration whose study may give insight into the broader questions of signal specificity in different developmental contexts. Formation of organs requires coordination of cell division and cell differentiation to insure appropriate size and organization. Signal integration between the RD gene networks and signaling pathways provides a mechanism to link differentiation and cell division such that differentiation does not occur before appropriate cell division takes place. Moreover the signaling back and forth between the RD gene network and signaling pathways provides a model for study of context specificity in activity and regulation, where identification of co-factors and targets may elucidate the mechanisms for this specificity.

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CHAPTER 2

A functional dissection of Eyes absent reveals new modes of regulation within the retinal determination gene network

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E.L.D. performed the analysis of the GAL4DBD-EYA fusion proteins and set up the S2-2H system, L.D. helped with transcription assays.

This chapter has been previously published:

Silver, S. J., Davies, E. L., Doyon, L., and Rebay, I. (2003). Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. Mol Cell Biol 23, 5989-5999.

Abstract

The Retinal Determination (RD) gene network encodes a group of transcription factors and cofactors necessary for eye development. Transcriptional and post-translational regulation of RD family members is achieved through interactions within the network and with extracellular signaling pathways, including Epidermal Growth Factor Receptor/RAS/MAPK, Transforming Growth Factor-β/DPP, Wingless, Hedgehog, and Notch. Here we present the results of structure-function analyses that reveal novel aspects of Eyes absent (EYA) function and regulation. We find that the conserved C-terminal EYA domain negatively regulates EYA transactivation potential, and that GROUCHO-SINE OCULIS (SO) interactions provide another mechanism for negative regulation of EYA-SO target genes. We have mapped the transactivation potential of EYA to an internal P/S/T-rich region that includes the EYA domain 2 (ED2) and two MAPK phosphorylation consensus sites, and demonstrate that activation of the RAS/MAPK pathway potentiates transcriptional output of EYA and the EYA-SO complex in certain contexts. *Drosophila* S2 cell two-hybrid assays were used to describe a novel homotypic interaction that is mediated by EYA's N-terminus. Our data suggest that EYA requires homo- and hetero-typic interactions and RAS/MAPK signaling responsiveness to ensure context appropriate RD gene network activity.

Introduction

Proper development of an organism requires a formidable amount of cell-cell communication, wherein successful information transfer is effected by signaling cascades that ultimately alter the gene expression profile of a cell. Additionally, cells must integrate signals from multiple pathways to coordinate morphogenesis and differentiation in a spatially and temporally appropriate manner. Examples of this combinatorial control paradigm have been elucidated using the *Drosophila* eye as a model system, and have demonstrated that unique combinations of general and tissue-specific transcription factors are required to specify and maintain distinct cell fates (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000).

Components of the retinal determination (RD) gene network, which includes *twin-of-eyeless* (*toy*), *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*), are essential for eye fate specification in metazoans. The RD gene network collectively encodes a cohort of nuclear transcription factors and/or cofactors whose expression is regulated by a conserved hierarchy of transcriptional regulation, such that TOY activates *ey* expression, EY induces *so* and *eya*, and EYA turns on *dac* (Chen et al., 1997). In addition to assuming pivotal roles during visual system development, the RD genes function in a variety of other contexts, including gonadogenesis (Bai and Montell, 2002), myogenesis (Heanue et al., 1999), limb formation (Galindo et al., 2002; Xu et al., 1997a), neurogenesis (Li et al., 2002), and the cell cycle (Kurusu et al., 2000). Consequently, null mutations are lethal and exhibit complex phenotypes that are reflective of the pleiotropic roles assumed by RD network proteins during development (Bonini et al., 1998; Cheyette et al., 1994; Kronhamn et al., 2002; Mardon et al., 1994). Additionally, the expression patterns of the RD genes are not wholly coincident (Bessa et al., 2002), suggesting

that reiterative deployment of the entire RD network module is not obligatory for the function of specific RD network proteins.

Much analysis of RD gene function and regulation has focused on the visual system, particularly in *Drosophila*, where gene activity can be manipulated without compromising viability or fertility of the animal. RD genes are best known for the "eyeless" phenotype associated with eye-specific hypomorphic mutations, and for the ability to induce formation of ectopic eye tissue upon overexpression (Bonini et al., 1997; Chen et al., 1997; Czerny et al., 1999; Halder et al., 1998; Pignoni et al., 1997; Shen and Mardon, 1997). Use of these two assays has revealed a complex system of positive feedback loops superimposed on the defined linear hierarchy of transcriptional regulation, which most likely amplifies and stabilizes expression of the RD gene products.

eya is the founding member of a novel gene family characterized by a highly conserved C-terminal motif that contains both SO (31) and DAC (9) binding sites, termed the EYA domain (ED; a.a. 486-760; Figure 1A). Vertebrate homologs, such as murine EYA1-4, are strikingly similar in their EYA Domain, yet their N-termini, with the exception of a small tyrosine rich region, the EYA Domain 2 (ED2; Figure 1A), are largely divergent (Xu et al., 1997b; Zimmerman et al., 1997). *so*, and vertebrate *Six* family genes, encode nuclear proteins with a homeobox type DNA binding domain and the conserved SIX domain (Kawakami et al., 2000). The latter is an important mediator of EYA-SO interactions (Pignoni et al., 1997). DAC, a novel nuclear protein, and its vertebrate DACH counterparts, contain two regions of high homology, the DACH-box-N and the DACH-box-C (Mardon et al., 1994). Like SO, DAC has been shown to physically interact with the EYA Domain, via the DACH-box-C (Heanue et al., 1999).

Analysis of loss-of-function *eya* mutants in *Drosophila* and in human patients suffering from Branchio-oto-renal (BOR) syndrome underscores the importance of EYA-SO and/or EYA-DAC interactions in vivo. BOR syndrome, a disease characterized by craniofacial, ear, and kidney defects, arises from mutations in the human *Eya1* gene (Abdelhak et al., 1997; Bui et al., 2000). In *Drosophila*, point mutations in the conserved EYA domain (ED) appear to cause loss-of-function phenotypes by impairing EYA's ability to interact with SO and DAC (Bui et al., 2000). Similarly, a recent study has shown that human BOR alleles that map to the EYA domain also have impaired interactions with SIX and DACH family members, emphasizing the evolutionarily conserved importance of interactions between these three RD family members in vivo (Ozaki et al., 2002).

The combined physical interaction, colocalization, and genetic data have led to a model whereby EYA and SO together constitute a functional transcription factor, with EYA providing the activation domain and SO contributing the DNA binding moiety. Consistent with this, in mammalian cell culture, SIX2, SIX4 and SIX5 are able to synergize with EYA to drive expression from a reporter construct (Ohto et al., 1999). The functional consequences of EYA-DAC interactions remain less well understood. Although initial characterization of *Drosophila dac* and its vertebrate counterparts, *Dach1* and *Dach2*, failed to identify a DNA binding domain, it has been postulated that the DACH-box-N may encode a novel DNA binding motif similar to the winged helix/forkhead subgroup (Kim et al., 2002). Thus, like SO, DAC may play a role in recruiting EYA to the promoters of target genes (Ikeda et al., 2002).

In addition to the complex interactions observed within the RD gene network, numerous signaling pathways, including Epidermal Growth Factor Receptor (EGFR)/RAS/MAPK (Hsiao et al., 2001), Transforming Growth Factor-β (TGFβ)/Dpp (Chen et al., 1999; Pignoni and

Zipursky, 1997), Wingless (Baonza and Freeman, 2002), Hedgehog (Curtiss and Mlodzik, 2000), and Notch (Bai and Montell, 2002; Kumar and Moses, 2001) have been shown to interact genetically with members of the RD network. However, with the exception of the EGFR pathway (Hsiao et al., 2001), little is known about how extracellular signaling pathways regulate RD gene expression and/or activity.

In order to better understand RD gene network regulation, we have performed an extensive structure-function analysis of the EYA protein. Using a series of *Drosophila* S2 cell based transcriptional activation assays, we have defined a P/S/T rich region of EYA, encompassing both the MAPK phosphorylation consensus sites and the tyrosine-rich ED2, that is necessary for EYA transactivation potential in cell culture and for ectopic eye induction ability in vivo. We demonstrate that RAS/MAPK signaling can positively regulate EYA transactivation, and that GROUCHO is a potent repressor of EYA-SO mediated transcription through its interactions with SO. Finally we show that EYA is able to self-associate, adding yet another layer of functional complexity to the elaborate hierarchy of interactions that exists among the RD gene products.

Materials and Methods

Construction of Transactivation Assay expression plasmids

pUAST-luciferase was constructed by amplifying the luciferase cDNA from pGL3luc (Promega) with primers LUC1, 5'-TTGGAATTCCAACATGGAAGACGCCAAAAAC-3,' and LUC2: 5' TTGGGTACCTTACACGGCGATCTTTCCGC-3', digesting with EcoRI and KpnI, and inserting into the EcoRI/KpnI sites of pUAST. All eya constructs were made using the eya1 cDNA. pRmHa3-Gal4DBD-eyawt, S-A, and S-D/E full length fusion constructs have a similar

design. The MAPK consensus sequence is defined as P-X-S/T-P, and Drosophila eya contains two adjacent phosphorylation sites at amino acids S402 and S407. The construct referred to as pRmHa3Gal4DBD-eyaS-A contains two S-A point mutations in place of the phosphoacceptor residues, and conversely the pRmHa3-Gal4DBD-eyaS-D/E construct contains S-D and S-E point mutations at these sites. A three-piece ligation was performed to insert a 660 bp, N-terminal eya PCR amplified fragment and a 1.6 kb, C-terminal eya restriction fragment containing either wildtype or mutated MAPK sites into pRmHa3-Gal4DBD, cut with KpnI and SalI. The N-terminal PCR product was generated using primers EYA1, 5'-tgggtaccttgtataatgtgccgtgctatc-3,' and EYA2, 5'-cgaagagttgaccgccactg–3,' and was digested with KpnI and BamHI. BamHI-SalI restriction fragments from pRmHa3-eya, pRmHa3-eyaS-A, and pRmHa3-eyaD/E (these constructs described previously in (Hsiao et al., 2001)) were then combined with the digested eya PCR product in a ligation with pRmHa3-Gal4DBD, cut with KpnI and SalI.

Similarly, three truncated pRmHa3-Gal4DBD-*eya* constructs that lack the conserved EYA domain (amino acids 486-760) were made containing each of the MAPK site variants described above. Primers EYA1 and EYA3, 5'-TTGGTCGACTTACACACTGCTGCCTCCGCTC –3,' were used to amplify a 1.3 kB product from a pRmHa3-*eya*, pRmHa3-$eya^{S\text{-}A}$, or pRmHa3-$eya^{D/E}$ template. PCR products were digested with KpnI and SalI, and ligated into the pRmHa3-Gal4DBD vector. A construct encoding the first 353 amino acids of EYA, including the N-terminal portion of the P/S/T-rich region, was also generated. This construct, pRmHa3-Gal4DBD-*eya 1-353*, was generated using primers EYA1 and EYA1545A, 5'-TTGGTCGACG TAGTTGGCCGGACTGTA-3'. The 920 bp PCR product and pRmHa3-Gal4DBD were digested with KpnI and SalI, and ligated directionally. An internal deletion construct that lacks the entire P/S/T-rich region, pRmHa3-Gal4DBD-*eya Δ223-438*, was made by inserting an annealed,

double-stranded linker with 5' BamHI and 3' KpnI sticky ends into pRmHa3-*myc-eya*, cut with the aforementioned enzymes. Primers EYAD1, 5'-GATCCATTTTGTACGGTACC-3,' and EYAD2, 5'-CGTACAAAATG-3,' were annealed and used in the directional ligation described above to generate pRmHa3-*myc-eya Δ223-438*. pRmHa3-Gal4DBD-*eya Δ223-438* resulted from a two piece ligation between pRmHa3-Gal4DBD-*eya*, cut with BamHI and SalI, and a complementary restriction fragment from pRmHa3-*myc-eya Δ223-438*. A construct encoding a truncated EYA in which the ED2 domain (amino acids 318-353) is also deleted, pRmHa3-Gal4DBD-*eya ΔED2 ΔED*, was made using the Stratagene Quikchange site-directed mutagenesis protocol. Primers dEya 1351/1455 (S), 5'-CAGCTGTACAGCAGTCCGTCACCGTATGCGGTCAGC3,' and dEya1351/1455(A), 5'-GCTGACCGCATACG GTGACGGACTGCTGTACAGCTG-3,' were used to generate pBSSK-*eya ΔED2*. A 600 bp BamHI-SacII *eya ΔED2* fragment was then shuttled into the truncated pRmHa3-Gal4DBD-*eya* construct in a directional ligation. pRmHa3-Gal4DBD-*eya 318-436* was made using primers KpnI-EYA D2 (S), 5'-TTGGGTACCTACGCCGGCTACAACAACTTC-3,' and EYA3 to amplify a 350 bp product. The PCR product and pRmHa3-Gal4DBD were digested with KpnI and SalI, and used in two-piece ligations.

Development of S2 Cell 2-Hybrid (S2-2H) Assay

A vector containing the DNA binding domain of yeast Gal4 (a.a. 1-147), pRMHa3-Gal4DBD, was constructed as follows. Gal4DBD was amplified from pCasperUbGal4 using primers 5'-TGGAATTCCAACATGAAGCTACTGTCTTCTATCG-3' and 5'-TGGGTACCCGATACAGTCAACTGTCTTTG-3', which contain 5' and 3' EcoRI and KpnI

sites, respectively. The digested PCR product was ligated into pRmHa3, a pUC9-derived vector containing a metallothionein responsive promoter upstream of the Multiple Cloning Site (MCS). pRmHa3-Gal4AD was made by amplification of the Gal4 activation domain (a.a. 768-874) from pCasperUbGal4 via PCR with primers Gal4AD S2302 5'-TGGAATTCCAACATGGCCAATTTTAATCAAAGTG-3' and Gal4AD A2673 5'-TTGGTACCGTATCTTCATCATCGAATAGA-3', cut with EcoRI and KpnI, and inserted into pRmHa3. To ensure nuclear expression of these constructs, an NLS was added using these two oligos, Gal4AD NLS S-50 5'-TGTGACCCCCCCCAAGAAGAAGCGCAAGGTGGAGGACGATGGTAC-3' and Gal4 AD NLS A-54 5'-CATCGTCCTCCACCTTGCGCTTCTTCTTGGGGGGGGTCACAGTAC-3'. Oligos were annealed and then ligated into the KpnI site of the pRmHa3-Gal4AD to make pRMHa3-Gal4AD-NLS, which we refer to as pRMHa3-Gal4AD. pRmHa3-Gal4AD-*eya* resulted from a three-piece ligation using KpnI-BamHI *eya* and BamHI-SalI *eya* restriction fragments, and pRmHa3-Gal4AD cut with KpnI and SalI. Primers SoS4 5'-TTGGTACCTTACAGCATCCCGCCACAG-3' and So1234 5'-TTGGTCGACTCATAAGTGCTGGTACTC-3' were used to amplify full length *so* cDNA from pBSSK *so* (gift of G. Mardon), with unique 5' KpnI and 3'SalI cut sites. The digested PCR product was inserted into pRmHa3-Gal4DBD in a directional, two piece ligation to make pRmHa3-Gal4DBD-*so*. To make pRmHa3-Gal4AD-*so*, *so* was cut out of pRmHa3-Gal4DBD-*so* with KpnI and SalI inserted into pRmHa3-Gal4AD cut with the same enzymes. To make *dac* constructs, DacS622 5'-TTGGTACCGATTCTGTGACAAGTGAAC-3' and DacA1370 5'-AAG TGCTTCAGGAAGAGCTCG-3' primers were used to amplify the 5' region of *dac* from pBSSK-*dac* (gift of G. Mardon), adding a 5'KpnI site. The PCR product was cut with Kpn I and

Stu I, which is found internally in the amplified *dac* fragment. A three-piece ligation was performed using KpnI/StuI *5'dac* and a StuI/SalI restriction of fragment from pBSSK-*dac*, into the KpnI/SalI sites in pRmHa3-Gal4DBD. For the AD construct, full length *dac* was cut out of pRmHa3-Gal4DBD-*dac* with KpnI and SalI and ligated into those sites in pRmHa3-Gal4AD to create pRmHa3-Gal4AD-*dac*. For pRmHa3-Gal4DBD-*eya ΔD2*, the N-terminal *eya ΔD2* construct described above was shuttled into full length pRmHa3-Gal4DBD-*eya* using BamHI and SacII. For pRmHa3-Gal4DBD-*eya domain*, the *eya domain* was PCR amplified using primers EYADS 5'-TTGGGTACCGAACGGGTGTTCGTCTGG-3'
and EYA DA 5'-TTGGGATCCTCATAAGAAGCCCATGTC-3', which contain KpnI and BamHI sites used to clone into the pRmHa3-Gal4DBD vector.

Construction of Transcription Assay expression plasmids

To construct *ARE-luciferase*, *luciferase* cDNA was isolated as an XhoI/XbaI fragment (~1.6Kb) from Promega's pGL3-Luciferase, and inserted into XhoI/XbaI sites of pBluescript-SK+. To add the hsp70 TATA box, oligos EBS link 1 5'-CCATATGATCTGCAGAGGGTATATAATGC-3' and EBS link 2 5'-TCGAGCATTATATACCCTCTGCAGATCATATGGGTAC-3' were annealed and inserted into the KpnI/XhoII sites of pBSSK-*luciferase*. KpnI and XhoI sites were retained and NdeI and PstI sites and a TATA box were inserted. *ARE-luciferase* was made by multimerizing the AREC3 (SIX4) binding site, as defined in (Kawakami et al., 1996) using oligos ARES 5'-TCGAGGGTGTCAGGTTGCG-3' and AREA 5'-TCGACGCAACCTGACACCC-3'. Oligos were annealed and ligated, then cut with XhoI and SalI A resultant 7mer was cloned into the MCS of pBSSK, and then shuttled into pBSSK-*TATA-luciferase*. To make pRmHa3-*flag-eya*, full length *eya1* cDNA was PCR amplified using primers

EYAI 5397 5'-TTGTATAATGTGCCGTCGTATC-3' and EYA STOP 5'-TTTCATAAGAAGCCCATGTCGAGG-3' and then digested with SmaI which cuts the *eya* cDNA internally. The 0.7 kb blunt/SmaI fragment was inserted into SmaI cut pBSSK + Flag vector (gift from R. Fehon) to produce an in-frame fusion of the FLAG epitope with the 2nd amino acid of EYA. A three piece ligation was done to join the 5' end of *flag-eya*, obtained as a SacI/SmaI fragment from pBSSK-*flag-eya*, with the 3' end of *eya*, (obtained from pRMHa3-*eya*) as a SmaI/SalI fragment. These were inserted into SacI/SalI cut pRMHa3. Similarly, pBSSK-*myc-eya* was generated by PCR amplification using primers EYAI and EYA STOP, and blunt ligated into the StuI site of pBSSKmyc. A three piece ligation between the EcoRI/BamHI fragment from pBSSK-*myc-eya*, and BamHI and SalI from pRmHa3-*eya*, into the EcoRI and SalI sites of pRMHa3, resulted in pRMHa3-*myc-eya*. In order to construct pRMHa3-*dac*, first a ClaI fragment was cut out of pBSSK-*dac* (gift from G. Mardon) and cloned into pBSSK to remove most of the large 5'UTR (Cla-*dac*). A 3' fragment was cloned into pBSSK using BamHI and HindIII sites (HI/HIII-*dac*). A full length construct was made by four piece ligation with EcoRI-StuI from Cla-*dac* plus StuI-BamHI fragment from pBSSK-*dac* plus BamHI-XhoI fragment from HI/HIII-*dac* into the EcoRI/XhoI sites of pUAST. The entire full length *dac* cDNA including approximately 45 bp of the 5'UTR and 245 bp of the 3'UTR was then excised with EcoRI and XhoI and ligated into the EcoRI/SalI sites of pRMHa3. pRMHa3-*myc-so* was generated by PCR amplification of full length *so* cDNA using primers So S805 5'-TTACAGCATCCCGCCACAGAT-3' and So A2268 5'-AACTAGAATCATAAGTGCTGG-3', and blunt end ligated into the StuI site of pBSSK-*myc*, resulting in an in-frame fusion of the MYC epitope to the 2nd amino acid of SO. This was moved into pUAST using EcoRI and XbaI . To move the full length tagged *so* into pRMHa3, *myc-so* was cut out of pUAST-*myc-so* with

XbaI, blunted with Klenow, then digested with EcoRI and subcloned into EcoRI-SmaI sites of pRMHa3. A full-length *groucho* expression construct, pMT-GROUCHO, was generated by PCR amplifying the N-terminus of Groucho, using the *groucho* cDNA clone LD33829 as template and primers Groucho-start 5'-ATGAATTCAACAACATGTATCCCTCACCGG-3' and Groucho-A879 5'-TGTGCGATACTTCTCACGATCGG-3', digesting with EcoRI and XbaI, and ligating with an XbaI/XhoI fragment from LD33829 into EcoRI/SalI cut pRMHa-3.

All regions generated by PCR were verified by sequencing, and all constructs were tested for expression and localization in S2 cells by immunohistochemistry. Further subcloning details available upon request.

Co-immunoprecipitation and Western Blots

Transfected cells were harvested, then lysed by rocking at 4°C for 20 minutes in 1 ml of lysis buffer (100 mM NaCl; 50 mM Tris, pH7.5; 2 mM EDTA; 2 mM EGTA; 1% NP-40 + one Complete, Mini protease inhibitor cocktail tablet(Roche)/10 ml). Clarified lysates were subjected to immunoprecipitation with anti-flag conjugated agarose beads (Sigma) for 1.5. hours at 4°C. Beads were washed 3X with lysis buffer. The immunoprecipitates were boiled in 40 µl of 2x SDS buffer, and western blotting was carried out as previously described (O'Neill et al., 1994) (mouse α-myc (1:300), mouse α-GRO (1:50), Rb α-flag (1:5000).

For western blots to analyze protein levels, half of the transfected cells were lysed as described below for β-galactosidase assays, and the other half were pelleted, vortexed briefly, and resuspended in 40ul 2X SDS buffer. Transfection efficiency was measured by βgal assays and appropriate amounts of crude lysate were run on the gel. Efficiency was confirmed by western

blotting for βgal (Rb anti-βgal, 1:20,000). Protein levels were examined with GP α-EYA (1:10000), mouse α-myc (1:300), and Rb α-flag (1:5000).

Generation of Transgenic Lines

pRmHa3-*flag-eya* and pRmHa3-*myc-eya ΔD2* were cut with SmaI and SalI, and a directional ligation was performed to create pRmHa3-*flag-eya ΔD2*. Likewise, a SmaI/SalI double digest was performed on pRmHa3-*myc-eya Δ223-438* to generate pRmHa3-*flag-eya Δ223-438*. EcoRI/SalI double digests were used to excise the *flag-eya*, *flag-eya ΔD2*, and *flag-eya Δ223-438* cDNAs from the pRmHa3 vector for insertion into pUAST, cut with EcoRI and XhoI. pUAST-*flag-eya*, pUAST-*flag-eya* ΔD2, and pUAST-*flag-eya Δ223-438* were subsequently used to generate transgenic lines as previously described (Rebay et al., 1993).

S2 Cell Transactivation and Transcription Assays

Drosophila S2 cells were transiently transfected using calcium phosphate as described previously (Pascal and Tjian, 1991). 2.5 ug of each construct indicated was transfected for 6 hours along with 2.5ug of *pUAST-luciferase* or 10ug of *ARE-luciferase* as the reporter gene, and 1 ug pActin 5.1-V5His-lacZ (Invitrogen) to normalize for transfection efficiency. Cells were allowed to recover for 17 hours, whereupon expression was induced by addition of 0.1M $CuSO_4$. After 24 hours, cells were harvested by spinning at 1000 rpm for 1 minute and lysed by rocking at 4°C for 20 minutes in 250 µl of lysis buffer (Tropix/Applied Biosystems). Samples were subsequently microfuged at 14K for one minute at 4°C, and supernatants transferred to fresh tubes. Luciferase and ß-galactosidase activities were quantified using a Luciferase Assay Kit (Tropix/Applied Biosystems) or Galacto-Star Assay kit (Tropix/Applied Biosystems). Assays

were performed in triplicate on whole cell extracts, according to the manufacturer's instructions (TROPIX/Applied Biosystems). A minimum of four independent transfections were performed for each condition. The average luciferase/β-galactosidase signal for Gal4DBD/*pUAST-luciferase* or *ARE-luciferase* alone was set to 1 and the experimental averages were normalized relative to this value. Data were analyzed and graphed using Microsoft Excel. Error bars denote one standard deviation above and below the mean for each construct. In Figures 3, 5, and 6, SO is always tagged with the FLAG epitope. In Figure 3, EYA constructs are tagged with the FLAG epitope, and in Figures 5 and 6, EYA constructs are tagged with the MYC epitope.

Results

Eya functions as a transactivator

To address the question of whether EYA can function as a transcriptional coactivator, we took advantage of the well-characterized yeast transcription factor Gal4 and its target sequence, UAS (Fields and Song, 1989), to design an assay for transactivation in *Drosophila* S2 cells. The DNA binding domain of Gal4 (Gal4DBD) was fused in frame to the *eya* coding region (Gal4DBD-Eya; Figure 1B) and subcloned into a vector containing a metallothionein promoter, which allows inducible expression in S2 cells. Gal4DBD-EYA fusion proteins were tested for their ability to activate expression of a *UAS-luciferase* reporter gene; co-transfection of a constitutively expressed, Actin-lacZ plasmid enabled us to normalize activity levels based on transfection efficiency. Immunohistochemistry with anti-Gal4DBD antibodies confirmed the expression and nuclear localization of all Gal4DBD-EYA fusion proteins in transfected S2 cells (data not shown).

First, we tested the full-length *eya* coding region fused in-frame to the Gal4DBD (Figure 1B). As shown in Figure 1B, constructs 1 and 2, Gal4DBD-EYA exhibits 3.5-fold greater activity than the Gal4DBD alone. A series of deletion and truncation constructs were designed to define a minimal and sufficient domain for this activity. Strikingly, a fusion protein expressing the N-terminal 485 amino acids of EYA but lacking the conserved EYA Domain, Gal4DBD-EYA ΔED, displays an approximate 70-fold increase in transactivation potential relative to the full length Gal4DBD-EYA construct (Figure 1B, constructs 3 versus 2, note scale). The converse fusion protein expressing only the C-terminal EYA domain, Gal4DBD-EYA Domain (a.a. 486-760) does not transactivate (data not shown). This difference is likely not due to changes in protein stability, as EYA and EYA ΔED are expressed at similar levels (Figure 1C). The discrepancy in activity levels of the full length versus Gal4DBD-EYA ΔED chimeras suggests that the EYA Domain may function as an autoregulatory inhibitor in this context.

A Proline, Serine, Threonine rich region is critical for Eya transactivation

EYA ΔED was dissected further to determine the regions critical for transactivation. EYA ΔED contains the conserved EYA Domain 2 (ED2, a.a. 318-353), a tyrosine-rich region that has not been functionally characterized (Figure 1A). ED2 lies within a larger Proline/ Serine/Threonine (P/S/T)-rich region (a.a. 223-438) that includes two consensus MAPK phosphorylation sites previously shown to be important for EYA regulation in vivo (Hsiao et al., 2001).

We found that Gal4DBD-EYA 2-353, an N-terminal construct that contains the ED2 but truncates the P/S/T-rich region, exhibits very low transactivation activity, suggesting a critical requirement for this latter domain (Figure 1B, construct 4). Consistent with these results, the

fusion protein that contains the last two-thirds of the P/S/T rich region, Gal4DBD-EYA 318-436, exhibits a 22-fold increase in transactivation potential relative to the Gal4DBD alone control (Figure 1B, construct 5 versus 1). Therefore the P/S/T-rich region of EYA is critical but not entirely sufficient for transcriptional co-activation, and upstream regions of the protein (a.a. 2-223) are required to achieve maximal transactivation levels.

Interestingly, deletion of the conserved ED2 (Gal4-DBD-EYA ΔED2, ΔED; a.a. 2-317; 353-485; Figure 1B, construct 6) results in a 6 fold reduction in transactivation potential relative to that of EYA ΔED (Figure 1B, construct 3) yet shows a 41-fold increase relative to the Gal4DBD control (Figure 1B, construct 1). Although the ED2 is not essential for EYA transactivation per se, our results suggest that it is needed to achieve maximal levels, and ascribes a function to this previously uncharacterized domain.

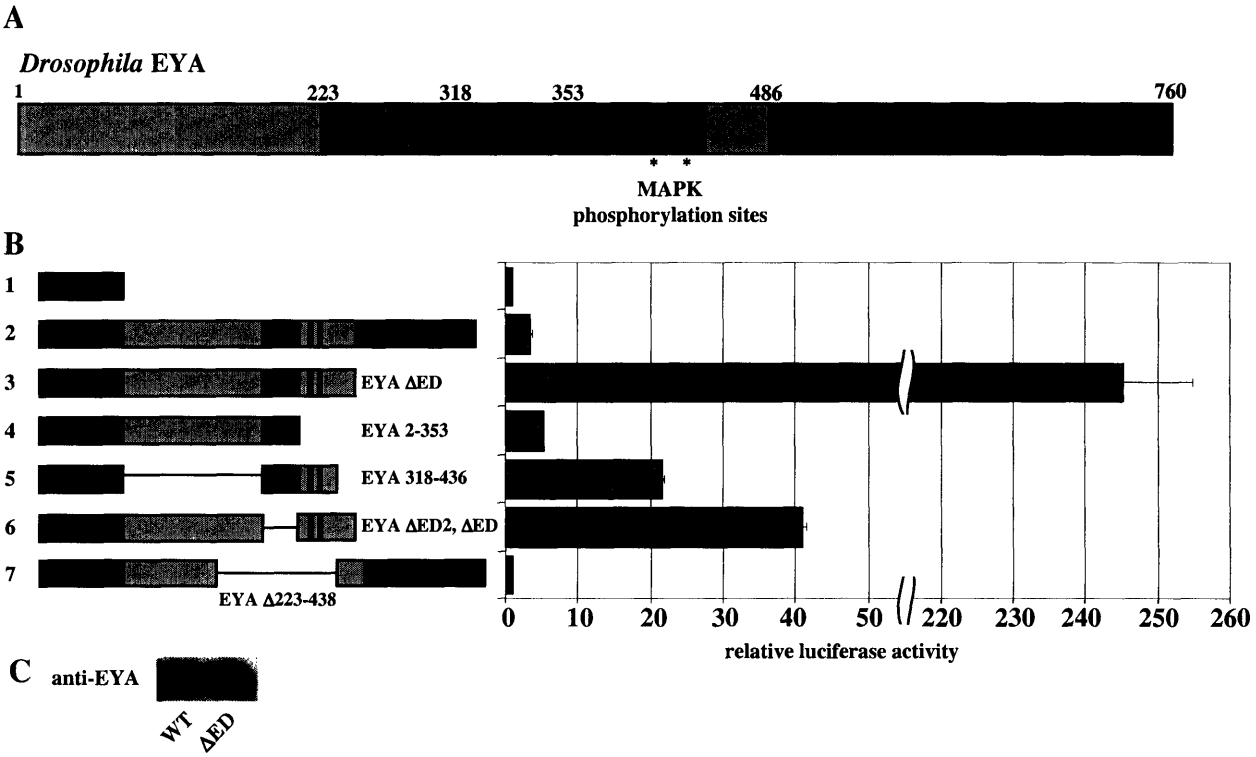
Although the full-length EYA is a weaker transactivator in this assay than any of the "active" deletion constructs that lack the C-terminal EYA domain, we wanted to determine whether the P/S/T-rich region is necessary for the trans-activation potential of full-length EYA. To address this question, the entire P/S/T rich region, including ED2, was deleted to generate Gal4DBD-EYA Δ223-438. We found that this internal deletion abrogates transcriptional activation in full length Eya (Figure 1B, construct 7 versus 3). The identical result, namely a complete lack of transactivation, was obtained when the C-terminal EYA Domain is also deleted from construct 7 (data not shown).

RAS/MAPK signaling increases EYA transactivation potential

As the P/S/T region important for EYA transactivation potential also contains the MAPK phosphorylation sites (Ser402 and Ser407) shown to affect EYA activity in vivo, we asked

Figure 1: The N-terminus of Eya is a potent transactivator

(A) *Drosophila* EYA contains two conserved regions, the EYA Domain 2 (ED2), and the EYA domain (ED). The P/S/T rich region includes both the ED2 and the two MAPK phosphoacceptor sites.
(B) Gal4DBD-Eya fusions were used to assay the ability of Eya to activate transcription from a UAS-luciferase reporter gene. Transactivation potentials were calculated by taking the ratio of luciferase/ß-gal activity for each construct, and were plotted relative to the activity of the Gal4DBD vector alone. As shown in B, construct 2, full length EYA can activate transcription 3.5 fold above Gal4DBD alone. The N-terminus of EYA (construct 3), a construct that lacks the EYA domain, is a potent transactivator, activating transcription over 250 fold (note scale change on axis). Gal4DBD-EYA 1-353 (construct 4), a truncation that contains the ED2 but removes part of the P/S/T rich region, reduces transactivation potential to only 5 fold. The ED2 plus part of the P/S/T rich region is able to activate transcription at low levels (construct 5), indicating that the entire N-terminus is necessary for full transactivation potential. Deletion of the conserved ED2 within the N-terminus of EYA (construct 6) sharply reduces transactivation to 41 fold above background, 1/5 the activity of the intact N-terminus. Strikingly, deletion of the entire P/S/T rich region (construct 7) results in complete loss of transactivation potential.
(C) Deletion of the EYA domain does not affect protein expression levels.



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(C) Deletion of the EYA domain does not affect protein expression levels.

whether RAS signaling modulates EYA's transactivation potential. To examine this possibility, we cotransfected activated *ras* (*ras*^{V12}) with the Gal4DBD-EYA fusion constructs. We did not observe an increase in the transactivation potential of Gal4DBD-EYA (data not shown), but observed a variable but significant increase in Gal4DBD-EYA ΔED transactivation potential in the presence of RAS^{V12} (Figure 2). In repeated trials, this increase in transactivation ranged from 10% to 66%, the latter of which is depicted in Figure 2.

As we had previously shown that site-specific mutations in the MAPK consensus phosphoacceptor residues affect EYA function in vivo (Hsiao et al., 2001), we wanted to test whether these mutations might also influence transactivation in the Gal4DBD assay system. Mutation of the two MAPK consensus sites S402 and S407 to alanine (EYA$^{S\text{-}A}$) significantly reduces EYA activity in ectopic eye induction assays. Conversely, mutation of the phosphoacceptor residues to aspartic or glutamic acid (EYA$^{S\text{-}D/E}$) produces a hyperactive protein, presumably by mimicking its phosphorylated state (Hsiao et al., 2001). Surprisingly, we found that both EYA$^{S\text{-}A}$ and EYA$^{S\text{-}D/E}$ point mutations increased transactivation activity of Gal4DBD-EYA ΔED to slightly higher levels than were seen upon addition of RAS^{V12} (Figure 2).

As EYA transactivation potential is increased upon cotransfection of *ras*^{V12} or mutation of the MAPK phosphoacceptor residues, it seems likely that these residues are important for proper regulation of EYA transactivation. Our finding that in this system both EYA$^{S\text{-}A}$ and EYA$^{S\text{-}D}$ mutations result in increased transactivation relative to wildtype Eya may reflect the complexity of the consequences of RAS/MAPK signaling. However, it is important to note that the chimeric Gal4DBD-EYA fusion proteins and/or the truncations we engineered may have an altered conformation relative to native EYA, such that MAPK phosphorylation events have different consequences in this context.

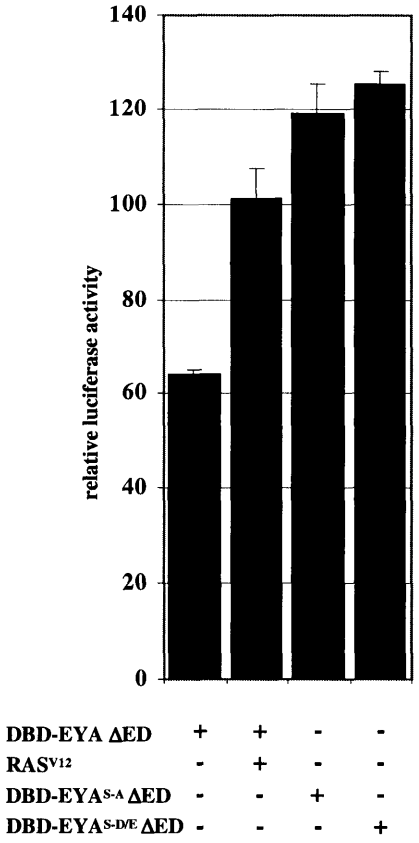


Figure 2: RAS/MAPK Signaling activates Eya transactivation
Transactivation via Gal4DBD-EYA ΔED is increased significantly but variably upon the addition of RAS^{V12}. Mutations of the MAPK phosphoacceptors to alanine (EYA^{S-A}) to prevent phosphorylation, or to aspartic or glutamic acid (EYA^{S-D}) to mimic phosphorylation, both increase Gal4DBD-EYA ΔED transactivation activity, suggesting that these sites are important for regulation of EYA transactivation potential. Taken with the increase seen upon addition of RAS^{V12}, we conclude that RAS signaling can increase EYA transactivation potential.

ARE-luciferase is responsive to the EYA-SO transcription factor

The synthetic assay system described above enabled us to map the regions crucial for EYA transactivation activity to the ED2 and the surrounding P/S/T rich region. RAS/MAPK-dependent effects on EYA transactivation levels indicate that RAS signaling can activate EYA transactivation but that such effects may not afford themselves to straightforward interpretation. In order to examine this question in a more native system, we designed a transcription assay system to measure the transactivation potential of EYA-SO complexes.

Currently, the genomic DNA target sequences bound by *Drosophila* SO are not known, but several studies in mammalian cells and tissues have identified SIX family response elements. In particular, the *Na, K-ATPase α1 subunit gene* (*ATP1a1*) Regulatory Element (ARE) has been shown to respond to SIX family members SIX2, SIX4 and SIX5 in vivo and in vitro (Kawakami et al., 1996). As SO and SIX2 belong to the same SIX family subgroup (SIX1/2) (Kawakami et al., 2000), and their homeodomains are 93% identical (Seo et al., 1999), we reasoned that they are likely to bind similar sequences. We therefore multimerized the core ARE binding site (see Materials and Methods) and placed this enhancer in front of a minimal promoter followed by *luciferase* cDNA, which we will refer to as *ARE-luciferase*.

We found that *ARE-luciferase* is responsive to co-transfection of *eya* and *so*, and together they activate *ARE-luciferase* 27-fold over the reporter alone (Figure 3A). *ARE-luciferase* is not appreciably activated alone, nor upon transfection of *eya* or *so* individually (Figure 3A). Therefore *ARE-luciferase* activation provides a measure of EYA's efficacy as a transcriptional co-activator when bound to SO. We also asked whether the addition of DAC affects *ARE-luciferase* transcription and found that DAC did not affect transactivation potential of the EYA-SO transcription factor (data not shown).

The P/S/T rich region of Eya is necessary for transactivation but not for EYA-SO interactions

We used the *ARE-luciferase* reporter and full length EYA and SO to ask whether the ED2 and the P/S/T rich region determined to be critical in the synthetic Gal4DBD assay system are essential in a more physiologically relevant context. In this assay, we used otherwise full length EYA constructs that lack either the ED2 or the entire P/S/T rich region, EYA ΔED2 and EYA Δ223-438. As shown in Figure 3A, deletion of the ED2 reduces transactivation to only 14-fold relative to controls, while deletion of the entire P/S/T-rich region, EYA Δ223-438, virtually eliminates transcriptional co-activation.

One possible explanation for this lack of activity might be a decrease in protein expression levels in the EYA deletion constructs. In order to test this, we performed quantitative Westerns blots to assay tagged EYA protein levels. Transient transfections were normalized for effieciency using Bgal assays and appropriate amounts of lysate were loaded. We found that while the ΔED2 deletion did not alter protein expression levels (Figure 3B, lane 2), EYA Δ223-438 was less abundantly expressed than full length EYA (Figure 3B, lane 3). In order to correct for this difference, we transfected sufficient EYA Δ223-438 plasmid to produce protein levels above that of EYA full length (Figure 3B, lanes 4 and 5). Using these higher amounts of EYA Δ223-438 plasmid we still observe only low levels of transactivation (Figure 3A), indicating that deletion of this region of EYA compromises activity.

To confirm that the loss of transactivation potential seen in these deletions was not due to a loss of EYA-SO binding, we developed a system to test for direct protein-protein interactions in S2 cells, which we term S2 cell two-hybrid (S2-2H) assays (refer to Methods for details). Use

of *Drosophila* cultured cells, rather than the more common yeast or mammalian cell based systems (14,15), allows interactions between *Drosophila* proteins to be assayed in a more physiologically native environment, thereby increasing the probability that necessary cofactors and/or protein modifications are present.

Using our S2-2H assay, Gal4DBD-EYA, EYA ΔED2, and EYA Δ223-438 fusions were each tested for interaction with a Gal4AD-SO fusion protein. As shown in Figure 3C, Gal4AD-SO is able to interact with all three Eya proteins, and demonstrates even stronger interactions with Gal4DBD-Eya Δ223-438. This, combined with our western analysis, indicates that the reduced transactivation observed upon deletion of the ED2 and the P/S/T-rich region (Figure 3A) reflects a change in activity levels of the EYA-SO transcription factor, rather than simply the loss of EYA protein or loss of the ability to form an EYA-SO complex.

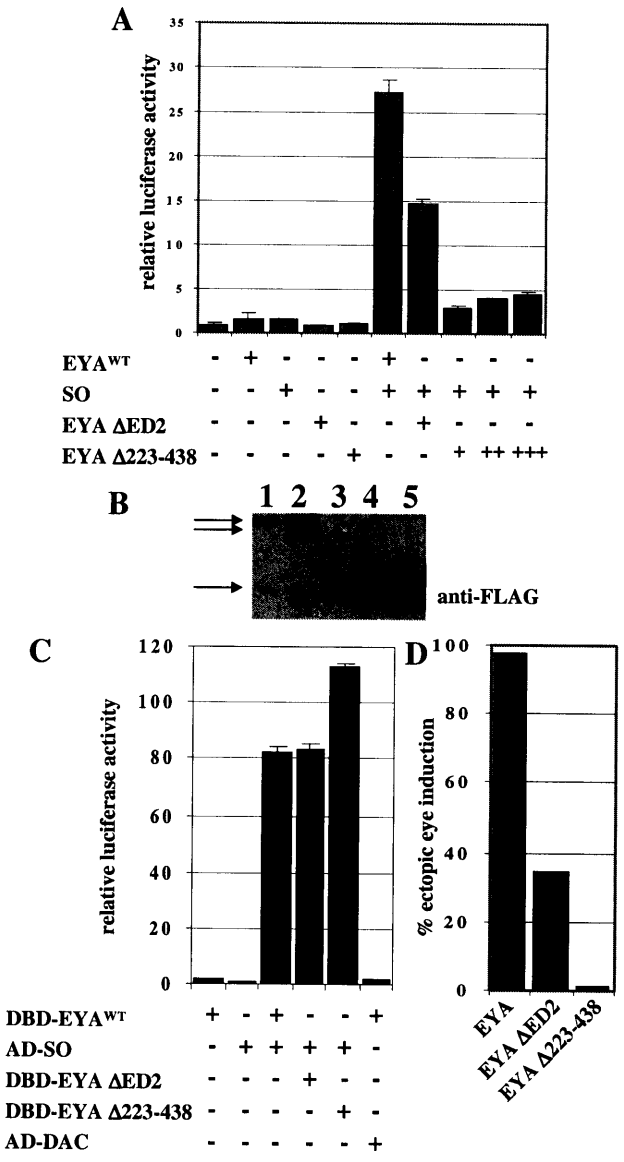
We confirmed that the P/S/T rich region and ED2 are necessary for EYA activity in vivo by assaying the ability of these deletions to induce ectopic eyes when overexpressed using the UAS/Gal4 system (Brand and Perrimon, 1993). We found that when driven by the 57A1dpp-Gal4 driver, EYA ΔED2 and EYA Δ223-438 exhibited drastically reduced activity relative to wildtype EYA (Figure 3D), though all constructs were expressed at comparable levels as assayed by western blots of Ub-Gal4 driven expression of UAS-EYA constructs in embryos (data not shown). Thus ED2 and the P/S/T rich region are critical for EYA function in vivo.

DAC does not interact with EYA or SO in S2-2H assays

As DAC did not affect EYA-SO mediated transcription of *ARE-luciferase*, we asked whether DAC was able to interact with EYA or SO using our S2-2H system. Gal4DBD and Gal4AD fusions were made with full length EYA and DAC and tests were performed in both

Figure 3: ARE-luciferase is responsive to the EYA-SO transcription factor
(A) EYA and SO alone do not affect transcription of the ARE-luciferase reporter gene, but together can activate transcription 27 fold. Full length $EYA^{\Delta ED2}$ is unable to fully activate transcription of this gene, and neither is a construct missing the entire P/S/T rich region, $EYA^{\Delta 223-438}$. This construct was not expressed at the same level as wildtype EYA, so we transfected two (indicated by ++) and three times (indicated by +++) the amount of plasmid to raise protein levels to and above the levels of EYA. These levels still do not activate ARE-luciferase.
(B) Quantitative Western blotting shows that EYA (lane 1) and $EYA^{\Delta ED2}$ (lane 2) are expressed at similar levels, while the same amount of $EYA^{\Delta 223-438}$ plasmid (lane 3) is not. However, transfection of two and three times more $EYA^{\Delta 223-438}$ plasmid results in robust expression as shown in lanes 4 and 5.
(C) EYA and SO show a strong interaction in an S2-2H assay. The deletions in EYA do not affect interactions with SO, and in fact $EYA^{\Delta 223-438}$ appears to have a stronger interaction with SO. DAC does not interact with EYA in S2-2H assays.
(D) Overexpression of EYA using the 57A1dpp-Gal4 driver causes ectopic eye induction in over 98% of animals (n=413). Overexpression of $EYA^{\Delta ED2}$ results in ectopic eyes in only 35% of animals (n=506). Deletion of the entire P/S/T rich region, $EYA^{\Delta 223-438}$, results in a protein that can only rarely induce ectopic eyes, seen in only 1.5% of animals examined (n=204).

Figure 3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DBD-EYA ΔED2 - - - + - -
DBD-EYA Δ223-438 - - - - + -
AD-DAC - - - - - +
D
% ectopic eye induction
100
80
60
40
20
0
EYA
EYA ΔED2
EYA Δ223-438

directions. Surprisingly, no interaction was observed in either direction (data shown in one direction, Figure 3C). We also asked whether SO might be required to nucleate an EYA-DAC complex, but did not observe an interaction (data not shown). To determine whether RAS/MAPK signaling might be required for formation of an EYA-DAC complex we cotransfected Ras^{V12} or used the $Eya^{S\text{-}A}$ and $Eya^{S\text{-}D}$ constructs described above, but still did not observe an EYA-DAC interaction (data not shown). SO and DAC also fail to interact in the S2-2H assay (data not shown).

EYA-EYA interactions are mediated via the N-terminus

As the RD gene network is known to function in reiterative feedback loops, we wondered if EYA might interact with itself to potentiate or restrict function. Using our S2-2H assay, we find that full length EYA shows a significant interaction with itself (using Gal4DBD-EYA and Gal4AD-EYA; Figure 4), at 7 fold above background. We then used our series of deletion and truncation constructs fused to the Gal4DBD domain coexpressed with the Gal4AD-EYA fusion to ask which domain mediates this EYA-EYA interaction. In order to distinguish between the activity of the fusion alone and the S2-2H interaction, we transfected each alone and with AD-EYA (Figure 4).

As shown in Figure 4, EYA 2-317 (construct 3) shows slightly greater interaction with AD-EYA than full length EYA, while a construct including the ED2, EYA 2-353 (construct 4) shows a striking increase in interaction relative to full length EYA. EYA 223-353 (construct 5) shows interaction levels comparable to full length EYA, however, deletion of the ED2 in the context of full length EYA also results in a striking increase in EYA-EYA interaction (Figure 4, construct 6). The EYA domain, which mediates EYA-SO interactions, does not interact above

background with full length EYA (Figure 4, construct 7). This result, coupled with the striking increase observed between EYA 2-353 and EYA, leads us to conclude that EYA-EYA interactions are likely mediated by amino acids in the N-terminus, and that the ED2 (a.a. 318-353, Figure 1A), and the regions immediately following it, including the MAPK phosphorylation sites, while not necessary for this interaction, may strongly potentiate it. We could not test other N-terminal EYA constructs in this assay, such as EYA ΔED and EYA ΔED2ΔED, as expressed alone they strongly activate the reporter (Figure 1B). As this interaction maps to regions we describe above as crucial for full EYA transactivation potential (Figure 1B) and for EYA function in vivo (Figure 3D), it seems likely that the EYA-EYA interaction is functionally significant and could contribute to regulated transcriptional activity.

Phosphorylation increases EYA transactivation potential in the context of the EYA-SO transcription factor

Having established an assay in which full-length EYA acts as a transcriptional co-activator when complexed to SO, we returned to the question of whether EYA activity is regulated by RAS/MAPK signaling. Although activated RAS did not affect EYA-SO mediated transcriptional regulation, nor did mutation of the EYA phosphoacceptor sites to Alanine, (Figure 5A), we found that the $EYA^{S\text{-}D/E}$-SO complex consistently exhibited a 50% increase in transactivation levels relative to the EYA-SO complex (Figure 5A). This result is consistent with previously reported transgenic analyses, wherein overexpression of $EYA^{S\text{-}D/E}$ led to stronger and more penetrant phenotypes than did EYA (Hsiao et al., 2001). Analysis of protein levels in flies expressing $EYA^{S\text{-}A}$ or $EYA^{S\text{-}D/E}$ transgenes indicated no differences in protein stability (Hsiao et al., 2001), and we repeated these results in cell culture with quantitative westerns as shown in

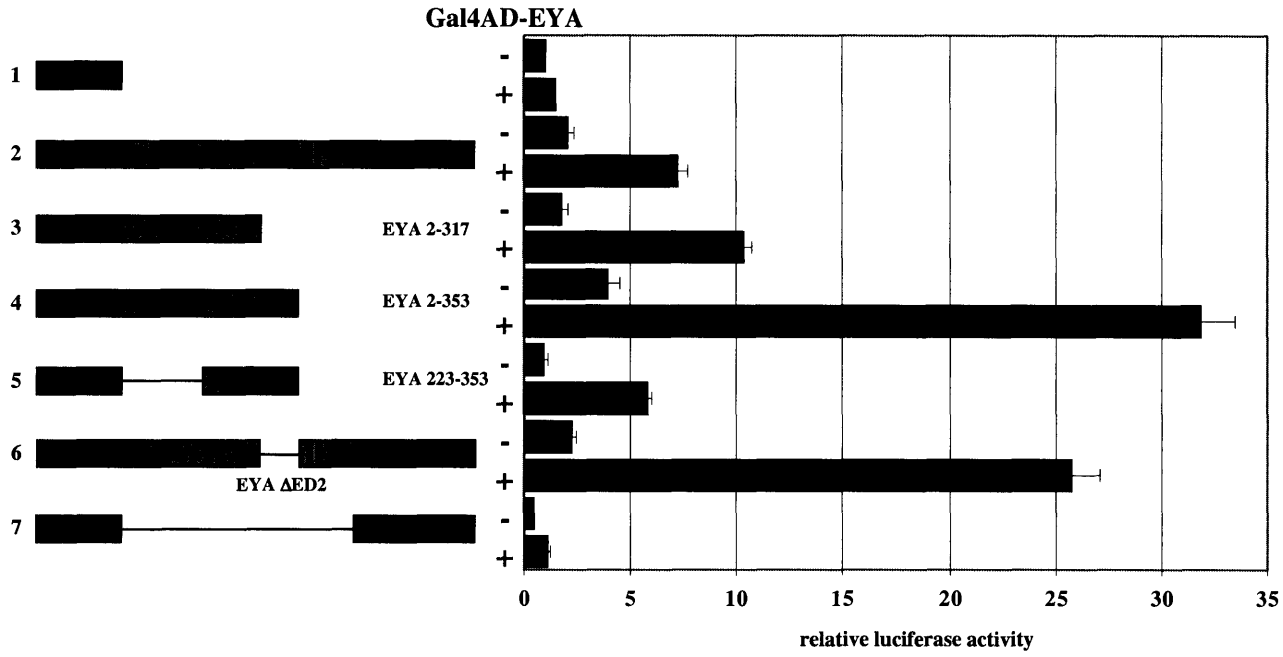

Figure 4: EYA-EYA interactions are mediated by the EYA N-terminus
Using the S2-2H system, Gal4DBD-EYA fusions shown on the left were co-expressed with (+) and without (-) full length Gal4AD-EYA. Full length EYA interacts with itself 7 fold above background. This interaction is mediated by amino acids 223-317, as all constructs that contain this minimal region can interact with Gal4AD-EYA. Strikingly, the Gal4DBD-EYA 2-353 and Gal4DBD-EYA ΔED2 (constructs 4 and 6) show more than three fold stronger interaction than that of full length EYA. The Gal4DBD-EYA domain fusion (construct 7) does not interact with Gal4AD-EYA, consistent with our finding that the EYA N-terminus mediates this interaction.

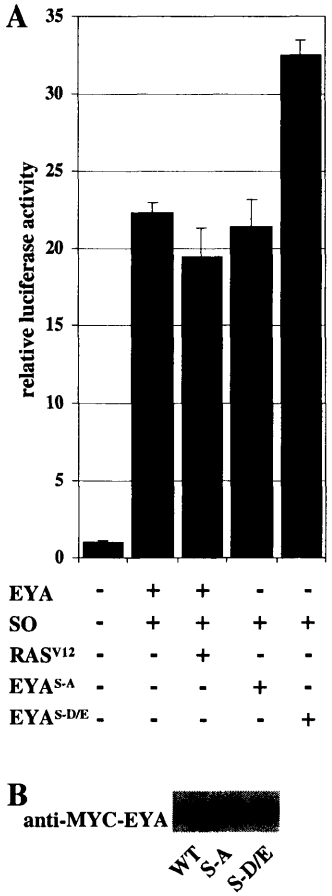

Figure 5: The EYA-SO transcription factor is regulated by phosphorylation
(A) As shown in Figure 3, the EYA-SO transcription factor can activate expression of *ARE-luciferase*. This expression is not affected by addition of RAS^{V12} nor upon mutation of the MAPK phosphoacceptor sites to alanine (EYA^{S-A}). A striking increase in activation is seen when the EYA^{S-D} phosphomimetic mutant is used, showing that phosphorylation acts to increase EYA transactivation potential. That RAS^{V12} itself does not produce the same increase on wildtype EYA in this assay suggests that RAS signaling may have multiple effects on the RD gene network, and in particular may negatively regulate SO.
(B) EYA phosphoacceptor mutations do not affect protein expression levels.

Figure 5B, where wildtype and mutant EYA are expressed at the same levels. The S2-2H system was used to rule out the possibility that the transactivation increase seen with the $EYA^{S\text{-}D/E}$-SO complex results from an increase in EYA-SO interactions (data not shown). Therefore the increase in transactivation exhibited by the $EYA^{S\text{-}D/E}$-SO complex suggests that phosphorylation directly increases EYA's transactivation potential.

As we see this result only upon mutation of the phosphoacceptor site, but not in response to RAS/MAPK activation, it remains possible that a different signaling pathway mediates this phosphorylation event. However, previously reported genetic and biochemical evidence (Hsiao et al., 2001) indicates that RAS/MAPK signaling is responsible for phosphorylation of this site in vivo. Thus we favor the interpretation that EYA transactivation is potentiated by MAPK phosphorylation, as evidenced by the increased activity of the $EYA^{S\text{-}D/E}$-SO complex, and that the lack of response to RAS stimulation likely reflects a more complex role for RAS within the RD gene network.

GROUCHO is a repressor of the EYA-SO transcription factor

Recent studies in zebrafish (Kobayashi et al., 2001), mice (Zhu et al., 2002) and medaka (Lopez-Rios et al., 2003) have revealed a functional role for SIX3 interactions with GROUCHO (GRO), a transcriptional co-repressor. One of these studies also demonstrates weak interactions between SO and a murine Groucho homolog, GRG5 (Zhu et al., 2002). As SO belongs to a different class of SIX homologs, and SIX3/SIX6 are distinct from other families in that they do not interact with EYA or EYA homologs, we wanted to ask if this SO-GRG5 interaction indicated a functional role for GRO in regulation of the EYA-SO transcription factor. We found that co-expression of GRO strongly reduces but does not eliminate activation via the EYA-SO

transcription factor. As shown in Figure 6A, EYA-SO activates transcription more than 33-fold, while co-expression of GRO abrogates activation to only 20-fold.

This co-repressor function of GRO may be mediated by interactions with SO through the previously characterized engrailed homology 1 (eh1) domains within the SIX domain (Kobayashi et al., 2001), or may be mediated through an eh1 domain found within the EYA domain (Ze'ev Paroush, personal communication). In order to address this question, we performed co-immunoprecipitation (CO-IP) to look at direct interactions between GRO and EYA or SO. Strikingly, we found that GRO can CO-IP with SO alone, but not in the presence of EYA (Figure 6B, lanes 5 and 4). This is not due to competition with EYA for GRO binding, as IP of EYA cannot CO-IP GRO (Figure 6B, lane 6). We therefore propose an additional negative regulatory mechanism for EYA-SO targets, whereby in the absence of high levels of EYA, GRO interactions with SO leads to repression and downregulation of target genes. Thus SO may function both as a transcriptional activator and repressor dependant upon the context specific expression levels of particular co-factors.

Discussion

The RD gene network encodes proteins that operate in multiple contexts to effect differentiation of various cell types. Inputs from extracellular signaling pathways, such as the RAS/MAPK cascade, may provide instructive, context-dependent cues that regulate the expression and/or activity of RD gene family members. We have shown that *Drosophila* EYA is a potent transactivator, either on a heterologous promoter or in conjunction with SO, and that that this activity maps to an internal P/S/T-rich region encompassing the ED2 and MAPK consensus sites. This activity is negatively regulated by the EYA domain, and positively

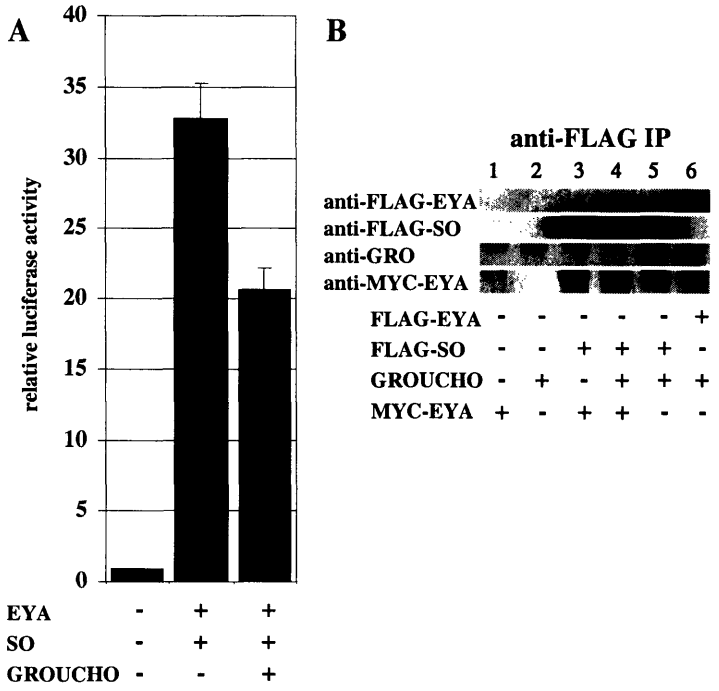

Figure 6: The EYA-SO transcription factor is negatively regulated by interactions with GROUCHO

(A) When co-expressed with EYA and SO, GRO is a repressor of the EYA-SO transcription factor.

(B) Lanes 1 and 2 show that myc-EYA and GRO are not pulled down by anti-Flag beads. In lane 3, IP of SO can co-IP EYA. Lane 4 shows that IP of SO can co-IP EYA but not GRO, however in lane 5 we see that without EYA, GRO can associate with SO. Thus co-immunoprecipitation of GRO with SO is disrupted by cotransfection of EYA. This result does not seem to be due to direct competition between EYA and SO for GRO, as IP of EYA cannot co-IP GRO. All proteins were expressed at similar levels in crude cell lysates (data not shown).

modulated by phosphorylation, likely through RAS/MAPK signaling. We also provide evidence for direct EYA-EYA interactions, and demonstrate that the ED2 may be critical in this context. Together our results suggest a complex cooperation and interplay among the distinct structural motifs of EYA that reflects the importance of proper regulation of the RD gene network.

Our transcription assay results correlate well with those obtained in mammalian cell culture studies of murine EYA homologs, mEYA1-4, which showed that their N-termini can function as transactivators on a heterologous promoter (Xu et al., 1997a). Our functional dissection of *Drosophila* EYA enables us to propose a role for a second, and previously uncharacterized, conserved domain in EYA, the EYA Domain 2 (ED2), in mediating EYA transactivation potential. The P/S/T rich region surrounding the ED2, which includes two MAPK phosphorylation consensus sites, is absolutely necessary for EYA transactivation, and both the ED2 and the P/S/T rich region are essential for EYA function in vivo.

Ectopic expression of EYA is associated with a wide range of deleterious phenotypes (Hsiao et al., 2001), suggesting that EYA activity must be precisely regulated to ensure appropriate growth and development. Our observation that the conserved EYA Domain functions as an autoregulatory inhibitor of EYA transactivation potential suggests that regulation of and by this domain is critical for proper EYA function. Relief of this inhibition in vivo may require co-factor binding or protein modification. Alternatively, the inhibition mediated by the EYA domain might be dependent on interactions with an unidentified negative regulator of EYA. In this context, it would be interesting to ask whether any of the BOR alleles that map to the Eya Domain (Ozaki et al., 2002) affect the transactivation potential of human EYA1.

Another negative regulatory component of the EYA-SO transcription factor arises from our finding that striking repression is achieved in the presence of GROUCHO. Furthermore, we

provide evidence that SO-GRO interactions are disrupted by EYA, providing an intriguing model for SO target gene regulation. As EYA and SO are not entirely co-expressed and GRO is widely expressed (Knust et al., 1987), a SO-GRO complex may provide tight regulation of EYA-SO targets, functioning as an OFF switch in the absence of EYA. This may explain the lack of ectopic eye induction seen upon overexpression of SO alone (Pignoni et al., 1997) compared to EYA alone (Bonini et al., 1997), and a mechanism for the cooperativitity observed when EYA and SO are coexpressed (Pignoni et al., 1997), as EYA is necessary to overcome SO-GRO mediated repression.

Our finding that DAC does not interact with EYA or SO in S2-2H assays is surprising but does not preclude their interaction in vivo. It is possible that the EYA-DAC interaction requires cofactors or modifications not made in *Drosophila* S2 cell culture, or that some factor present in S2 cells inhibits the interaction. As well, the use of Gal4DBD and Gal4AD fusions may in some way disrupt an EYA-DAC interaction. It will be interesting to ask whether cotransfection of one known mammalian cofactor, CREB binding protein (Ikeda et al., 2002), can nucleate an EYA-DAC interaction in S2 cells. Alternatively, EYA may indirectly associate with DAC in the context of an as yet uncharacterized macromolecular complex that may vary according to the particular transcriptional target being regulated.

The observation that EYA interacts with itself reveals yet another potential mechanism for complex and reiterative interactions within the RD gene network. This interaction appears to be mediated by the N-terminal half of EYA, a region that we have found necessary for full transactivation potential. Furthermore, we find that the ED2 potentiates EYA-EYA interactions. As the EYA-EYA interaction maps to regions of EYA necessary for transactivation, we propose that homotypic interactions may contribute to EYA function as a transcription factor in vivo.

Determination of cell fate is dependent on both the presence of a particular complement of transcription factors and the appropriate activation state of these factors. Here we provide evidence that while RAS/MAPK activation is not necessary for Eya transactivation potential, it can potentiate EYA mediated transactivation. This leads to an intriguing mechanism for modulation of the EYA-SO transcription factor, whereby in the absence of RAS/MAPK signaling, it is competent to activate some transcription, but in the presence of signal, this function is potentiated such that target genes may be activated to higher levels. An alternative but not mutually exclusive role for RAS/MAPK activation of EYA may be to allow the higher activation potential of EYA to overcome negative regulation of specific target genes.

Activation of EYA by RAS/MAPK signaling provides a direct point of crosstalk between a signal transduction module and the RD gene network. Our results suggest that RAS/MAPK signaling may regulate multiple aspects of RD network function. In the context of Gal4DBD-Eya fusions acting on *UAS-luciferase*, RAS signaling clearly increases transactivation activity. Surprisingly, we found that mutation of the MAPK phosphoacceptor sites in this fusion protein to either Alanine, to prevent phosphorylation, or a negatively charged residue, to mimic phosphorylation, result in higher transactivation levels. We believe that these results may be due the nature of this assay, which uses chimeric and truncated Gal4DBD-EYA ΔED fusions, as we do not observe the same effect in the context of full length EYA working with SO to promote transcription. Rather, in the context of an EYA-SO complex acting on *ARE-luciferase,* RAS^{V12} does not affect transcription yet there is a consistently strong increase in activation when the phosphomimetic $EYA^{S\text{-}D}$ is used. One possible explanation for the lack of RAS responsiveness is that RAS signaling could simultaneously upregulate EYA but downregulate SO. This would provide a mechanism for fine-tuned transcriptional regulation, whereby RAS signaling can

activate the EYA-SO transcription factor through phosphorylation of EYA but negatively regulates SO to prevent sustained high levels of activation. Such dual and conflicting inputs by the RAS/MAPK pathway are consistent with previous genetic observations. Specifically, our previous work has demonstrated a positive role for the pathway with respect to RD network function using EYA as a point of cross-talk, whereas work by others has implicated the RAS pathway as antagonizing RD gene function, although in this case the molecular mechanisms underlying the inhibitory regulation is unknown (Kumar and Moses, 2001). Thus it remains to be seen how RAS/MAPK signaling regulates SO or other members of the RD gene network, or if this direct interaction is unique to EYA.

In addition to RAS/MAPK signaling, Notch, Hedgehog, Wingless, and TGFβ/DPP signaling all play important roles in eye development (Treisman and Heberlein, 1998). The integration of multiple signaling pathway inputs with our existing knowledge of RD gene network transcriptional regulatory loops suggests a mechanism for unique specification of multiple cell fates in the eye. Our work provides evidence that EYA is a crucial modulator of its own activity, through autoinhibition and homo-typic interactions. We also show evidence that the RAS/MAPK pathway directly enhances the transactivation potential of EYA, and that the co-repressor GROUCHO inhibits EYA-SO mediated transcription. It will be important to discover whether other signaling pathways interact directly with RD gene network proteins, and how such inputs effect the expression of distinct cadres of target genes, thereby establishing and/or reinforcing unique cell fates.

Acknowledgements

We thank S. Artavanis-Tsakonas for GRO monoclonal antibody, Z. Paroush for *gro* cDNA, G. Mardon for *so* and *dac* plasmids and R. Fehon and T. Orr-Weaver for helpful comments on the manuscript. We thank all Rebay Lab members for their valuable advice on this project and manuscript. S.J.S. is a Howard Hughes Medical Institute Predoctoral Fellow. I.R. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences and is a Rita Allen Foundation Scholar. This work was supported in part by National Institutes of Health Grant RO1 EY-12549.

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Chapter 3

Novel function for the transcription factor Eyes absent as a protein tyrosine phosphatase

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S.J.S. performed analysis of HAD family members, performed transcription assays, and developed and performed kinetic phosphatase assays with T.T.

This Chapter was previously published as a *Letter to Nature*:

Tootle, T. L., Silver, S. J., Davies, E. L., Newman, V., Latek, R. R., Mills, I. A., Selengut, J. D., Parlikar, B. E., and Rebay, I. (2003). The transcription factor Eyes absent is a protein tyrosine phosphatase. Nature *426*, 299-302.

Abstract

Post-translational modifications provide sensitive and flexible mechanisms to dynamically modulate protein function in response to specific signaling inputs(Hunter, 2000). In the case of transcription factors, changes in phosphorylation state can influence protein stability, conformation, subcellular localization, interactions with cofactors, transactivation potential and transcriptional output (Hunter, 2000). Here we show that the evolutionarily conserved transcription factor Eyes absent (Treisman, 1999; Wawersik and Maas, 2000) belongs to the phosphatase subgroup of the haloacid dehalogenase superfamily (Collet et al., 1998; Thaller et al., 1998) and propose a novel function for it as a non-thiol based protein tyrosine phosphatase. In vitro assays demonstrate that Eyes absent has intrinsic phosphatase activity that is blocked by mutations altering the active site. Experiments performed in Drosophila cultured cells and in vitro indicate that Eyes absent has protein tyrosine phosphatase capability and may act autocatalytically to dephosphorylate itself. Confirming the biological significance of this function, mutations that disrupt the phosphatase active site severely compromise Eyes absent's ability to promote eye specification and development in Drosophila. Given the functional importance of phosphorylation-dependent modulation of transcription factor activity, our evidence of a nuclear transcriptional coactivator with intrinsic phosphatase activity suggests exciting new mechanisms for fine-tuning transcriptional regulation.

Introduction

The transcriptional coactivator Eyes absent (EYA) is a member of an evolutionarily conserved set of nuclear transcription factors and cofactors collectively termed the retinal determination (RD) gene network (Bonini et al., 1997; Treisman, 1999; Wawersik and Maas, 2000). While RD network members are perhaps best known for their roles in eye specification, redeployment of these genes, either individually or as a network, contributes to a diverse array of essential developmental processes in all metazoans (Treisman, 1999; Wawersik and Maas, 2000). EYA family members are defined by a conserved ~275 amino acid motif, referred to as the EYA domain (ED), that has been shown to bind two other RD members, Sine oculis (SO) and Dachshund (DAC) (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997). Together, EYA and SO form a potent transcriptional activator (Silver et al., 2003), while the mechanistic implications of EYA-DAC interactions are less clear (Ikeda et al., 2002; Kim et al., 2002). Emphasizing the functional conservation among EYA homologs, mammalian EYA transgenes can rescue the "eyeless" phenotype of Drosophila *eya* mutations (Bonini et al., 1997; Bui et al., 2000).

Results and Discussion

We have explored a new function for EYA's C-terminal ED that is suggested by protein motif searches and structural modeling studies. These investigations place EYA within the phosphatase subgroup of the Haloacid dehalogenase (HAD) superfamily (Fig. 1a and Supplementary Fig. S1a). HAD family members constitute a diverse collection of enzymes found in all organisms ranging from bacteria and archaea to humans, that includes dehalogenases,

ATPases, phosphonatases, phosphomutases, epoxy hydrolases and a growing number of magnesium-dependent phosphatases (Collet et al., 1999; Collet et al., 1998; Thaller et al., 1998). Understanding of the in vivo function of HAD family phosphatases remains extremely limited, particularly in eukaryotic systems.

X-ray crystallography combined with mutagenesis studies of several HAD family proteins has revealed a conserved α/β-hydrolase fold that unites three non-contiguous sequence motifs to form the catalytic core of the enzyme (Aravind et al., 1998; Collet et al., 1999; Selengut, 2001). Structural modeling studies predict that the ED will form a HAD α/β-hydrolase-like fold (Fig. 1b). Five conserved residues brought together by this tripartite configuration surround the active site and are essential for catalysis (Aravind et al., 1998; Collet et al., 1999; Selengut, 2001). These residues are strikingly conserved in the ED of all EYA proteins (Fig. 1a and Supplementary Fig. S1b). In Motif 1 (DXDX(T/V)) the invariant first aspartic acid serves as the nucleophile in all HAD family proteins and likely forms a phospho-aspartate intermediate (Cho et al., 2001; Ridder and Dijkstra, 1999). The second aspartic acid distinguishes the phosphatase/phosphohydrolase subgroup from other branches of the HAD superfamily (Collet et al., 1998; Selengut, 2001; Thaller et al., 1998) and is strictly conserved in all EYA homologs. Motif 2 contains an essential Serine/Threonine at the end of the β-strand and Motif 3 contributes at least three required residues, a lysine and two aspartic acids, the second of which has undergone a conservative substitution to glutamic acid in EYA proteins. Requirement for the two acidic residues within Motif 3 appears strictest within the phosphatase/ phosphohydrolase branch of the HAD superfamily (Thaller et al., 1998). The high degree of conservation of this catalytic quintet (D, S/T, K, D, E) in invertebrate, vertebrate and plant EYA homologs suggests that EYA belongs to the phosphatase subgroup of the HAD superfamily.

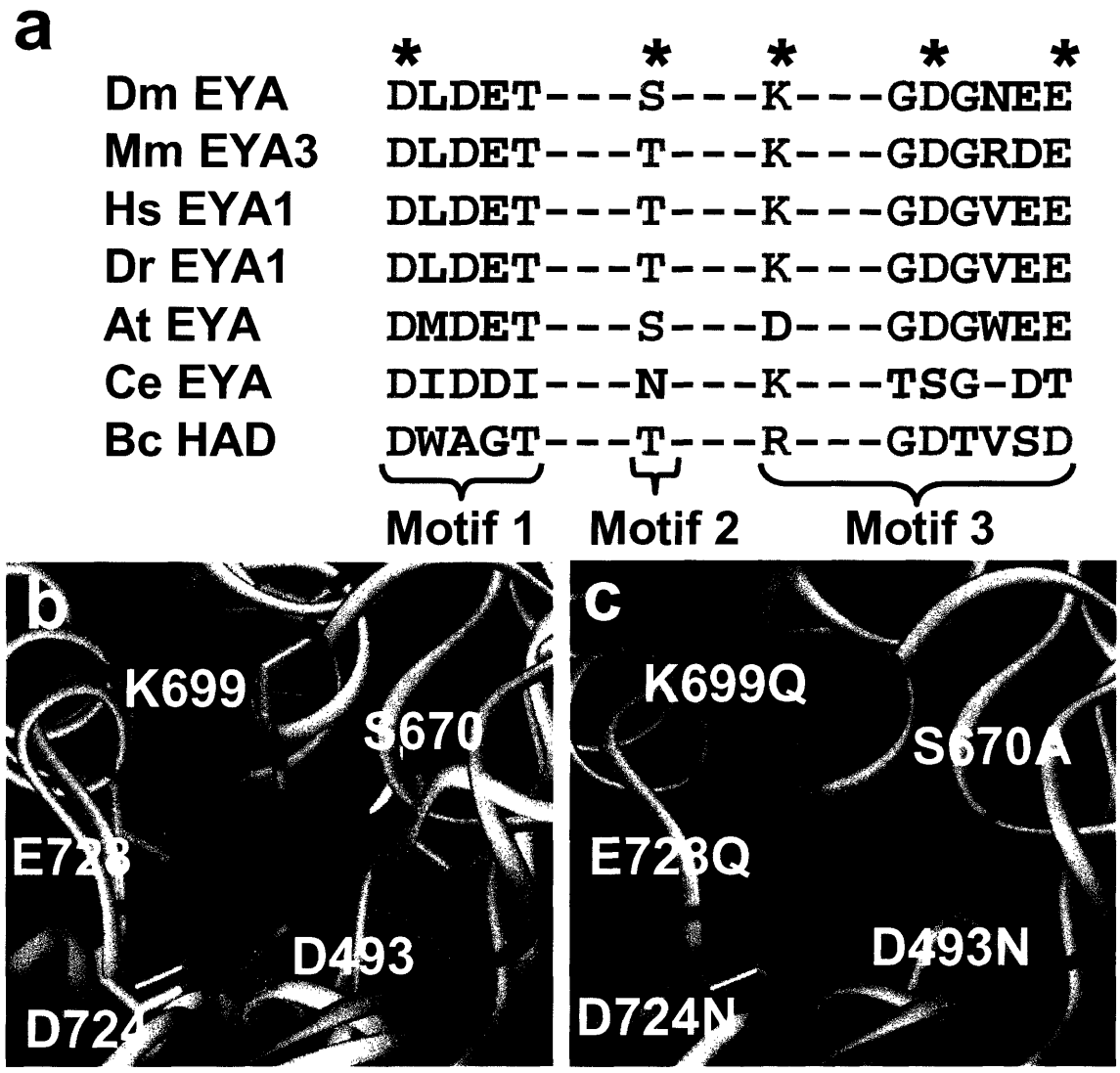


Figure 1: EYA is a member of the phosphatase subgroup of the HAD superfamily.
a, The non-contiguous sequences comprising the HAD Motifs 1, 2, and 3. Pink residues define the HAD motif, with those mutated in this study marked with a "*". Blue residues are most strongly conserved among the phosphatase subgroup of the HAD superfamily. Green residues are highly conserved in both ATPases and phosphatases (Aravind and Koonin, 1998; Collet et al., 1999).
b, Structural modeling studies predict a similar active site configuration for Drosophila EYA and other HAD proteins. The HAD template backbone is identified with a white ribbon and the EYA model backbone is rendered with a cyan ribbon. Key active site residues are highlighted as sticks, either white for the HAD or yellow for EYA.
c, Superimposition of mutant DmEYAHAD residues on the DmEYA model. Alignment of the substitutions (in magenta) and their wild type counterparts (in yellow) is shown.

To investigate whether EYA has intrinsic phosphatase activity, we tested the ability of recombinant GST-tagged ED fusions to dephosphorylate the synthetic substrate para-nitrophenyl phosphate (pNPP). Using a murine homolog, we demonstrate that EYA can function as a phosphatase (Fig. 2). Mutations altering the presumptive HAD active site residues severely compromise activity (Fig. 2a; see Supplementary Information for details). Sensitivity to the tyrosine phosphatase inhibitor vanadate, but not to inhibitors of serine/threonine phosphatases (Fig. 2a and b), and a requirement for Mg^{++} are consistent with EYA being a HAD family phosphatase (Selengut and Levine, 2000). We also tested recombinant Drosophila EYA in these assays, and although its activity is significantly lower, it remains Mg^{++}-dependent and vanadate sensitive (Fig. 2c). While the most likely explanation for the weak in vitro activity of the fly ED is that we have not identified appropriate conditions for purifying properly folded and active protein, we cannot rule out the possibility that Drosophila EYA, although it retains all the conserved residues comprising the HAD motif (Fig. 1), may have only limited ability to function as a phosphatase. However, the fact that the mouse EYA isoform used in our in vitro assays is able to substitute for Drosophila EYA in vivo (Bui et al., 2000), when considered together with the results of the in vivo experiments described below, leads us to propose that EYA proteins possess a conserved phosphatase function.

To investigate whether EYA might have protein phosphatase capability, something that has not been definitively demonstrated for any other HAD family protein (Selengut, 2001), several phosphotyrosine or phosphothreonine containing synthetic peptides were tested as substrates. We find that EYA exhibits robust activity toward one of the tyrosyl phosphorylated peptides, with a Km significantly lower than that measured using pNPP as a substrate (Fig. 2d).

Figure 2: EYA exhibits phosphatase activity in vitro.

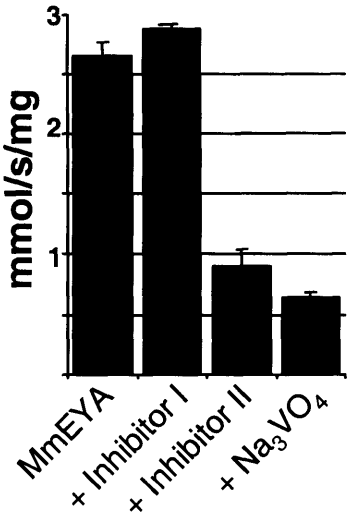
a, Kinetics for mouse EYA3 GST-ED fusion proteins (GST-MmEYA). D246N, T420A, K449Q, D474N and E478Q are mutations analogous to the D493N, S670A, K699Q, D724N and E728Q described for Drosophila EYA. For those mutant enzymes whose activity was too low to be measured, >>> indicates a Km significantly higher and an efficiency (kcat/Km) significantly lower than that measured for D246N.**b,** Like the HAD family phosphatase MDP1 (Selengut and Levine, 2000), phosphatase activity of MmEYA is blocked by tyrosine phosphatase inhibitors (Inhibitor II and Na3VO4) but not serine/threonine phosphatase inhibitors (Inhibitor I).**c,** Phosphatase activity of Drosophila EYA (DmEYA), although significantly weaker than that obtained with MmEYA, is also blocked by addition of the tyrosine phosphatase inhibitor Na3VO4.**d,** Kinetics for mouse EYA3 GST-ED fusion proteins (GST-MmEYA) with respect to the tyrosyl phosphorylated peptide substrate I(pY)GEF.

Figure 2

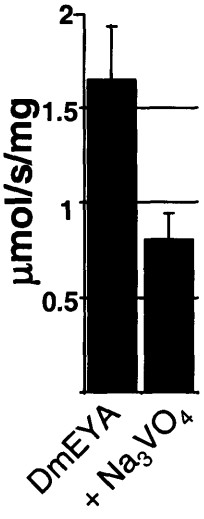
a

GST-MmEYA	Km (mM)	kcat (min^{-1})	kcat/Km ($M^{-1}min^{-1}$)
Mm EYA	93	0.017	0.18
+ Na_3VO_4	190	0.011	0.058
D246N	680	0.0012	0.0018
T420A	290	0.010	0.034
K449Q	590	0.021	0.035
D474N	66	0.019	0.29
E478Q	>>>	<<<	<<<
D246N + D724N	>>>	<<<	<<<

b



c



d

GST-MmEYA	Km (µM)	kcat (min^{-1})	kcat/Km (M^{-1} min^{-1})
Mm EYA	99	2.7×10^{-4}	2.71
D246N	560	9.0×10^{-5}	0.16

No measurable activity was detected with the phosphothreonine or other phosphotyrosine containing peptides (data not shown; see Methods for details). These results demonstrate that EYA has protein tyrosine phosphatase (PTP) capability, although they do not rule the possibility that EYA could dephosphorylate other substrates as well. The fact that not all tyrosyl phosphorylated peptides were hydrolyzed suggests EYA has specific sequence preferences with respect to its putative protein substrates. Because HAD family phosphatases employ a catalytic aspartate (Cho et al., 2001; Ridder and Dijkstra, 1999) as the nucleophile rather than the cysteine residue used by standard PTPs (Andersen et al., 2001), these results suggest EYA is the founding member of a new class of non-thiol-based PTPs.

We have used the genetically tractable Drosophila system to investigate the physiological relevance of EYA's putative PTP activity. For these experiments, site-directed mutagenesis was used to target the five HAD active site residues in Drosophila EYA (Fig. 1c). Five single and four double mutant combinations were generated and will be referred to collectively as the EYA^{HAD} mutants. These EYA^{HAD} mutants were first tested in transfected Drosophila S2 cultured cells where immunostaining and western blotting analyses revealed no apparent changes in subcellular localization (data not shown) or expression levels (Supplementary Fig. S2a) relative to EYA^{WT}.

EYA, like most other RD genes, induces formation of eye tissue outside the normal eye field when ectopically expressed (Bonini et al., 1997; Hsiao et al., 2001; Treisman, 1999; Wawersik and Maas, 2000). Scoring the percentage of flies exhibiting ectopic eye formation provides a sensitive measurement of EYA activity (Hsiao et al., 2001). To determine whether the HAD active site mutants compromise EYA's ectopic eye induction potential, we generated transgenic lines carrying full-length EYA^{HAD} mutant expression constructs. All EYA^{HAD} mutants

exhibit strikingly reduced ectopic eye induction relative to EYA^{WT} (Fig. 3a). Protein expression levels from the EYA^{HAD} transgenes were comparable to those from EYA^{WT} lines (Supplementary Fig. S2b), indicating that the reduction in ectopic eye inducing potential reflects a change in protein activity rather than reduced expression. Comparable reductions in EYA activity were also observed with EYA^{HAD} transgenes in which two of the five HAD active site residues were mutated simultaneously (data not shown).

Because the HAD motif active site mutants compromise EYA's ability to induce ectopic eye formation, we asked whether an intact HAD motif is required for normal EYA function during eye development. We compared the ability of EYA^{WT} versus EYA^{HAD} transgenes to complement the eye-specific loss-of-function *eya*^{2} allele. *eya*^{2} homozygous mutant flies exhibit a completely penetrant "eyeless" phenotype, in which the entire eye is missing (Fig. 3c, d). For these experiments we define "rescue" as the ability of a given transgene to produce recognizable eye tissue within the normal eye field of an adult fly. We also estimate the size of the rescued eye tissue relative to that of a wild type eye in order to compare the extent of rescue.

Expression of EYA^{WT} transgenes rescues the *eya*^{2} "eyeless" phenotype with complete penetrance (Fig. 3b, e) in both eyes of each individual fly (data not shown). In striking contrast, all EYA^{HAD} mutant transgenes exhibit a significantly reduced frequency and extent of rescue, with rescue usually occurring in only one of the two eye fields of an individual (Fig. 3b, f). For all EYA^{HAD} transgenes tested, even in cases where rescue efficiency is only two to three fold lower than that of EYA^{WT}, the size of the rescued eye tissue is always significantly (5-10 fold) reduced relative to that obtained with EYA^{WT} lines (Fig. 3b, e, f). Western blot analyses of eye imaginal discs again ruled out the possibility that reduced protein expression might be responsible for this result (Supplementary Fig. S2c). In combination with the ectopic eye

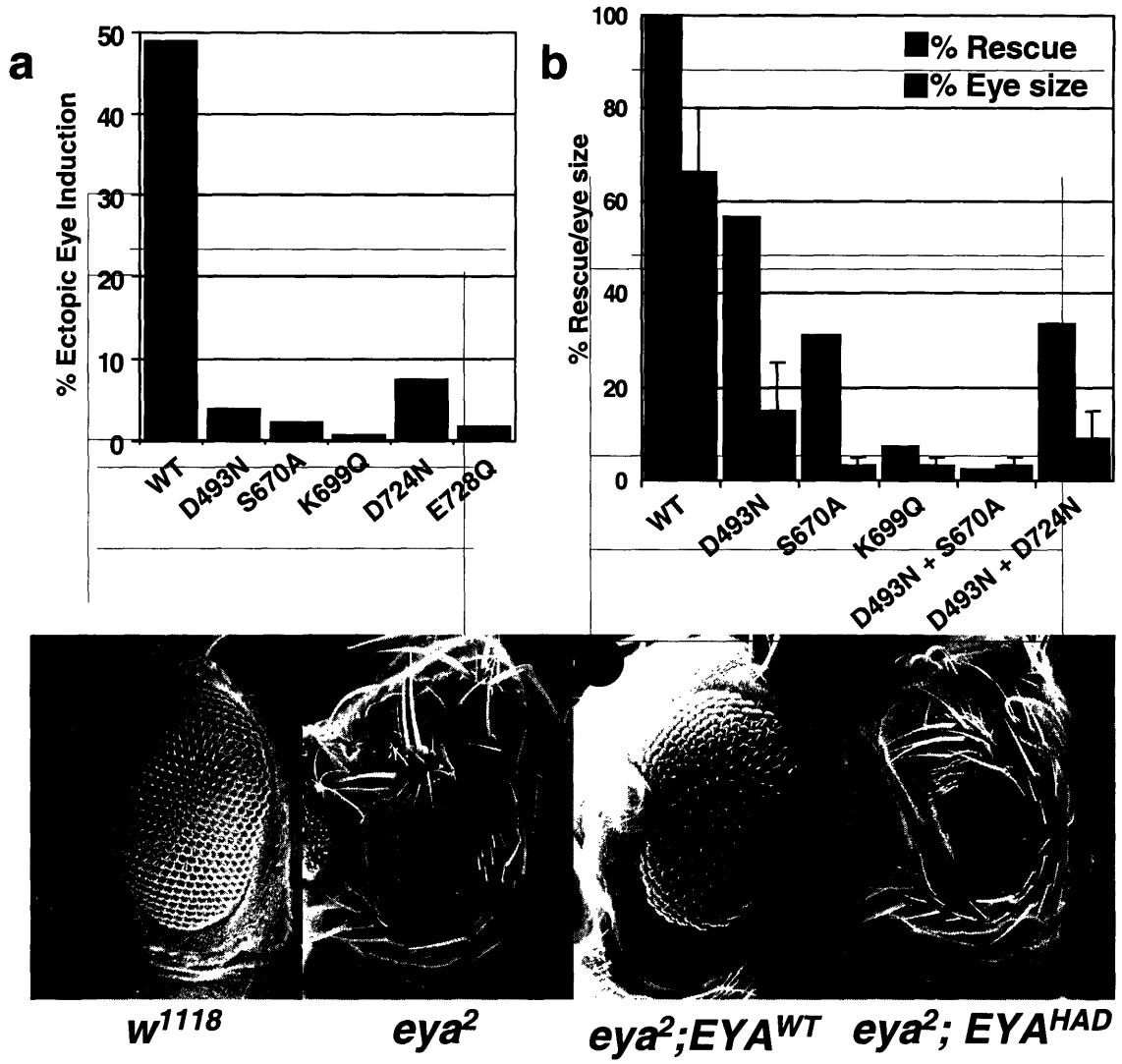


Figure 3: EYA^{HAD} mutants exhibit severely reduced activity relative to EYA^{WT} in ectopic eye induction and genetic rescue assays.

a, The frequency of ectopic eye induction associated with expression of EYA transgenes was calculated from multiple independent transgenic lines: EYA^{WT}, 2465 flies from 8 lines (Hsiao et al., 2001); EYA^{D493N}, 1502 flies from 5 lines; EYA^{S670A}, 955 flies from 3 lines; EYA^{K699Q}, 953 flies from 3 lines; EYA^{D724N}, 265 flies from a single line; EYA^{E728Q} - 1239 flies from 4 lines.

b, The percentage of eyes from flies of the genotype *eya2;UAS-EYA/dpp-GAL4* exhibiting rescue of the *eya2* "eyeless" phenotype (black bars) and average size of the rescued tissue relative to a wild type eye (grey bars) is plotted. Data derives from the following lines: EYA^{WT}, 155 flies from two independent lines; EYA^{D493N}, 124 flies from a single line; EYA^{S670A}, 281 flies from a single line; EYA^{K699Q}, 176 flies from a single line; EYA$^{D493N+S670A}$, 209 flies from two independent lines; EYA$^{D493N+D724N}$, 151 flies from two independent lines.

c-f, Scanning electron micrographs of adult eyes. **c,** *w1118*. **d,** *eya2*. **e,** *eya2;UAS-EYA^{WT}/dpp-GAL4*. **f,** *eya2;UAS-EYA^{HAD}/dpp-GAL4*, arrow points to a small patch of rescued eye tissue.

induction assay data, the results of these rescue experiments argue strongly that EYA's activity as a putative HAD family phosphatase is required to promote normal eye development in Drosophila.

Because the region of the ED that binds to the RD gene network protein SO (Bui et al., 2000; Pignoni et al., 1997) partially overlaps with Motif 1 of the HAD domain (Supplementary Fig. S1b), we checked whether the EYA^{HAD} missense mutations compromise EYA's ability to interact productively with SO. EYA and SO interact to form a potent transcriptional activator required for eye specification, in which SO contributes the DNA binding domain and EYA provides the transactivation potential (Ikeda et al., 2002; Pignoni et al., 1997; Silver et al., 2003). Using a transcription assay in Drosophila S2 cultured cells (Silver et al., 2003), we find that the ability of EYA^{HAD} mutant proteins to synergize with SO to activate transcription of a reporter gene is comparable to that of EYA^{WT} (Fig. 4 and Supplementary Fig. S3). Although we cannot rule out the formal possibility that in vivo the EYA^{HAD} mutations disrupt interactions with other proteins rather than blocking phosphatase activity, the finding that mutational disruption of the HAD motif active site does not abrogate EYA's ability to function as a transcriptional coactivator in conjunction with SO leads us to propose that EYA proteins have two essential functions: a previously described role as a transcription factor and a novel role as a protein tyrosine phosphatase.

To investigate further EYA's intrinsic PTP capability with respect to physiologically relevant substrate candidates, we exploited our finding that EYA can be tyrosine phosphorylated in Drosophila S2 cells (Fig. 5a; see Supplementary Information for discussion) by affinity purifying full-length EYA from these cells and using it as a protein substrate in an in vitro phosphatase reaction. Because the phosphotyrosine signal associated with the EYA^{HAD} mutant

proteins was consistently elevated relative to EYA^{WT} (Fig. 5a; see Supplementary Information for discussion), the EYA^{HAD} protein was used as the substrate. We find that incubation of EYA^{HAD} protein with recombinant murine GST-ED fusion protein strongly reduces the phosphotyrosine signal (Fig. 5b). HAD active site mutants that exhibit impaired activity both in vitro and in vivo (Figs. 2 and 3) also have severely reduced activity in this assay (Fig. 5b). These results demonstrate that EYA has PTP capability with respect to a full-length endogenous protein substrate and that such activity depends on an intact HAD motif. Although we do not yet understand the physiological relevance of tyrosine phosphorylation and dephosphorylation of EYA, the results of these experiments (Fig. 5), together with our previous demonstration that EYA is able to self-associate (Silver et al., 2003), suggest that EYA may act autocatalytically to dephosphorylate itself.

In conclusion, we propose that EYA is both the founding member of a novel class of non-thiol based PTPs and to our knowledge, the first example of a transcription factor with intrinsic phosphatase activity. Further work will be required to understand how tyrosine phosphorylation and dephosphorylation regulates EYA function in vivo, and what substrates, potentially including EYA itself, may be regulated by its PTP activity. Elucidation of the biochemical regulatory mechanisms that coordinate EYA's dual functions as transactivator and phosphatase during eye specification will provide new insights into the function of the RD gene network, and more generally a new paradigm for transcriptional regulatory strategies. Although preliminary analyses have not identified other HAD-motif containing proteins that are annotated as transcriptional regulators (R. R. L. and I. R., unpublished observation), it seems likely that dual function mechanisms analogous to that we propose for EYA may prove to be a general strategy for fine-tuning transcriptional output, particularly in highly regulated developmental systems.

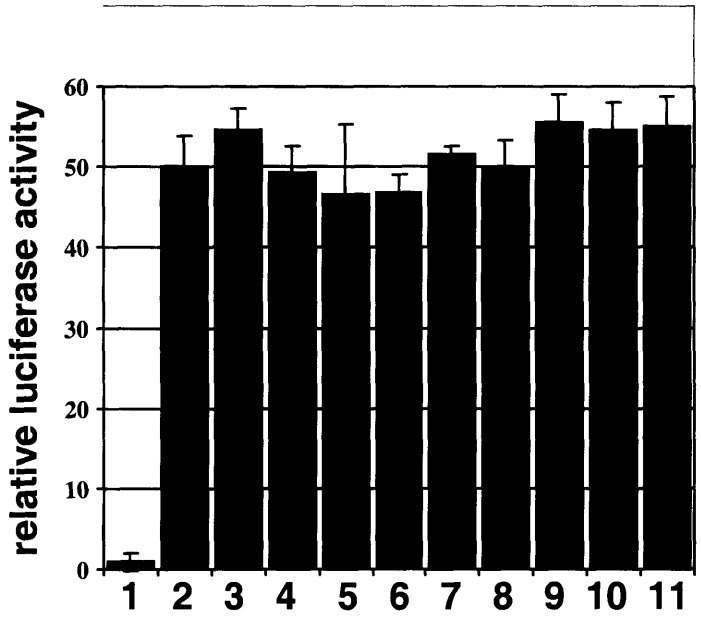


Figure 4: EYAHAD mutations do not disrupt EYA's role as a transcriptional coactivator in conjunction with Sine oculis.

The Drosophila cell culture based transcription assays were performed as recently described (Silver et al., 2003). Lanes: 1, Are-Luciferase; 2, WT; 3, D493N; 3, S670A; 4, K699Q; 5, D724N; 5, E728Q; 6, D493N + S670A; 7, D493N + K699Q; D493N + D724N; D493N + E728Q. See Supplementary Figure S3 for further details.

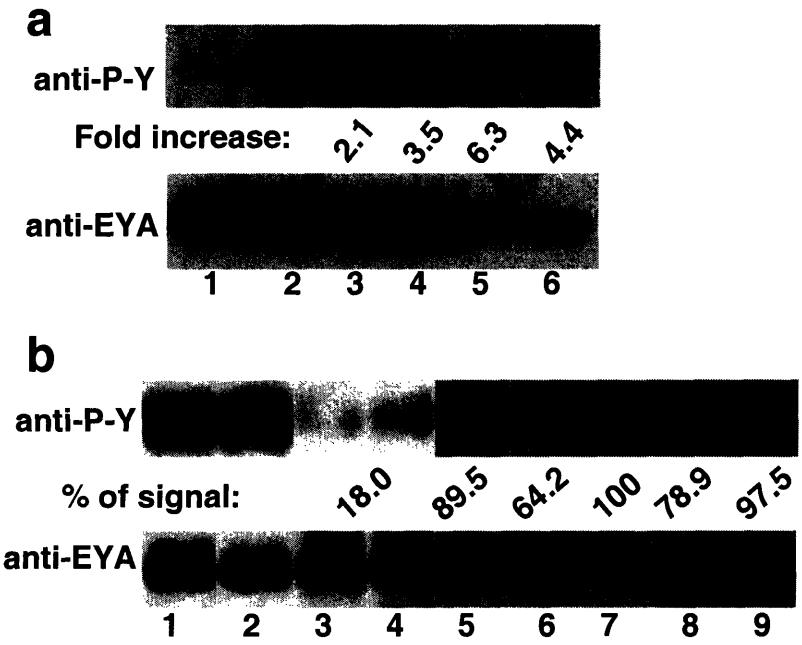


Figure 5: EYA has protein tyrosine phosphatase capability.

Top panels show immunoblots probed with anti-phosphotyrosine (anti-P-Y); bottom panels show immunoblots of the same samples probed with anti-Flag to detect EYA (anti-EYA).

a, Lanes: 1-2, independent transfections of EYA^{WT}; 3, $EYA^{D493N + S670A}$; 4,$EYA^{D493N + K699Q}$; 5, $EYA^{D493N + D724N}$; 6, $EYA^{D493N + E728Q}$. Fold increase in P-Y levels for the EYA^{HAD} mutants relative to an average of the P-Y signal in the two EYA^{WT} lanes, and corrected relative to the strength of the anti-EYA signal, is indicated underneath the anti-P-Y blot.

b, Dephosphorylation of Drosophila EYA by recombinant GST-ED. Full length tyrosine phosphorylated Drosophila $EYA^{D493N + D724N}$ (all lanes) was immunoprecipitated and incubated with recombinant mouse GST-ED, either wild type (WT) or HAD mutant variants (Lanes: 1-2, control; 3-4, WT; 5, D246N, 6, T420A; 7, K449Q; 8, D474N; 9, E478Q). The percentage of anti-P-Y signal on $EYA^{D493N + D724N}$ relative to controls and corrected for relative protein levels is indicated. Numbers shown are an average from two independent experiments for each GST-ED tested; results from only one of the two experiments are shown for the GST-ED HAD mutants. Samples were run on the same gel to allow quantitative comparisons.

Acknowledgements We thank M. Voas, D. Doroquez, L. Doyon and B. Chaffee for technical assistance, J. Flynn, S. Neher and S. Flaugh for advice, reagents and technical help, D. Maas for murine EYA cDNAs, the entire Rebay lab for helpful advice and discussions, F. Pignoni for the *eya*^{2} stocks used in the rescue assay, and R. Hegde for sharing unpublished information. We are grateful for advice and encouragement from R. Fehon, G. Fink, T. Orr-Weaver, S. Shenolikar, F. Solomon and J. York. This manuscript was improved by comments from R. Fehon, T. Orr-Weaver, F. Solomon and J. York. T. L. T., S. J. S., I. A. M. and B. E. W. P. are grateful for support from the Ludwig Foundation, the Howard Hughes Medical Institution, the Packard Foundation and the National Science Foundation, respectively. I.R. acknowledges financial support from the National Eye Institute and the Burroughs Wellcome Fund.

Methods

Bioinformatics

The phylogenetic tree of a number of different hydrolase family members (PFAM 00702.6) was generated by the neighbor-joining method using ClustalX(Thompson et al., 1997). EYA protein sequences from Drosophila (Dm, gi:17737399), mouse (Mm, gi:6753794), human (Hs, gi:3183005), zebrafish (Dr, gi:18858653), arabidopsis (At, gi:21593200) and nematode (Ce, gi:3875091) were aligned with the Bacillus cereus phosphonoacetaldehyde hydrolase (Bc, gi:10835405) HAD protein using ClustalX (Thompson et al., 1997). For the structural modeling analyses, Prospect Pro (Xu and Xu, 2000) was used to thread the Drosophila EYA ED sequence (DmEYA) against structures in the protein data bank. From these comparisons, Phosphonoacetaldehyde hydrolase (PDB:1FEZ) was selected as a suitable modeling template. DmEYA and the template initially were aligned according to threading results and then modified by anchoring several phosphonatase active site residues to their synonymous positions within DmEYA. The structural model of DmEYA was created with Modeler (Sali and Blundell, 1993) employing the alignment and using the coordinates of the Phosphonoacetaldehyde hydrolase structure. The original template, 1FEZ, and the DmEYA model were aligned using CCP4 (Otwinowski, 1993). Key active site positions within the DmEYA model were replaced with the variants described in this study using the Builder module within InsightII (Accelrys ™, 2001).

Phosphatase assays

Phosphatase assays were performed using GST-ED fusion proteins (purification protocol described in Supplementary Information). For enzyme kinetics with the synthetic substrate p-Nitrophenyl Phosphate (pNPP, Sigma), assays were done in triplicatewith six substrate

concentrations over six timepoints. 80ul reactions performed in microfuge tubes at 30°C in 200mM PIPES pH 7.0, 5mM EDTA and 10mM $MgCl_2$ were quenched by addition of 40ul of 10M NaOH. PNP anion was detected at 405 nm (extinction coefficient ε_M= 1.78 x 10^4 /cm M) using a Tecan GENios plate reader. Reactions were normalized to buffer alone controls and the results analyzed by Lineweaver-Burk plot using Microsoft Excel. Synthetic peptide substrates tested were: I(pY)GEF and TSTGPE(pY)EPGENL (Calbiochem); END(pY)INASL, DADE(pY)LIPQQG and RRA(pT)VA (Promega). 50ul reactions were performed at 25°C in 200mM HEPES pH7.0, 10mM MgCl2, 5mM EDTA and quenched with 50µl of Molybdate Dye Solution (Promega). Malachite Green/Ammonium Molybdate-phosphate complex was detected at 595 nm and converted to moles of free phosphate using a phosphate standard curve. Assays with I(pY)GEF were carried out for five substrate concentrations over five timepoints and the results were analyzed as described for pNPP.

Phosphatase Inhibitor Cocktail Sets I and Set II (Calbiochem) were used at 1:50 in pNPP phosphatase assays. Sodium Orthovanadate was used at 4mM final concentration in pNPP phosphatase assays.

Amino terminally flag epitope tagged EYA constructs were subcloned into the copper inducible metallothionein promoter vector. 5 µg of DNA for each construct was transfected into S2 cells as previously described(Tootle et al., 2003). Following published protocols(Cohen et al., 1997; Huyer et al., 1997; Imbert et al., 1994; Ruff et al., 1997; Scanga et al., 2000), cells were treated with 100µM $NaVO_3$, 200µM H_2O_2 for 15 minutes prior to lysis in 100mM NaCl, 50mM Tris, pH 7.5, 2mM EDTA, 2mM EGTA, 1% NP-40, 1mM Na_3VO_4, and one mini-complete protease inhibitor tablet (Roche) per 10 ml. All subsequent solutions include 1mM Na_3VO_4. Clarified lysates were incubated with 25µl of anti-flag M2 agarose affinity gel (Sigma) for 1.5 hours at

4°C. Beads were washed twice in lysis buffer and twice in 10mM Tris, pH 7.5, 150mM NaCl, resuspended in 30µl of 2x SDS sample buffer, boiled and 10µl were loaded per lane. Westerns were performed as previously described(O'Neill et al., 1994) except that blocking and antibody incubations were performed in 1% Casein According to Hammarsten (EM Science). Antibodies: guinea pig anti-Eya 1:16,000, rabbit anti-phosphotyrosine 1:400 (.21 mg/ml, Upstate); HRP-conjugated goat anti-guinea pig and anti-rabbit 1:5000 (Jackson ImmunoResearch). Determination of fold increase in phospho-tyrosine signal relative to EYA protein amounts was performed using NIH Image software; samples analyzed in this way were always run together on the same gel.

To obtain sufficient tyrosine phosphorylated Drosophila EYA to use as a substrate in the in vitro phosphatase assay, a stable cell line expressing flag-tagged $Eya^{D493N + D724N}$ was generated. 500µl of cells were immunoprecipitated for each reaction as described above, except the 1mM Na_3VO_4 was omitted from the wash buffer. The washed immunoprecipitates were incubated in phosphatase assay reaction buffer (as described above but without pNPP), either with GST agarose or with 100µg GST-ED proteins for 1 hour at 30°C, processed for western blotting and analyzed as described above.

Molecular Biology and Genetics

Site-directed mutagenesis, subcloning, generation of transgenics, crosses, ectopic eye scoring, calculation of % ectopic eye induction and scanning electron microscopy were performed as previously described (Hsiao et al., 2001; Tootle et al., 2003). Fly crosses were at 25°C with the exception of the genetic rescue assays which were performed at 20°C.

The cell culture based transcription assays were performed as recently described (Silver et al., 2003). For western blot analyses of protein expression levels, equivalent samples of either S2 cells, embryos or dissected eye-imaginal discs were lysed in SDS-sample buffer, separated by SDS-PAGE and immunoblotted with GP anti-EYA antiserum diluted 1:10,000.

Protein Purification

GST-MmEYA3 ED (aa 237-510 of mouse EYA3) or GST-DmEYA ED (aa 438-760 of Drosophila EYA) fusion proteins were purified from BL21 E. coli cells grown to an OD_{600} of 1.0 and then induced with IPTG for 2.5 hours at 18°C. Cells were harvested by centrifugation, resuspended in 50mM Tris buffer pH 8.0, 1mM EDTA and 100mM NaCl plus protease inhibitor cocktail (1 tablet per 50 mls, Roche) and lysed by three passes through a French Press at 1000 psi. Clarified lysates were rocked overnight at 4°C with glutathione-agarose beads. Beads were washed five times with 50mM HEPES, 300mM NaCl, and left in a final 1:1 slurry. Protein concentrations were estimated by Coomassie Blue staining of an SDS-PAGE gel run with a dilution series of GST-ED and a BSA standard curve. Depending on the protein concentration, appropriate amounts of beads were diluted for the phosphatase assays. To determine Km and enzyme efficiency, GST-ED was eluted by rocking for 20 minutes at 4°C with 300mM reduced glutathione in 50mM Tris pH 7.0, 150mM NaCl. For peptide assays, 10mM reduced glutathione was used in the elutions. Protein concentrations were determined by Bradford Assay.

Phosphatase Inhibitor Cocktail Ingredients

Inhibitor Cocktail Set I (Calbiochem) contains p-Bromotetramisole (inhibits alkaline phosphatase), Oxalate 2.5mM, Cantharidin 500uM (inhibits serine/threonine protein

phosphatase), Microcystin LR 500nM (inhibits serine/threonine protein phosphatase). Inhibitor Cocktail Set II (Calbiochem) contains 200mM Imidazole (inhibits alkaline phosphatase), 100mM Sodium Fluoride (inhibits acid phosphatase), 115 mM Sodium Molybdate (inhibits acid phosphatase), 100mM Sodium Orthovanadate (inhibits protein tyrosine phosphatase and alkaline phosphatase), and 400mM Sodium Tartrate Dihydrate (inhibits acid phosphatase).

Supplementary Information

Discussion

We have found that EYA is a novel protein tyrosine phosphatase member of the HAD superfamily. Specific point mutations shown previously to compromise the HAD active site in other superfamily members severely reduce EYA's phosphatase activity. The in vitro assays, one using pNPP or a tyrosyl phosphorylated peptide as artificial substrates (Fig. 2) and the other using tyrosine phosphorylated Drosophila EYA protein as a physiologically relevant substrate (Fig. 5), and the in vivo assays, ectopic eye induction and genetic rescue (Fig. 3), consistently reveal this trend. However there are subtle activity differences between the various mutants tested, as well as slight variations between the same mutants tested in different assays.

The most notable example results from our analyses of the D493N mutant (D246N in murine EYA3). This mutation alters the aspartic acid residue that serves as nucleophile and forms a phospho-aspartate intermediate in the reaction(Cho et al., 2001; Ridder and Dijkstra, 1999). Given its critical role in catalysis, the prediction is that a mutation in this residue should severely compromise activity. In fact, in all the in vitro assays (Fig. 2 and Fig. 5b) and in the ectopic eye induction assays (Fig. 3a), the activity of D246N is greatly compromised. However in the genetic rescue experiment, although activity is significantly reduced relative to EYA^{WT}, the residual activity is greater than that measured for any of the other HAD mutants (Fig. 3b). While the reason for this is unclear, it presumably reflects the complexity of assaying function in the context of normal eye development, where both functions of EYA as a phosphatase and as a transcriptional cofactor are required. Elucidation of the mechanisms whereby EYA's dual

functions as transcriptional coactivator and phosphatase are coordinated will be required to fully understand such subtle distinctions in activity between the different HAD active site mutants in vivo and how these correlate with their relative activities in vitro.

A second example is the D724N mutant (D474N in murine EYA3), which unlike all the other mutants tested and consistent with its previously characterized role in Mg^{++} binding rather than substrate binding(Cho et al., 2001), does not increase the Km measured in the pNPP assay (Fig. 2). Activity in both the PTP and ectopic eye induction assays is comparable, although perhaps slightly elevated, to that measured for the other HAD active site mutants. In general, interpreting modest differences in activities between different mutants in the various assays must be performed with caution because we do not yet understand the biochemical mechanisms that coordinate and regulate EYA's dual functions as transcription factor and phosphatase.

The Drosophila EYA^{HAD} protein used in the PTP assay (Fig. 5b) was purified from a stably transfected S2 cell line that had been artificially stimulated with pervanadate. Confirming that EYA is tyrosine phosphorylated in the absence of pervanadate, we find that EYA^{HAD} is tyrosine phosphorylated in unstimulated cells, although the signal is reduced relative to that observed in stimulated cells (Fig. S4a). We have been unable to immunoprecipitate sufficient EYA^{WT} protein from transient transfections to detect a signal in the absence of pervanadate and efforts to generate a stable cell line have not yet been successful(Clark et al., 2002). Therefore, to facilitate detection of PTP activity in our assay (Fig. 5b), we elected to purify EYA^{HAD} protein from pervanadate stimulated cells in order to increase the pool of tyrosine phosphorylated protein substrate.

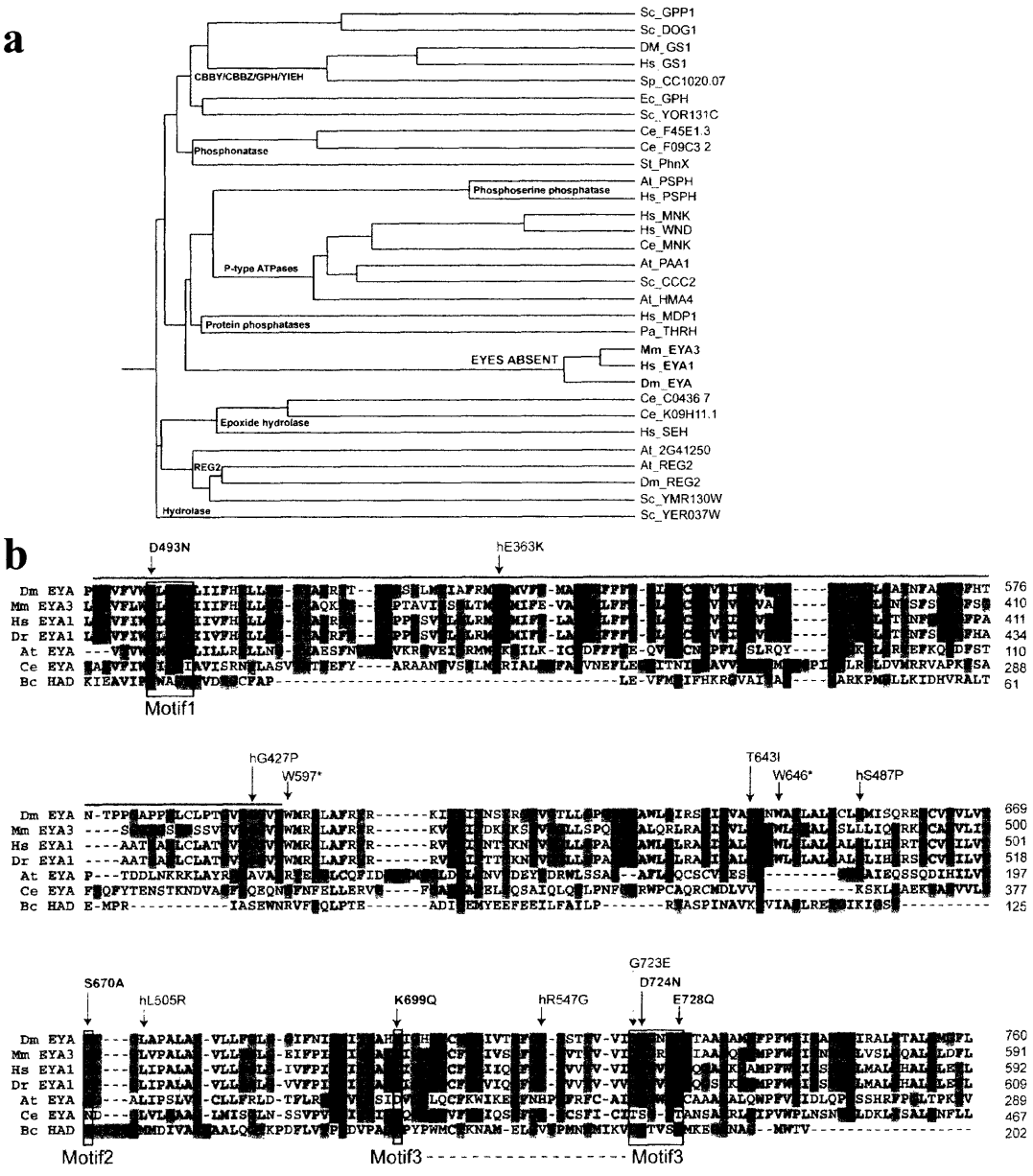
The most likely explanation as to why we require the use of pervanadate to detect tyrosine phosphorylation of EYA in transiently transfected cells (Fig. 5a) is that EYA has

autocatalytic activity and actively dephosphorylates itself. Drosophila S2 cells express significant levels of endogenous wild-type EYA. We have recently shown that EYA has the ability to self-associate(Silver et al., 2003), leading us to postulate that endogenous EYA may associate with the transfected EYA and dephosphorylate it. Addition of pervanadate presumably greatly impairs, but does not totally knock out phosphatase activity resulting in a weak but detectable signal for EYA^{WT} (Fig. 5a, Lanes 1 and 2). This interpretation is consistent with our finding that addition of vanadate in the pNPP assay doubles the apparent Km, but does not completely inactivate the enzyme (Fig. 2a). EYA^{HAD} mutants exhibit increased phosphotyrosine signal in this assay (Fig. 5a), likely reflecting their reduced activity as a phosphatase (Fig. 2). In the case of the stable cell lines expressing the catalytically inactive EYA^{HAD} mutants, in the absence of pervanadate, the endogenous EYA is sufficiently active to dephosphorylate a significant portion, but not all, of the overexpressed EYA^{HAD} mutant protein (Fig. S4a). However, because these experiments were carried out in Drosophila cells rather than in vitro, it is possible that rather than reflecting impairment of intrinsic PTP activity in the EYA^{HAD} mutants, the increased phosphotyrosine signal resulted from a second coprecipitating PTP that interacts more strongly with EYA^{WT} than with EYA^{HAD}, or from the EYA^{HAD} proteins serving as better substrates for the relevant tyrosine kinase. Arguing against this interpretation, we find that incubation with recombinant EYA^{WT} fusion protein, but not EYA^{HAD} fusion protein, strongly reduces the phosphotyrosine signal associated with Drosophila EYA^{HAD} (Fig. 5b). This suggests that EYA may serve as its own substrate, likely acting in trans to dephosphorylate itself. Based on our genetic analyses indicating that EYA's phosphatase activity is required for eye specification and development (Fig. 3), we propose that EYA may autoactivate by dephosphorylating itself on specific tyrosine residues.

Figure S1: EYA is a member of the phosphatase subgroup of the HAD superfamily.

a, A HAD superfamily phylogenetic tree suggests EYA proteins are related to protein phosphatases. Branches are labeled with the two letter species abbreviation and common protein name. The EYA branch is highlighted in red.**b,** Multiple Sequence Alignment reveals that the conserved EYA domain shares similarity with the HAD hydrolase domain, particularly in those regions implicated in forming the active site (PFAM 00702.6). Residues comprising Motifs 1-3 of the HAD domain are boxed. Labeled arrows designate the positions of variant residues used in this study or identified in Drosophila and human EYA mutations. Site-directed mutations generated in this study are shown in red. Additional variants associated with specific Drosophila *eya* alleles (Bui et al., 2000) or derived from human patients suffering from the EYA1-specific branchio-oto-renal syndrome (Azuma et al., 2000) are shown in black, with the human mutations prefaced with the letter "h". While none of these variants overlap with the five residues focused on in this study, one, the Drosophila G723E mutation, affects a conserved glycine residue in Motif 3 of the HAD domain. The putative SINE OCULIS binding site (Bui et al., 2000; Pignoni et al., 1997) is denoted with a solid black line.

Figure S1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Sc_YER037W
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Phosphonatase
Phosphoserine phosphatase
P-type ATPases
Protein phosphatases
EYES ABSENT
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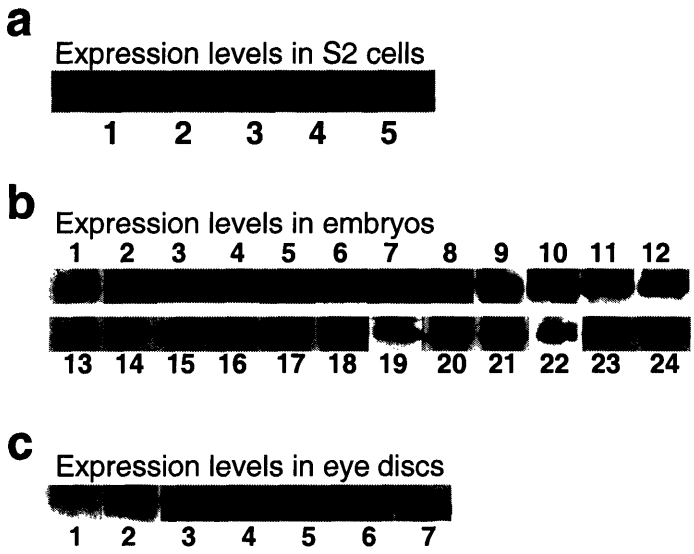

Figure S2: HAD active site mutations do not result in appreciable changes in protein levels relative to wild type EYA.

a, Western blot of transfected S2 cells showing comparable expression of EYA^{HAD} mutants relative to EYA^{WT}. Equivalent samples from pools of stably transfected S2 cell lines expressing EYA^{WT} and four different EYA^{HAD} mutants. Lanes 1-5: EYA^{WT}; $^{EYAD493N + S670A}$; $EYA^{D493N + K699Q}$; $EYA^{D493N + D724N}$; $EYA^{D493N + E728Q}$.

b, Western blots of equivalent samples of embryos in which the EYA transgenes have been expressed using a *ubiquitin*-GAL4 driver line reveal comparable expression levels in EYA^{HAD} lines relative to EYA^{WT}. Each lane represents an independent transgenic line. Lanes: 1-4, EYA^{WT}; 5-9, EYA^{D493N}; 10-13, EYA^{S670A}; 14-17, EYA^{K699Q}; 18, EYA^{D724N}; 19-22, EYA^{E728Q}; 23-24, $EYA^{D493N + D724N}$.

c, Western blots of equivalent samples of eye imaginal discs in which the EYA transgenes have been expressed using a *dpp*-GAL4 driver line reveal comparable expression levels in EYA^{HAD} lines relative to EYA^{WT}. Each lane represents an independent transgenic line used in the rescue assay. EYA sometimes runs as a doublet (for example, lanes 1 and 2 and lanes 16-20 of panel 3c). Lanes: 1, EYA^{WT}; 2, EYA^{D493N}; 3, EYA^{S670A}; 4, EYA^{K699Q}; 5&6, $EYA^{D493N + S670A}$; 7, $EYA^{D493N + D724N}$.

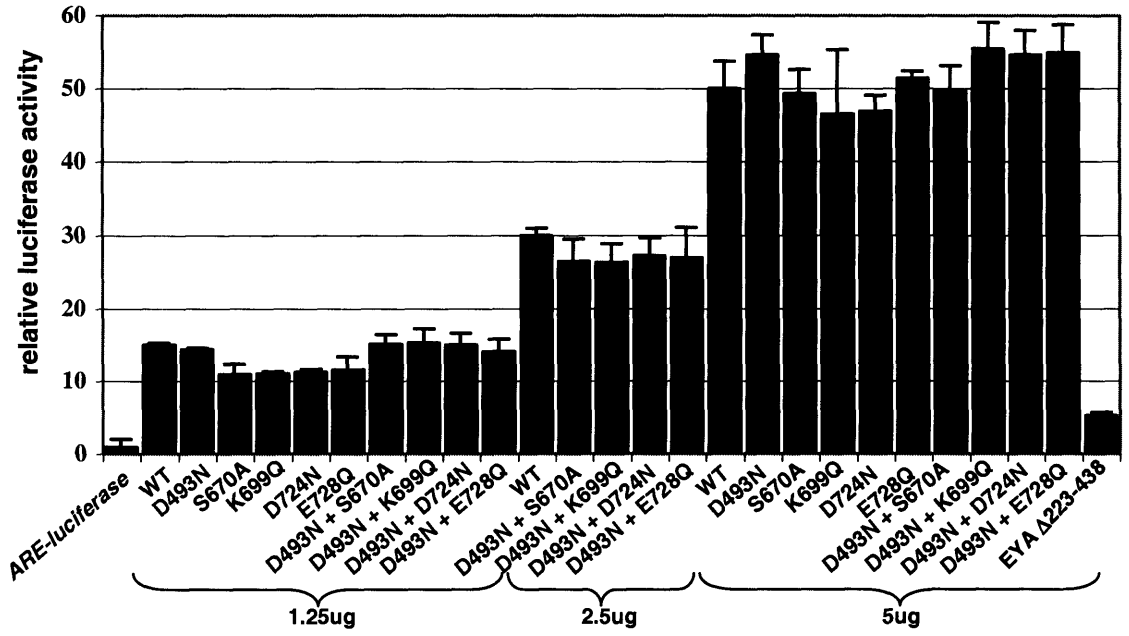

Figure S3: EYA^{HAD} mutations do not disrupt EYA's role as a transcriptional coactivator in conjunction with SO.

EYA^{WT} versus EYA^{HAD} activity was tested at three different concentrations of EYA DNA (1.25 ug, 2.5 ug and 5 ug) to confirm the linearity of response. A mutation (Δ223-438; last sample in graph) that deletes the transactivation domain of EYA, but can still bind SO (Silver et al., 2003), demonstrates that it is possible to block activity in this assay system. The reporter baseline level in the absence of EYA and SO is shown (ARE-luciferase; first sample in graph).

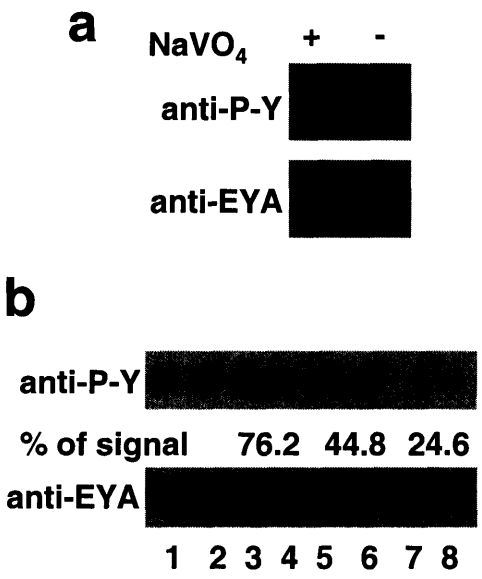

Figure S4: Using tyrosine phosphorylated EYAHAD as a substrate in an in vitro phosphatase reaction.

a, Tyrosine phosphorylation of EYA^{HAD} in the absence of pervanadate is reduced relative to levels achieved in the presence of pervanadate. Quantitation was not performed because the exposure time necessary to obtain a sufficiently strong signal in the (-) pervanadate lane placed the signal in the (+) pervanadate range outside of the linear range of detection. **b,** A titration curve of amounts of GST-ED necessary to achieve maximal activity in the PTP assay was performed. Duplicate experiments are shown. Lanes: 1-2, 1ug, set at 100%; 3-4, 5ug; 5,6, 10ug; 7,8, 50ug. $EYA^{D493N+D724N}$ was used as the substrate as in Figure 4b.

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Chapter 4

The lozenge minimal eye enhancer can be used to examine the function of the EYA-SO transcription factor on a native promoter

Serena J. Silver and Ilaria Rebay

Introduction

Studies of the EYA-SO transcription factor have been limited by lack of known *in vivo* targets in *Drosophila*. Recent work from Utpal Banerjee's lab has identified the *lozenge* (*lz*) minimal eye enhancer (LMEE) as a regulatory target of SO (Yan et al., 2003). The LMEE is a 250 bp region which requires DNA binding sites for SO and GLASS to recapitulate the eye specific expression of LZ (Yan et al., 2003; Figure 1A).

The SO binding site identified in the LMEE, TGATAT, is similar to the site identified *in vitro* through SO homeodomain interactions with plasmid DNA, GATAC (Hazbun et al., 1997). More striking perhaps is the physical arrangement of the two SO binding sites, which are found in an almost perfect palindrome separated by 8 bp, and are almost two hundred bp downstream of the GLASS binding sites. This promoter architecture may be crucial to accommodate cofactors or multiprotein complexes for endogenous gene regulation.

The LMEE therefore provides an exciting new tool for examination of the EYA-SO transcription factor in a more native setting. By inserting this genomic region upstream of a luciferase reporter gene, we can ask whether activation of this reporter is modulated by other signaling networks or mutations in EYA and SO. We can also compare the effects of these changes on *LMEE-luciferase* to those we see with *ARE-luciferase*, a reporter gene with 7 tandem copies of the mouse SIX binding site AREC3, which we have previously used to study the EYA-SO transcription factor (Silver et al., 2003).

Results and Discussion

LMEE-luciferase can be used as readout of EYA-SO activity in S2 cell culture

LMEE-luciferase is activated to low levels when expressed alone in S2 cells, or with EYA or SO alone, but when co-expressed with both EYA and SO, 11 fold activation is observed (Figure 1B). This activation is due to direct binding of SO to the *LMEE* sequence, as mutation of the SO binding sites sharply decreases this activity while mutation of the GLASS binding sites does not (data not shown). Furthermore, activation of *LMEE-luciferase* requires the transactivation potential of the PST-rich region of EYA, implicating the EYA-SO complex in regulation of this native promoter (Figure 1B).

DAC is a co-activator of the EYA-SO transcription factor, independent of EYA-mediated phosphatase activity

We then examined whether the RD gene member DAC might affect regulation of this promoter, as DAC is thought to physically interact with EYA and has been suggested to act as a co-repressor or co-activator (Ikeda et al., 2002; Li et al., 2003; Li et al., 2002). DAC appears to co-activate the *LMEE-luciferase* reporter gene (Figure 2). This is unlike our observations with the *ARE-luciferase* reporter gene, where we did not observe any effect upon contransfection of DAC (Silver et al., 2003).

Recent work from our lab and others identified a new function for EYA as a protein phosphatase (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003), which we have shown to be independent from its role as a transactivator (Tootle et al., 2003). However, it has been suggested that the phosphatase activity of EYA might act to switch the function of a SIX1-DACH complex from repression to activation (Li et al., 2003). These experiments showed

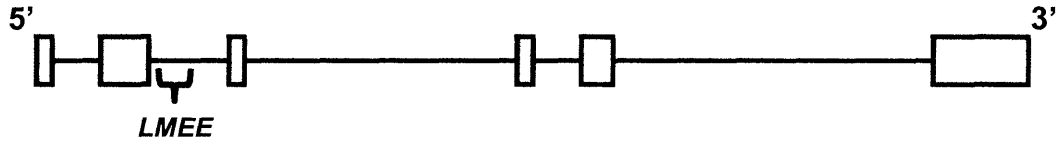
SIX1-DACH mediated repression of a heterologous promoter, which could be relieved by coexpression of wildtype but not phosphatase mutant EYA (Li et al., 2003). As the *LMEE-luciferase* provides a new readout of DAC cooperativity with the EYA-SO transcription factor, we asked if the EYA phosphatase function affects this coactivation. From the model outlined above, one would predict that EYA phosphatase activity would be required for DAC coactivation of *LMEE-luciferase*, or that EYA phosphatase activity might be required for even EYA-SO activation of *LMEE-luciferase*.

In order to remove EYA phosphatase activity we used point mutations in each of the five key residues for HAD family member function, which we have previously shown to be crucial for EYA phosphatase function *in vitro* (Tootle et al., 2003). As mentioned above, these point mutations, singly or doubly, did not affect EYA-SO mediated activation of *ARE-luciferase* (Tootle et al., 2003). Upon testing with *LMEE-luciferase*, we found that some retained full activity while others were compromised (Figure 3). More strikingly, we found that all could be activated further by co-expression of DAC (Figure 3), indicating that EYA phosphatase activity is not required for DAC to function as a co-activator in S2 cells. It remains to be understood why this result differs from that found in mammalian cell culture, although cell context specific differences as well as promoter specific attributes are likely to play important roles. It is also intriguing that addition of DAC can activate even compromised EYA mutants to similar levels as wildtype (Figure 3), suggesting that high levels of DAC may in some way compensate for these point mutations.

It is also quite striking that we observe different results using *ARE-luciferase* and *LMEE-luciferase*. As mentioned above, we do not observe any effect of DAC on *ARE-luciferase* activation assays (Silver et al., 2003). It is intriguing to ask whether DAC might require contact

A

lozenge locus



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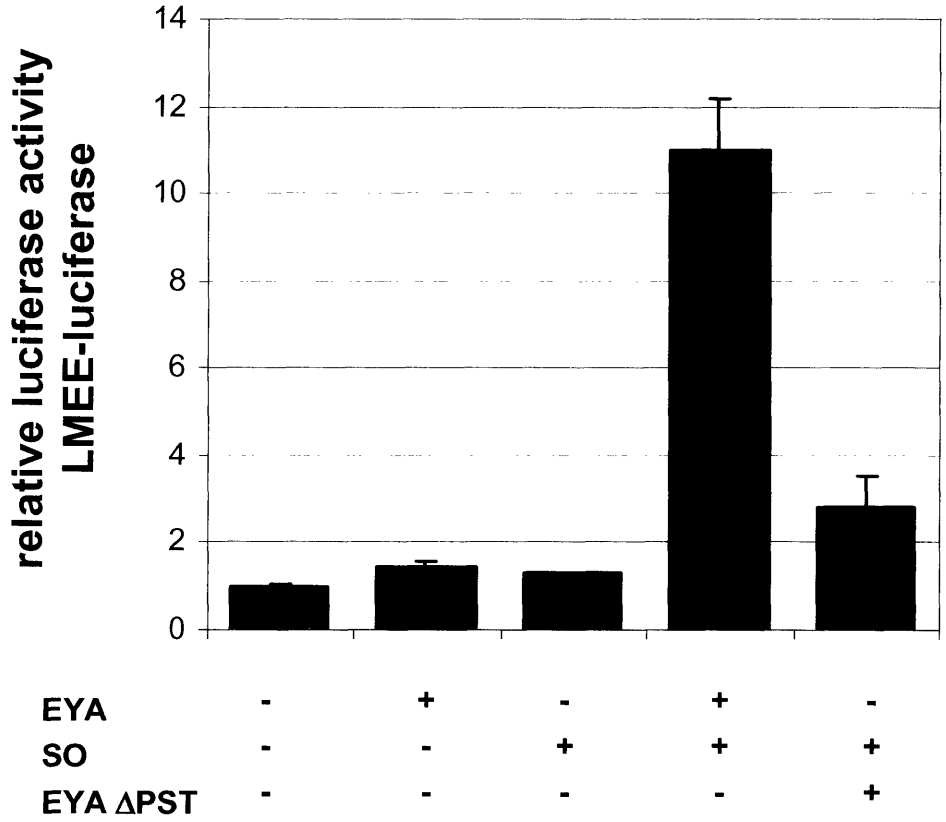

Figure 1: The *lozenge* minimal eye enhancer (LMEE) is a SO target
In A, the LMEE is found in the second intron of the *lozenge* gene, and contains two palindromic SO binding sites, shown here in red, within a larger footprint of SO, shown in bold (adapted from Yan et al.). Upstream GLASS binding site is shown in blue. In B, transient transfection reporter assays show that LMEE-luciferase can be used in S2 cells to measure EYA-SO activity, with 11-fold induction above background when both are present. This readout is dependent on EYA transactivation ability, as deletion of the PST rich region of EYA abrogates activation.

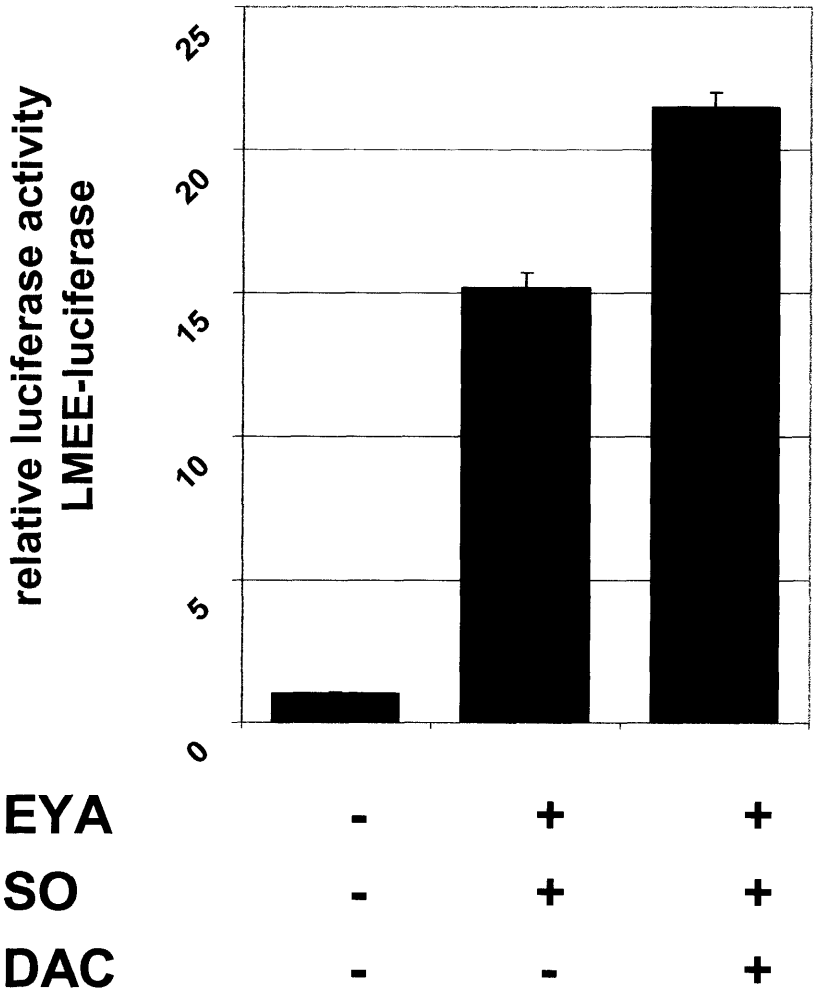

Figure 2: DAC can potentiate EYA-SO mediated transcription
Co-transfection of DAC leads to greater *LMEE-luciferase* activitation by EYA and SO.

with the DNA independently of EYA and SO, which the architecture of the *ARE-luciferase* reporter gene with 7 tandem SO binding sites (compared with *LMEE-luciferase* in Table 1) might not allow. In contrast, *LMEE-luciferase* contains only two SO binding sites found as a palindrome, with over 200 bp of native promoter sequence which might contain DAC binding sites.

RAS-MAPK signaling activates EYA-SO mediated transcription

Previously we have shown that phosphorylation of EYA, likely by MAPK, can activate the EYA-SO transcription factor (Silver et al., 2003). In order to ask whether this is also the case in the context of *LMEE-luciferase*, we made use of several tools to examine the effects of RAS activity on EYA; coexpression of activated RAS, RAS^{V12}, and mutation of the phosphoacceptor serines in EYA to alanine (EYA^{S-A}) to prevent phosphorylation, and to aspartic or glutamic acid (EYA^{S-D}) to mimic phosphorylation. We found that serine phosphorylation is not required for EYA-SO activation of *LMEE-luciferase*, but that activation is increased upon cotransfection of Ras^{V12} or use of the phosphomimetic EYA mutant (Figure 4). We observe a much greater impact upon using RAS^{V12}, which may reflect more complex affects of RAS activation on the RD gene network as a whole, or even on EYA. It is likely that the two previously identified MAPK phosphoacceptor sites are not the only input point for RAS activation of the EYA-SO transcription factor, as activation is also observed when RAS is cotransfected with mutant EYA^{S-A} or $EYA^{S-D/E}$ (Figure 4).

TABLE 1: A Comparison of ARE and LMEE luciferase reporter constructs

ARE-luciferase	*LMEE-luciferase*
	2 palindromic binding sites
	TGATAT
	8 bases between binding sites
	glass binding sites
	Ets binding sites
	250 bp total length
	Drosophila *lozenge*

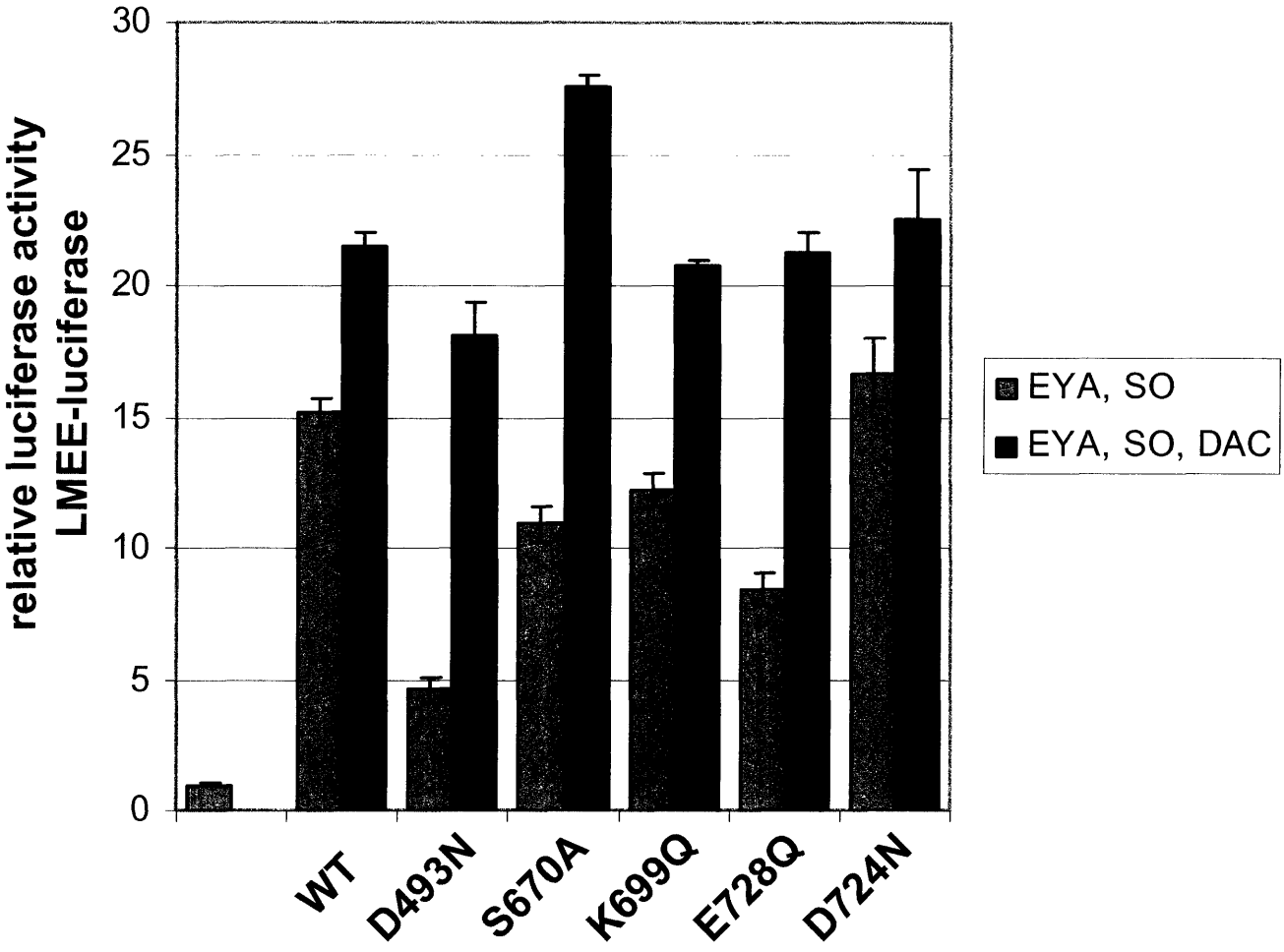

Figure 3: EYA phosphatase activity is not required for DAC coactivation
Some of the EYA^{HAD} mutants which are defective for phosphatase function have lower levels of transcriptional activation (blue). However, all are co-activated by DAC expression to levels close to that of wildtype EYA (red), suggesting that DAC expression might overcome the problem caused by these point mutations.

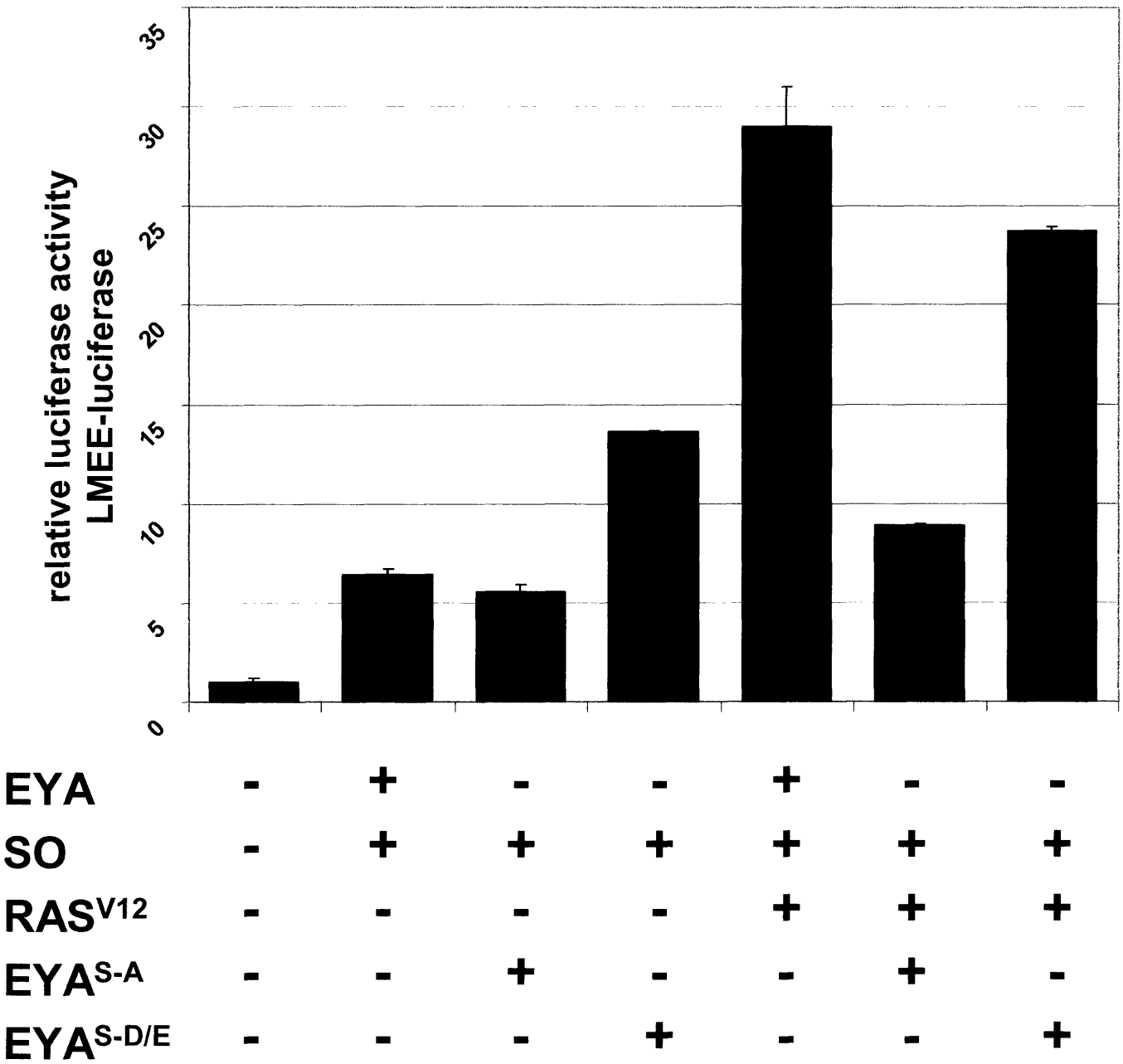

Figure 4: RAS activates the EYA-SO transcription factor
Mutation of the EYA phosphoacceptor residues to alanine does not affect transactivation, but mutation of the residues to glutamic or aspartic acid to mimic phosphorylation increases EYA transcription factor activity. Activated RAS can also stimulate the EYA-SO transcription factor, likely through additional residues or functions as stimulation can also be seen for the phosphoacceptor mutants.

Chromatin IP experiments show SO directly binds to the *LMEE in vivo*

Chromatin immunoprecipitation (ChIP) experiments can be used to ask whether nuclear factors that activate promoters do so by direct binding to that promoter sequence. The *LMEE* might provide an important tool to study RD gene network member association with target DNA. Our initial focus was to ask whether SO is associated with the *LMEE* in Drosophila cell culture using S2 cells, and if we could detect SO association with the *LMEE* in *Drosophila* eye discs, where genetic and molecular evidence suggested it would bind (Yan et al., 2003).

We found that immunoprecipitation of endogenous SO, from eye discs and from S2 cells, showed association with the *LMEE* (Figure 5). This suggested that this promoter might be used to examine association of other RD gene network members, such as EYA and DAC, that are thought to complex with SO to affect transcription of target genes.

In summary, the *LMEE* provides a useful new tool for study of the EYA-SO transcription factor in a more native setting. Already this promoter can be clearly seen to behave differently from less native constructs such as *ARE-luciferase*, and cell culture studies of different promoters in vertebrates, differences which may help us to better understand the regulatory mechanisms governing the EYA-SO transcription factor *in vivo*. Through a combination of ChIP experiments and transcription assays, we may also be able to differentiate between signals and mutations which affect EYA-SO localization to DNA versus their ability to activate transcription.

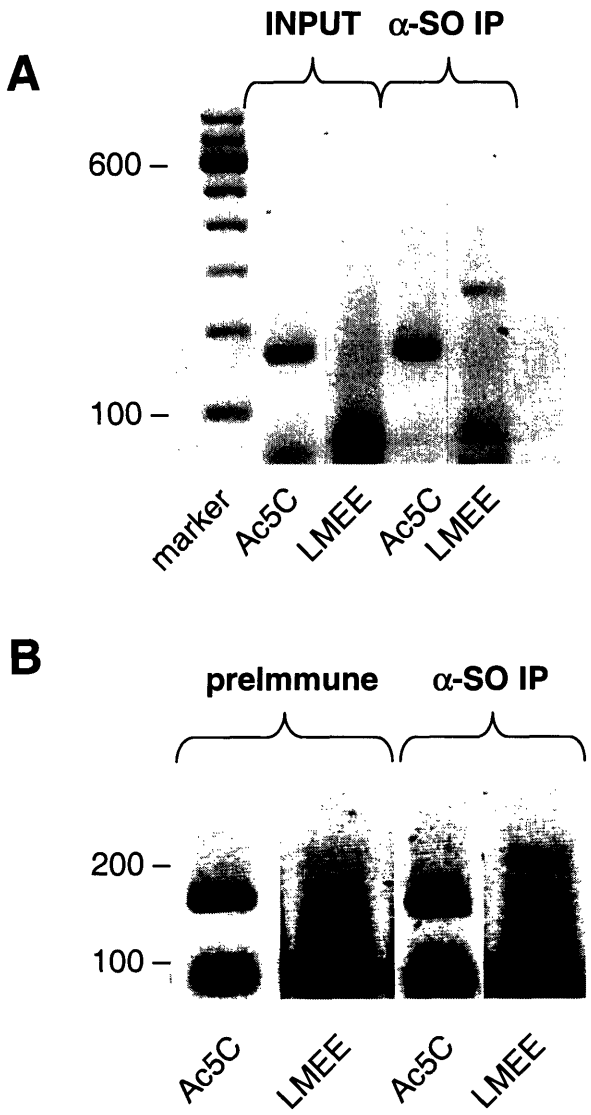

Figure 5: SO is bound to the LMEE *in vivo*
PCR from wildtype eye discs following ChIP with SO antibody. The LMEE is 4.5x enriched in the SO IP as compared to input using Ac5c as a control (A; analysis performed using NIH image). Endogenous SO can also associate with the LMEE in S2 cells, compare LMEE association after SO IP to preimmune sera IP (B). Numbers on the left indicate basepairs on ladder.

Materials and Methods

LMEE-luciferase subcloning and transcription assays

The 250 bp LMEE was amplified from the LMEE-lacZ plasmid or LMEE^{so}-lacZ or LMEE^{gl}-lacZ plasmids (gifts from U. Banerjee, described in Yan et al., 2003) by PCR using the Universal primer and a LMEE A PstI 5'-CTGCAGCATTAACAAAATAAAAAAGGGG-3'. This PCR product includes an endogenous KpnI site upstream of the LMEE, and using that site and the PCR produced PstI site was cloned into the Kpn1/PstI sites upstream of the hsp70 TATA box in BSSK-TATA-luciferase. Transcription assays were performed in duplicate as previously described in Silver et al., 2003, using 5ug per assay of the reporter gene and each expression plasmid (expression plasmids previously described in Silver et al., 2003 and Tootle et al., 2003, and normalized using 1ug of Ac-lacZ per assay.

ChromatinIP experiments

For S2 cells: Protocol used as for Kc cells from D. MacAlpine, Bell lab, with the exception that a Quiagen PCR purification kit was used to purify DNA after immunoprecipitation, and DNA eluted in 30ul.

For eye discs: Protocol adapted from D. MacAlpine and R. Austin protocols for Kc cells and egg chambers, respectively.

1. Approximately 70 eye disc pairs, some with brains attached, were dissected and fixed in 2% formaldehyde for 15 minutes, rocking.
2. Add 2.5 M glycine to 125 mM (if you suspend discs in 1ml S2 cell media to fix, adding 54 ul of 37% formaldehyde, just add 50ul of 2.5M glycine here). Incubate 5 minutes. KEEP ON ICE.

3. Was twice with 1 ml cold TBS. (You may be able to freeze at this point but I have not tried it – you can certainly keep it in the cold room for a bit if you're dissecting more samples)
4. Add protease inhibitors to hypotonic and ChIP lysis buffers before use.
5. Resuspend pellet in 1ml hypotonic buffer, and let sit for 20 minutes on ice.
6. Remove all but 100ul of hypotonic buffer from the discs. Use a tight dounce to crush the disc about 30 times. After douncing add another 900 ul of hypotonic buffer, and pellet at low speed (1000K on table top) for 10 minutes. Wash with 1 ml hypotonic buffer and pellet again at low speed.
7. Resuspend pellet in 500 ul ChIP lysis buffer – let cells sit for 15-30 minutes on ice.
8. Sonicate three times for about 15 seconds each at power setting 1.5, 100% duty cycle – make sure the sonicator is at 1.5 or your sample will fly all over the place!!! Keep samples on ice when not sonicating.
9. Spin at 14K for 10 minutes at 4 degrees and put supernatant in a fresh tube. Freeze at -80 until you have enough samples to do your experiment.
10. Add antibody. SO immunoprecipitation was performed using 2ul of GP anti-SO per 500ul lysate. Controls were performed using the same amount of preImmune sera from GP. Rock overnight at 4 degrees.
11. Wash G protein coupled sepharose beads (also called Gamma-bind beads) twice in 1X PBS. Add a 30ul slurry of 1:1 beads to each sample and incubate at 4 degrees, rocking for 3 hours.
12. Pellet the beads at 2500K for 1 minute (4 degree centrifuge), remove 200ul for your INPUT, save. Wash beads 3X five minutes each time in 1X ChIP lysis buffer.

13. Wash beads once in high salt ChIP lysis buffer – 500mM NaCl for 5 minutes
14. Wash beads with TE for five minutes
15. Resuspend beads in 150ul TE/SDS buffer and incubate at 65 degrees for 10 minutes, vortexing every couple of minutes. Pellet beads by spinning 14K for 1 minutes, transfer the supe to a fresh tube as your IP.
16. Add 10ul of INPUT to 140ul TE/SDS
17. Seal tubes with parafilm and put at 65 degrees overnight to reverse crosslinks.
18. Use Quiagen PCR purification kit to extract DNA. Elute sample in 30ul elution buffer. Perform PCR with appropriate primers. Ac5c is a good control reaction; primers are in the common primer box.
19. Run PCR products on NuSieve 2% gel

PCR protocol:
Step 1: 95 degrees 2 minutes
Step 2: 95 degrees 30 seconds
Step 3: 55 degrees 30 seconds
Step 4: 72 degrees 1 minute
Step 5: Return to step 2, 29 times
(adjust cycle number depending on DNA yield)

Buffers for ChIP assays

Add protease inhibitors prior to use!

Hypotonic buffer
20mM K-Hepes pH 7.8 (adjust pH using KOH)
5mM KOAc
0.5 mM $MgCl_2$
0.5 mM DTT

ChIP lysis buffer
50 mM KHepes 7.8
140 mM NaCl
1 mM EGTA
1 mM EDTA
1% Triton-X 100
0.1 % Na-deoxycholate

High Salt ChIP lysis buffer
Same as above but with 500 mM NaCl

TE/SDS
10 mM Tris pH 8
1 mM EDTA
1% SDS

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Chapter 5

Discussion and Future Directions

Our functional analysis of the Retinal Determination (RD) gene network member EYES ABSENT (EYA), a novel nuclear factor, identified the regions necessary for EYA's function as transactivator and pointed to roles of RAS and GROUCHO in regulation of this activity (Silver et al., 2003). We have also examined a new and essential role for EYA as a protein tyrosine phosphatase (Tootle et al., 2003). Thus EYA represents the first identified eukaryotic transcription factor to also possess phosphatase activity, a dual function whose coordination is not understood. Each of these functions, transcription factor and phosphatase, can be performed independently, but both are required for EYA *in vivo*, suggesting that they may work together to specify eye fate. However, the targets for both of these functions, and thus the molecular steps by which EYA promotes organ specification, are largely still unknown.

Identification of phosphatase targets of EYA is hampered by the non-conventional aspartic acid-based catalytic mechanism of HAD family members, which rules out use of the elegant substrate trapping methodologies employed to identify substrates of phosphatases which use cysteine-based catalysis. Instead, targets of the EYA phosphatase will have to be identified through direct testing of candidates or novel proteomic approaches, perhaps including a version of substrate trapping for this family of enzymes.

However, several techniques can be used to identify transcriptional targets of EYA on the genomic level, which may allow us to assay directly the importance of EYA phosphatase function in the context of target gene transcriptional regulation. As EYA is recruited to the DNA through interactions with the homeodomain containing protein, SINE OCULIS (SO), identification of SO binding sites in the *Drosophila* genome should include many EYA-SO targets. One method for identifying these targets would be to perform chromatin immunoprecipitation (ChIP) using antibodies raised to SO (described in Appendix B), and

hybridization of purified DNA to a genomic microarray to look for enriched regions. This type of analysis might also be possible to perform using commercially available expression arrays, as the sheared DNA will include genomic regulatory regions and likely enough coding sequence to hybridize to arrays. Initial attempts to identify targets using a genomic microarray developed by the Bell lab were unsuccessful (data not shown), but optimization of the protocol including amplification procedures may prove more fruitful.

Another less direct method for finding targets of EYA is to overexpress EYA in a non-eye larval tissue, such as the wing or leg disc, and perform microarray analysis of induced genes compared to the expression profile of wildtype wing or leg discs, similar to the approach taken by (Michaut et al., 2003). Microarray data from this type of analysis should reveal both direct and indirect targets of EYA transcriptional regulation. One way to filter the large data set produced by this method is to employ computational strategies to identify common promoter elements, which can then be compared to the known SO family binding sites to predict direct targets.

While identification of many targets may help in understanding the mechanism behind EYA-SO mediated eye formation, as well as common promoter architecture, such as number of SO binding sites or motifs recognized by other transcription factors, the recent identification of *lozenge* as a SO target (Yan et al., 2003) provides a good starting place for direct analysis of promoter regulation by RD genes. SO binds to two imperfect palindromes in the *lozenge* minimal eye enhancer (LMEE) (Yan et al., 2003), and this region can be used to study the EYA-SO transcription factor in a native context.

Recently we showed that we can observe SO localization to the LMEE in both *Drosophila* eye discs and in S2 cell culture by ChIP experiments (Chapter 4). This provides a

tool to ask whether perturbation of signaling pathways or of other members of the RD gene network affects SO localization to a promoter. For example, SIX1 association with target promoters is regulated by cell cycle dependent phosphorylation, such that at the G_2/M transition it does not associate with DNA (Ford et al., 2000). SO from synchronized cell culture populations could be assayed by ChIP to ask whether its DNA association is also regulated by the cell cycle.

While it is not known if SO is phosphorylated in a cell cycle dependent manner, one source of SO phosphorylation may be the stress response pathway (I. Mills, personal communication). This pathway can be activated in S2 cells or dissected eye discs using osmotic or heat shock, and SO-LMEE interaction levels analyzed to ask whether stress affects the ability of SO to bind DNA. These experiments may give us insight as to the regulation of SO-DNA complexes, and by extension, EYA-SO-DNA complexes.

As we observe DAC co-activation of the EYA-SO regulation of LMEE in cell culture assays, it would be interesting to ask whether DAC associates with this promoter *in vivo*, by ChIP directly of DAC using the available monoclonal antibody. This would provide support for the *in vivo* relevance of DAC co-activator function, as thus far it has only been localized *in vivo* to a promoter in the context of repression (Li et al., 2002). This experiment could also be performed in eye discs with lower or higher levels of EYA and SO, through use of mutations or overexpression, to ask if they are required for DAC association with a promoter.

Another important set of experiments would be to examine association of EYA with the LMEE. Given the observation that point mutations in the EYA DOMAIN reduce EYA transactivation potential without affecting the EYA-SO interaction (Chapter 4 and data not shown), it would be interesting to ask if these mutant EYA proteins have altered levels of

enhancer association. If these proteins associate to similar levels as wildtype EYA, they may indicate key residues for interaction with or regulation by other factors, while if they display defects in promoter localization, they may indicate points of regulation for association of the EYA-SO transcription factor with DNA. One caveat to these experiments is that they must be done using overexpression of tagged EYA proteins in cell culture or in eye discs to distinguish wildtype EYA protein from mutant protein, and this overexpression will have to be carefully titrated to allow for meaningful results.

Another avenue of regulation for the EYA-SO transcription factor comes from interactions with the co-repressor GROUCHO (GRO). Our work has shown that in cell culture, GRO can compete with EYA for SO binding, leading to lower levels of target gene transcription (Silver et al., 2003). It remains to be understood how SO-GRO interactions *in vivo* contribute to development, and whether DAC plays a role in SO mediated repression.

GRO has been implicated in regulation of and by many signaling pathways, functioning as an active corepressor through its interactions with DNA binding proteins such as HAIRY, DORSAL, ENGRAILED, RUNT (for review see Fisher and Caudy, 1998) and more recently BRINKER (Hasson et al., 2001). Of particular note is the GRO interaction with RUNX family members (Fisher and Caudy, 1998), of which the SO target gene LOZENGE is a member. While GRO has not been shown to interact with LZ, it is intriguing to ask whether SO activation of *lz* expression may contribute to a positive feedback loop by LZ protein competing GRO away from SO and therefore allowing greater EYA-SO activation of target genes.

SO is the only member of the RD gene network which does not induce ectopic eyes when overexpressed, although it can synergize with EYA to induce larger and more frequent eyes

(Pignoni et al., 1997). One possible explanation for this observation may be the existence of a SO-GRO complex which prevents eye formation when SO alone is expressed but that is broken up only by the addition of ectopic EYA, a model which is supported by our co-immunoprecipitation data (Silver et al., 2003). In addition to the many *gro* mutant lines available in the fly community, there is an inducible groRNAi line which effectively knocks down GRO expression (Nagel et al., 2002), thereby allowing directed removal of the *gro* gene product, which is necessary globally for viability. This can be used in conjunction with transgenes driving SO to assess ectopic eye induction in the absence of GRO. As the ectopic eye induction system is highly quantitative, we can also ask whether reduction of GRO affects the frequency of eyes induced by EYA alone and synergistically by EYA and SO.

As discussed in Chapter 1, there are many links between the RD gene network and control of cell proliferation. One of the best understood aspects of this relationship is downregulation of SIX1 at the G_2/M transition via phosphorylation by Casein Kinase II (Ford et al., 2000). This phosphorylation event prevents SIX1 binding to target DNA (Ford et al., 2000), and may represent an important checkpoint control in cell proliferation. *Drosophila* SO is also a nuclear phosphoprotein (E. Davies, personal communication), and a clear triplet of SO similar to that observed for SIX1 is observed on western blots (Appendix B).

Drosophila, with its varied genetic tools, would provide an excellent model system for analysis of SIX family roles in cell cycle control. If SO both regulates and is regulated by the cell cycle similarly to SIX1, the triplet observed by western blot should be due to phosphorylation. Treatment of cell lysates with a strong phosphatase such as lambda or CIP may result in collapse of the triplet. In addition, analysis of the SO by western in synchronously

dividing cells may reveal if phosphorylation state is linked to the cell cycle. It is also intriguing to ask if EYA, which has been proposed by one group to be a dual-specificity phosphatase (Li et al., 2003), might itself regulate the phosphorylation state of SO.

It is also worth noting that EYA protein runs much larger than its predicted size on a western blot, indicating that it is likely highly modified posttranslationally. We have previously observed both serine-threonine (Hsiao et al., 2001) and tyrosine phosphorylation (Tootle et al., 2003) of EYA, but particularly the sites and consequences of tyrosine phosphorylation are not well understood. As EYA has been shown to associate with CKIIβ (Li et al., 2004), the regulatory subunit of the dual specificity protein phosphatase CKII, perhaps CKII regulates both EYA and SO, EYA through tyrosine phosphorylation and SO through serine/threonine phosphorylation.

Another link between the cell cycle and EYA is the finding that EYA expression can be turned off in the absence of cell proliferation (Kenyon et al., 2003). As there is at least one putative SO binding site in the EYA eye enhancer (Zimmerman et al., 2000) it is intriguing to ask if EYA expression might be negatively regulated by SO in the absence of cell division, providing a feedback loop to prevent differentiation through the RD network without appropriate proliferation.

The RD gene network is deployed in many different developmental contexts, including oogenesis, myogenesis, and eye development. Yet our understanding of the mechanisms which provide context specific clues to ensure appropriate target gene activation still contains many gaps. Identification of the targets of these proteins during eye development is an important first step to understanding how signal integration might take place, as the promoters of target genes

are likely be coregulated by many pathways in addition to the RD gene network. Another important role is likely played by posttranslational modification, perhaps dictating when EYA uses its phosphatase function rather than its transcription factor function, or when SO acts as a transcriptional activator versus a repressor. Better understanding of the modifications of these proteins and subsequent effects on activity or association with proteins and DNA will provide details as to context specific control of RD gene network function.

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Appendix A:

Characterization of a new class of Drosophila *Son-of-sevenless* (*Sos*) alleles highlights the complexities of Sos regulation and function in higher eukaryotes

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F.C. and A.Z. mapped the EY2-3 alleles to the SOS locus and performed initial characterization of the Ras^{V12} and yan^{act} genetic interactions, and performed sequencing of two alleles. L.D. helped with eye sections and with sequencing the remainder of SOS alleles.

This manuscript is currently in press at Genesis.

Abstract

The guanine nucleotide exchange factor (GEF) *Son-of-sevenless* (*Sos*) encodes a complex multidomain protein best known for its role in activating the small GTPase RAS in response to Receptor Tyrosine Kinase (RTK) stimulation. Much less well understood is SOS' role in modulating RAC activity via a separate GEF domain, or how its parallel functions in RAS and RAC mediated signaling events might be integrated in vivo. In the course of a genetic modifier screen designed to investigate the complexities of RTK/RAS signal transduction, a complementation group of eleven alleles was isolated and mapped to the *Sos* locus. Molecular characterization of these alleles indicates that they specifically affect individual domains of the protein, including both enzymatic GEF motifs, one specific for RAS and the other for RAC. While most appear to be hypomorphic mutations, one of these alleles, Sos^{M98}, which contains a single amino acid substitution in the RacGEF motif, functions as a dominant negative *in vivo* to downregulate RTK signaling. These alleles provide new tools for future investigations of role of SOS in modulating both RAS and RAC activation and how these dual roles are coordinated and coregulated during development.

Introduction

Receptor Tyrosine Kinase (RTK) signaling provides an important growth and differentiation cue which is used reiteratively during development to initiate and maintain cell fates. In Drosophila, proper regulation of RTK signaling is crucial for many processes including eye development, wing vein formation, and embryonic cell fate determination (Schweitzer and Shilo, 1997).

A crucial link between RTK signaling and its downstream effector, the small GTPase RAS, is the RAS guanine nucleotide exchange factor (RasGEF), *Son-of-sevenless (Sos)*. Upon RTK activation, tyrosine phosphorylation of the receptor allows recruitment of the adaptor protein GRB2/DRK, which brings with it SOS (Buday and Downward, 1993). Once localized to the membrane, the RasGEF activity of SOS facilitates release of guanine nucleotide, allowing RAS to bind GTP instead of GDP and thus become activated (Boriack-Sjodin et al., 1998; Liu et al., 1993). Activated RAS then activates RAF, initiating activation of the MAPK cascade (for review see (Avruch et al., 2001). Once phosphorylated by MAPKK, MAPK translocates into the nucleus where it phosphorylates many nuclear factors, including the ETS domain transcription factors *pointed* (*pnt*) and *yan* (O'Neill et al., 1994), which then effect changes in the transcriptional program of the cell (Gabay et al., 1996). PNT is a transcriptional activator that is stimulated by phosphorylation, while YAN is a transcriptional repressor that is exported from the nucleus and thus downregulated upon phosphorylation (O'Neill et al., 1994; Rebay and Rubin, 1995; Tootle et al., 2003).

As outlined above, RTK/RAS-mediated signals cannot be transduced to the nucleus without activation of RAS by SOS. While RasGEF activity is the best characterized function of SOS, its complex protein structure, with several distinct catalytic and protein-protein interaction

domains (Figure 1), suggests additional roles for SOS in the cell. Current knowledge of each domain, and how it may allow SOS to coordinate RAS activation with other signaling events, is outlined below.

The N-terminus of SOS has homology to histones and has recently been shown to fold into a histone-like pseudodimer (Sondermann et al., 2003). As crystals of the SOS N-terminus form as nonamers, and oligomerization can be detected *in vitro* by size exclusion chromatography (Sondermann et al., 2003), it is likely that this region mediates SOS self-association. Functionally, the N-terminus has been implicated as an autoinhibitory region which may interfere with the function of the Dbl homology (DH) and Pleckstrin Homology (PH) domains that directly follow (Das et al., 2000). Regulation of this autoinhibitory function may be mediated by signaling events, as inhibition appears to be relieved by tyrosine phosphorylation (Das et al., 2000). The role of SOS self-association in mediating these events is currently not known.

The DH domain is a catalytic guanine nucleotide exchange factor motif for Rho/Rac/cdc42-family GTPases (RhoGEF), and this domain in mammalian SOS1 specifically catalyzes exchange on RAC (Nimnual et al., 1998). RAC function has been linked to many important cellular processes, including motility, invasion, and axon pathfinding (Hall, 1998). DH domains are invariably followed by PH domains, which mediate protein-protein and protein-lipid interaction. In the case of SOS, the PH domain may be a point of protein-protein interaction, auto-inhibition (Das et al., 2000) and lipid binding (Chen et al., 1997).

The DH and PH motifs of SOS are followed by the domains important for RasGEF activity (Figure 4A). The RAS exchanger motif (REM) is important for full RasGEF function (Chen et al., 2000), and recently has been shown to enable an allosteric interaction between

Figure 1:

SOS encodes a 1600 amino acid protein with a diverse domain structure.

The SOS protein contains two types of GEF domain, one specific for catalysis of guanine nucleotide exchange on the small GTPase RAC, and the other specific for exchange on the small GTPase RAS. There are also many protein-protein interaction motifs in SOS, including the PH domain, REM, and PXXP motifs.

Figure 1

activated RAS and SOS which stimulates SOS to be a more potent RasGEF for a second RAS molecule (Margarit et al., 2003). Following the REM, the Cdc25 homology region encodes the catalytic RasGEF activity (Lai et al., 1993; Liu et al., 1993). Structural modeling and affinity studies have suggested a model whereby SOS binding to RAS through the Cdc25 homology region leads to distortion of the RAS nucleotide binding site, thus promoting release of guanine nucleotide (Boriack-Sjodin et al., 1998; Lai et al., 1993).

The C-terminus of SOS regulates SOS localization and perhaps even function. Signaling through RTKs recruits SOS to the plasma membrane through binding of the SH3 domains of the adaptor protein GRB2/DRK (Buday and Downward, 1993; Olivier et al., 1993) to the C-terminal PXXP motifs of SOS (Figure 4A). Recruitment of SOS to the membrane allows it to catalyze GDP/GTP exchange to stimulate RAS. In a parallel mechanism, SOS appears to be targeted to actin filaments by interaction of the C-terminal PXXP motifs with the SH3 domains of ABI-1 in an ABI-1/EPS-8 complex, where SOS may then act as a RacGEF (Scita et al., 1999; Scita et al., 2001).

A third possible role for the C-terminus of SOS as an autoinhibitory domain (Corbalan-Garcia et al., 1998) stems from observations of MAPK hyperactivation in response to ligand after transfection with a Sos C-term truncation (Byrne et al., 1996), and from phenotypic analysis of overexpression of a SOS C-term truncation in the fly eye (Karlovich et al., 1995). This autoinhibition may be regulated by a negative feedback loop whereby activated MAPK phosphorylates SOS leading to its downregulation. Supporting this idea, ERK-1 MAPK phosphorylates mammalian SOS1 in vitro and EGF stimulation of cultured fibroblasts induces a SOS1 mobility shift consistent with phosphorylation (Buday et al., 1995). Although the phosphorylated residues have yet to be mapped, the C-terminus of *Drosophila* SOS contains five

putative MAPK phosphorylation sites, while the C-terminus of mammalian SOS contains six putative MAPK phosphorylation sites (defined as P-X-S/T-P, Supplementary Figure 1).

Although SOS is ubiquitously expressed, it is tightly regulated by signaling events and unable to activate RAS or RAC without proper targeting to specific subcellular locations (Corbalan-Garcia et al., 1998; Scita et al., 1999). For example, studies constitutively targeting mouse and human SOS1 to the cell membrane observe a higher transforming potential of these constructs (Quilliam et al., 1994). SOS function also may be limited by availability of binding partners, as has been shown for ABI-1, where that protein is the limiting factor in targeting SOS to sites where it may activate RAC (Innocenti et al., 2002). Thus SOS function appears to be regulated both by heterologous protein-protein associations and by autoinhibitory interactions. A recent study has implicated phosphorylation of SOS by the non-receptor tyrosine kinase Abl as a mechanism for activation of SOS RacGEF activity (Sini et al., 2004). While phosphorylation of the various functional domains provides a potential mechanism for signal dependent activation or repression of SOS function, much remains unknown about the complex interactions of the different SOS domains, and how these different regulatory elements are coordinated *in vivo*.

Here we present the characterization of a novel class of *Sos* alleles isolated as dominant enhancers of eye specific expression of yan^{act}, a constitutively active allele that is no longer subject to downregulation via MAPK (Rebay et al., 2000). These *Sos* alleles, referred to collectively as $Sos^{EY2\text{-}3}$, comprise a series of point and deletion mutations throughout the *Sos* locus that individually disrupt many of the different domains important for SOS function. We have used these new alleles, together with previously characterized null alleles, to explore SOS function during development and have found defects in both gastrulation and later development. Sequencing and further *in vivo* characterization of the $Sos^{EY2\text{-}3}$ alleles has shown that they are not

null alleles and that at least one, Sos^{M98}, acts as a dominant negative. The amino acids and functional motifs affected by these mutations are all conserved from *Drosophila* to human SOS1, suggesting that our findings may have implications for SOS regulation and function in higher eukaryotes.

Results and Discussion

Isolation of new *Sos* alleles

Previously, we isolated eleven alleles of *Sos* as dominant enhancers of the phenotype induced by eye specific expression of yan^{act}, a dominant active allele of the transcriptional repressor *yan* (Rebay et al., 2000). Enhancement of the yan^{act} phenotype suggests that these mutations, referred to as $Sos^{EY2\text{-}3}$ alleles, impair endogenous RTK signaling, consistent with *Sos* functioning as a positive component of the pathway. However, as described below, subsequent genetic and molecular analyses suggest that the $Sos^{EY2\text{-}3}$ alleles behave differently than previously characterized null mutations, Sos^{e2h} and Sos^{e4G}, which contain early termination codons at amino acids 579 and 421, respectively (Simon et al., 1991).

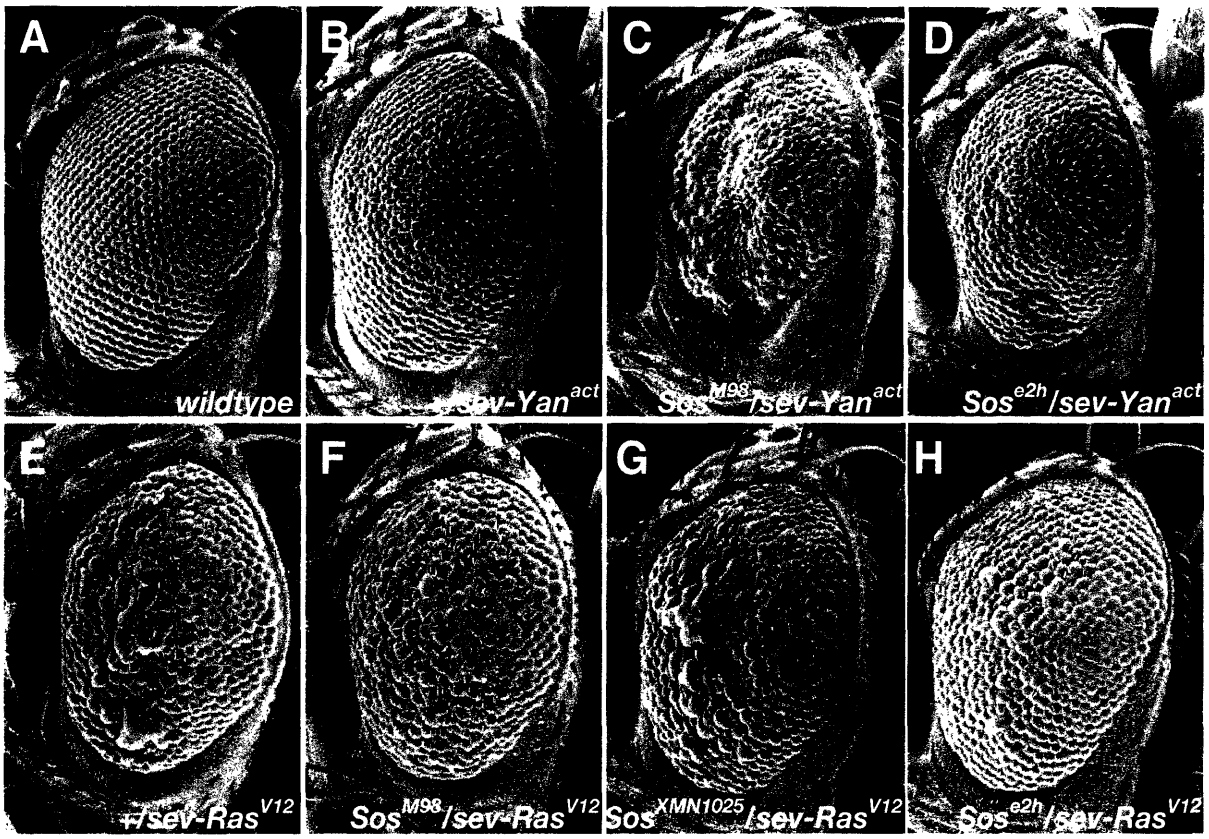
For example, while the $Sos^{EY2\text{-}3}$ complementation group contains many strong enhancers of $sev\text{-}Yan^{act}$ (Figure 2C, compare to Figure 2B, and wildtype in Figure 2A), Sos^{null} alleles do not enhance the phenotype (Figure 2D). Further discrepancies emerge when genetic interactions with a $sev\text{-}Ras^{V12}$ (activated *Ras*) transgene were examined. Sos^{null} alleles strongly suppress the $sev\text{-}Ras^{V12}$ rough eye phenotype (Figure 2H, compare to Figure 2E), while $Sos^{EY2\text{-}3}$ alleles only weakly suppress (Figure 2F-G, (Rebay et al., 2000). Transverse sections revealed that the extra R7 photoreceptors produced by $sev\text{-}Ras^{V12}$, on average 2.6 per ommatidia, (Figure 3B-C,

Figure 2:

$Sos^{EY2\text{-}3}$ alleles are strong enhancers of $sev\text{-}yan^{act}$

Scanning electron micrographs (SEM) of Drosophila eyes show the wildtype (A) compared to the screen starting phenotype with $sev\text{-}yan^{act}$ (B), and a *Sos* allele isolated by the screen which dominantly enhances this phenotype (C). In contrast, a null allele of *Sos* does not enhance the $sev\text{-}yan^{act}$ phenotype (D). The $sev\text{-}Ras^{V12}$ phenotype is shown in (E). The rough eye is only mildly suppressed by $Sos^{EY2\text{-}3}$ alleles (F,G), unlike null alleles of Sos which strongly suppress $sev\text{-}Ras^{V12}$ (H).

Figure 2


A
wildtype
B
+/sev-Yan^{act}
C
Sos^{M98}/sev-Yan^{act}
D
Sos^{e2h}/sev-Yan^{act}
E
+/sev-Ras^{V12}
F
Sos^{M98}/sev-Ras^{V12}
G
Sos^{XMN1025}/sev-Ras^{V12}
H
Sos^{e2h}/sev-Ras^{V12}

compare to wildtype in Figure 3A) are dominantly suppressed by Sos^{null} alleles to only 1.4 R7 cells per ommatidia and to a lesser extent, to 1.9 R7 cells, by $Sos^{EY2\text{-}3}$ (Figure 3D). Moreover, the overall disorganization of ommatidia observed in the *sev-Ras*^{V12} (Figure 3B) background is suppressed by the null allele of *Sos* (Figure 3C) but not $Sos^{EY2\text{-}3}$ (Figure 3D). Thus our screen for modifiers of yan^{act} isolated a distinct class of *Sos* alleles, illustrating the potential of different genetic modifier screens to isolate different subsets of alleles of a given gene.

Molecular Characterization of $Sos^{EY2\text{-}3}$ alleles

To investigate whether the $Sos^{EY2\text{-}3}$ alleles might provide useful tools for analyzing Sos regulation and function, we determined the molecular nature of these mutations (for details, see Materials and Methods). Sequencing of eight $Sos^{EY2\text{-}3}$ alleles revealed mutations in conserved domains that would be predicted to specifically affect activity levels of Sos (Figure 4A).

Sos^{M98} (D296N), a point mutation in the DH domain, disrupts a highly charged surface (Soisson et al., 1998), that may be a point of protein-protein interaction. The DH domain of SOS is the site of RacGEF activity (Nimnual et al., 1998), and a disruption of protein-protein interaction there might interfere with or promote GEF activity. Strikingly, the residue affected by Sos^{M98} is conserved not only amongst SOS homologs from *Drosophila* to humans, but also in the DH domains of distantly related GEFs such as TIAM-1 and TRIO (Figure 4B). Another mutation that maps to the DH domain, Sos^{XEQ410}, is a small in frame deletion that replaces K385 and L386 with a single Methionine (Figure 4A). As this region of SOS forms an alpha-helix (Soisson et al., 1998), loss of an amino acid likely disrupts the remainder of the helix.

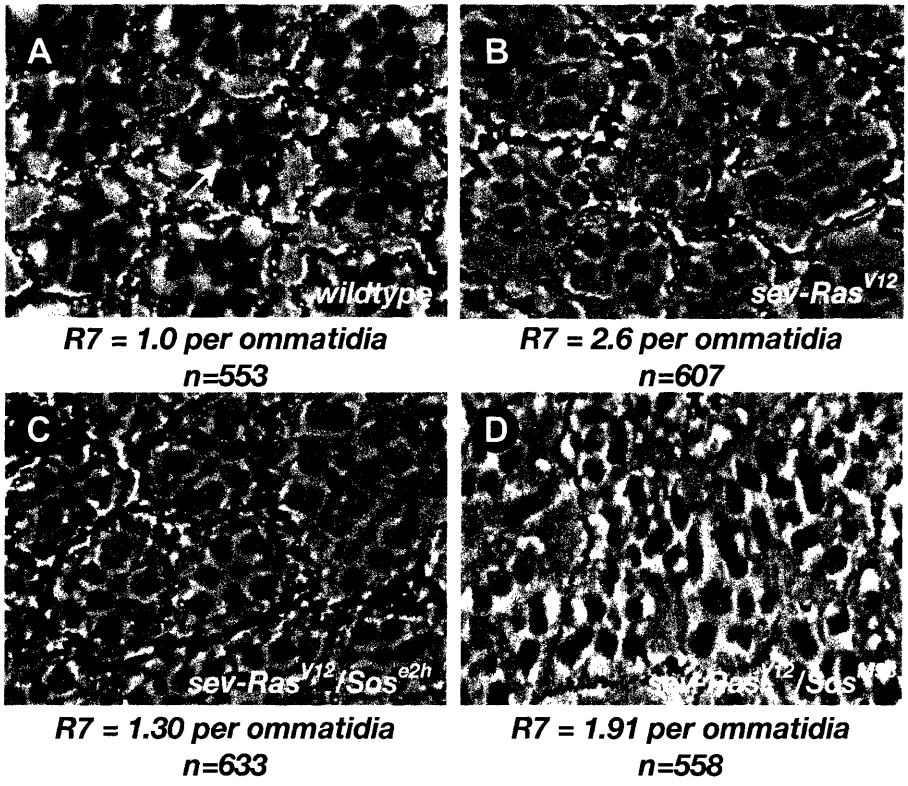
Five $Sos^{EY2\text{-}3}$ alleles appear likely to impair RasGEF function based on their molecular lesions. Sos^{EK1069} and Sos^{XER522} have early termination codons, before and after the Pleckstrin

Figure 3:

$Sos^{EY2\text{-}3}$ alleles mildly suppress $sev\text{-}Ras^{V12}$

sev-Ras^{V12} flies have disorganized ommatidia and extra R7 photoreceptors (B, 2.6 per ommatidia, versus 1.0 per ommatidia in wildtype eyes (arrow indicates R7, A). This phenotype is strongly suppressed by heterozygosity for *Sos* (C), with greater organization and fewer R7s (1.3 per ommatidia). In contrast, heterozygosity for *Sos*^{M98} leads to some suppression of extra R7s (1.9 per ommatidia) but the eye remains disorganized.

Figure 3



R7 = 1.0 per ommatidia
n=553

R7 = 2.6 per ommatidia
n=607

R7 = 1.30 per ommatidia
n=633

R7 = 1.91 per ommatidia
n=558

Homology (PH) domain, respectively (Figure 4A), leaving both without the RasGEF domains. $Sos^{XMN1025}$ and Sos^{UV349} both have missense mutations in the REM of SOS, an integral domain for RasGEF function (Chen et al., 2000) and a point of SOS-RAS interaction (Margarit et al., 2003). The most direct lesion to the RasGEF catalytic domain is in Sos^{XB10}, where a six base pair deletion removes two conserved residues, V1011 and A1012 (Figure 4A).

Sos^{XF9} is a small deletion near the C-terminus that leads to truncation of the PXXP motifs (Figure 4A). These motifs, which appear multiple times in the last several hundred amino acids of SOS, are important for SOS binding to the SH3 motif of DRK/GRB2 (Chardin et al., 1993), and for SOS binding to the SH3 domain of ABI-1, which may target SOS to RAC (Innocenti et al., 2002; Scita et al., 1999). Our finding that this allele contains intact catalytic domains yet displays defects in RTK signaling, indicates the importance of the full complement of PXXP motifs for SOS function *in vivo*.

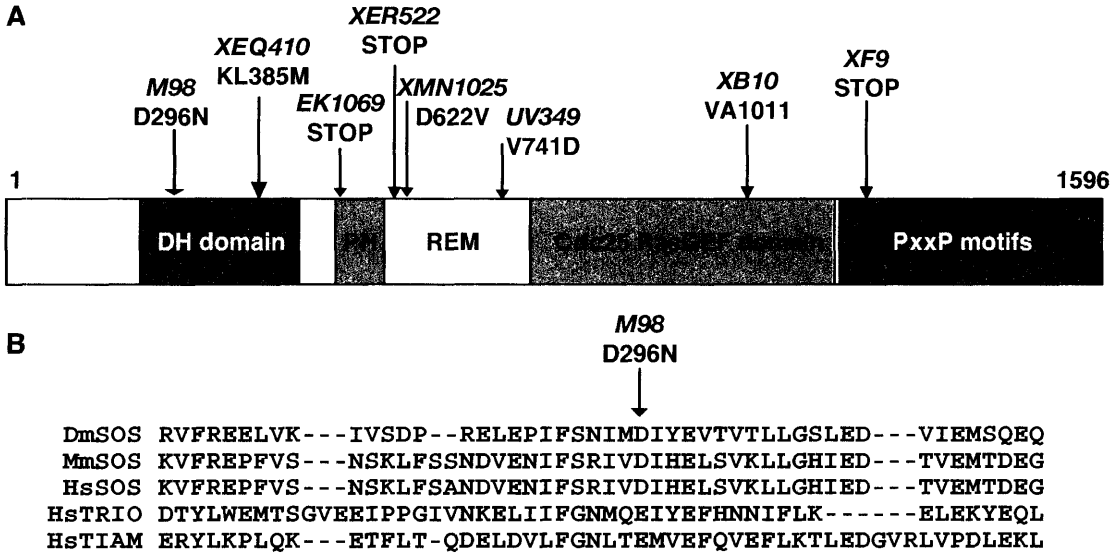
Emphasizing their relevance to understanding SOS function in higher eukaryotes, each of our alleles map to residues conserved in SOS homologs from *Drosophila* to humans (Supplementary Figure 1). Our alleles span the SOS protein, suggesting that multiple domains are necessary for proper function, and that mutations enhancing $sev\text{-}yan^{act}$ are not specific to any particular domain. In addition, the alleles that lack the RasGEF domain, Sos^{EK1069} and Sos^{XER522}, but still do not suppress the $sev\text{-}Ras^{V12}$ eye phenotype are of particular note as they indicate that the genetic suppression of $sev\text{-}Ras^{V12}$ by *Sos* requires inactivation of both the RAS and RAC GEF domains (Figure 1). This may reflect the complex interactions between RAS and RAC signaling, where RAS activation has often been linked to activation of RAC (Scita et al., 2000), and may indicate that activation of RAC contributes to the $sev\text{-}Ras^{V12}$ phenotype.

Figure 4:

Molecular characterization of *Sos*$^{EY2-3}$ alleles

Sos^{EY2-3} alleles map to discrete domains along the SOS protein (A). Allele name is in italics and the molecular lesion is indicated below. The residue affected in the *Sos*^{M98} allele is conserved amongst SOS homologs and in DH domains from unrelated proteins TIAM-1 and TRIO (B).

Figure 4


A
XER522
STOP
XEQ410
KL385M
M98
D296N
EK1069
STOP
XMN1025
D622V
UV349
V741D
XB10
VA1011
XF9
STOP
1
1596
DH domain
REM
PxxP motifs
B
M98
D296N
DmSOS RVFREELVK---IVSDP--RELEPIFSNIMDIYEVTVTLLGSLED---VIEMSQEQ
MmSOS KVFREPFVS---NSKLFSSNDVENIFSRIVDIHELSVKLLGHIED---TVEMTDEG
HsSOS KVFREPFVS---NSKLFSANDVENIFSRIVDIHELSVKLLGHIED---TVEMTDEG
HsTRIO DTYLWEMTSGVEEIPPGIVNKELIIFGNMQEIYEFHNNIFLK------ELEKYEQL
HsTIAM ERYLKPLQK---ETFLT-QDELDVLFGNLTEMVEFQVEFLKTLEDGVRLVPDLEKL

Analysis of *Sos* mutant phenotypes in the eye and wing

In order to understand how these mutations affect SOS function, we analyzed the mutant phenotype of our $Sos^{EY2\text{-}3}$ alleles, focusing on tissues in which RTK signaling plays well-documented roles. For example, RTK signaling has been shown to be crucial for proper recruitment and survival of photoreceptors in the Drosophila eye (Freeman, 1997) and for growth, patterning and specification of vein fate in the wing (Diaz-Benjumea and Hafen, 1994); (Nagaraj et al., 1999). Although all of the *Sos* alleles identified by our screen are homozygous lethal, several viable or partially viable transheterozygote combinations allowed us to examine phenotypes in the adult animal. In the wing, transheterozygotes exhibit wing size reduction and loss of wing vein material (Figure 5B, compare to wildtype in 5A). In addition, the $Sos^{EY2\text{-}3}$ transheterozygotes have small rough eyes with missing photoreceptors (Figure 5D, compare to wildtype in 5C). Photoreceptor R7 appears most severely affected and is lost in 60% of ommatidia. Both the eye and wing phenotypes are consistent with reduced RTK/RAS/MAPK signaling and with the $Sos^{EY2\text{-}3}$ alleles behaving as partial loss-of-function mutations.

Because interallelic complementation in the transheterozygous combinations might partially mask $Sos^{EY2\text{-}3}$ phenotypes, the FLP-FRT system (Xu and Rubin, 1993) was used to produce marked clonal patches of homozygous mutant tissue in the eye. For this analysis, two Sos^{null} alleles, Sos^{e2H} and Sos^{e4G} (Simon et al., 1991), and two *EY2-3* alleles, Sos^{M98}, a mutation in the DH domain, and $Sos^{XMN1025}$, a mutation in the REM, were examined. For these analyses, only one representative of each class is shown, as the same results were observed for both.

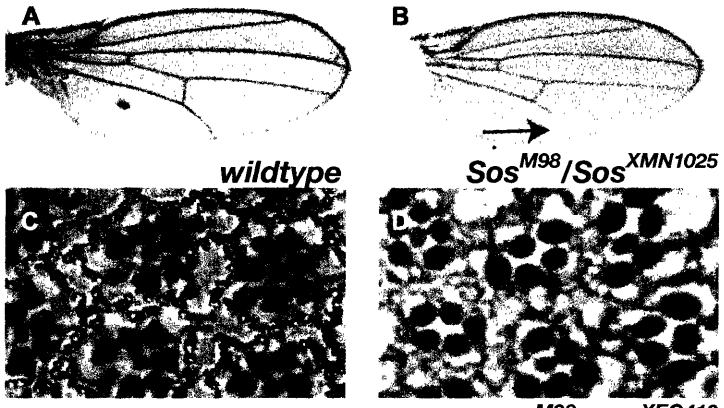
Sos^{null} cells do not survive to become part of the adult eye, resulting in ommatidia with fewer than the normal complement of photoreceptors (Figure 6A). In contrast, $Sos^{EY2\text{-}3}$ mutant tissue survives to form large clones in which many ommatidia are missing photoreceptors, with

Figure 5:

***Sos*$^{EY2-3}$ mutant tissue displays defects in wing vein fate and photoreceptor recruitment**

Transheterozygotes of *Sos*$^{EY2-3}$ alleles have shortened wing veins (arrow, B), and lack R7 photoreceptors in 60% of ommatidia (D). Wild type controls are shown in (A, C).

Figure 5


A
B
wildtype
Sos^M98/Sos^XMN1025
C
D
wildtype
Sos^M98/Sos^XEQ410
R7=1.0 per ommatidia
n=553
R7=0.41 per ommatidia
n=603

R7 lost at a comparable frequency to that seen in transheterozygotes (Figure 6B). The lower penetrance of photoreceptor loss observed in Sos^{EY2-3} mutant tissue compared with complete penetrance of photoreceptor loss observed in null alleles suggests that, consistent with the molecular nature of these alleles and with the transheterozygote phenotypes, Sos^{M98} and $Sos^{XMN1025}$ represent hypomorphic mutations in the *Sos* gene.

To ask whether photoreceptor recruitment occurs in the complete absence of *Sos* function, Sos^{null} clones were analyzed earlier in eye development by examining elav expression in the 3rd instar eye discs. As shown in Figure 6C, Sos^{null} tissue does not support specification of photoreceptor neurons. The lack of Sos^{null} tissue in the adult eye suggests the undifferentiated mutant tissue is removed by cell death, consistent with RAS signaling being required for cell survival in the eye (Bergmann et al., 2002).

A role for SOS in early embryonic patterning

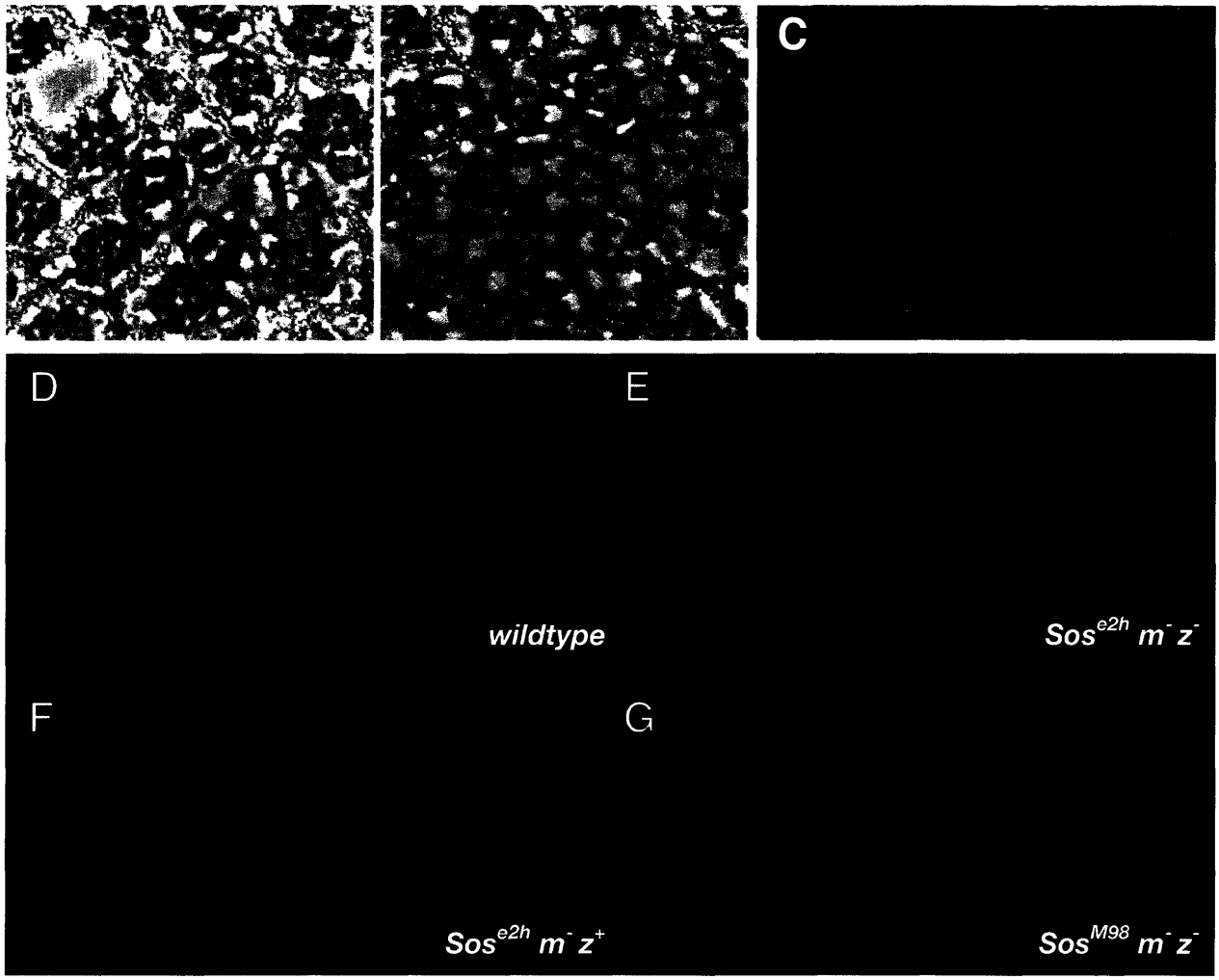
Zygotic mutant embryos of *Sos*, EY2-3 or null, have no obvious defects associated with their lethality. Many survive embryonic development, presumably due to high levels of maternal *Sos*, but die as 1st instar larvae. To examine the phenotype of *Sos* mutants without maternal contribution, we used the FLP-FRT ovo^{D1} system (Chou and Perrimon, 1996) to produce homozygous mutant germline clones. Embryos which lack maternal and zygotic Sos^{null} show a striking twisted or "u-shaped" phenotype, which is readily observed using the segmentally patterned marker anti-ENGRAILED (Figure 6E, compared to wildtype in 6D). This phenotype is similar to that seen in *corkscrew* mutants (Perkins et al., 1996; Perkins et al., 1992), a component of the RTK signaling pathway, and is likely to result from defects in gastrulation and germband retraction. Interestingly, the patterning defects but not the lethality of *Sos* maternal

Figure 6:

***Sos^{null}* tissue displays defects in photoreceptor specification and gastrulation**

Sos^{null} clones do not contain any mutant photoreceptors (A), while *Sos^{M98}* clones contain some photoreceptors (clone marked by lack of pigment, B). In third instar larvae, *Sos^{null}* clones are larger than seen in the adult but cannot specify photoreceptor cell fate (clones marked by lack of GFP, C). *Sos* maternal and zygotic null embryos have striking "corkscrew" phenotypes, shown by anti-engrailed staining in E; compare to wildtype embryo in (D). These patterning defects are rescued by zygotic contribution of wildtype SOS (F). Maternal and zygotic *Sos$^{EY2\text{-}3}$* embryos are homozygous lethal but do not display obvious patterning defects (G). Embryo shown is *Sos^{M98}*; similar results were observed for *Sos^{XMN1025}* germline clones.

Figure 6



and zygotic null embryos can be rescued by zygotic contributions of *Sos* (Figure 6F), indicating that maternal *Sos* is not essential for early embryonic patterning. The striking "corkscrew" phenotype has not previously been reported for *Sos* mutant embryos, and reveals the importance of maternally derived stores of SOS during embryogenesis. In contrast, germline clones of $Sos^{EY2\text{-}3}$ alleles are grossly normal (Figure 6G), though lethal late in embryogenesis/early 1st larval stages. This lethality can be rescued by zygotic *Sos*, suggesting again that the $Sos^{EY2\text{-}3}$ mutations are partial loss of function alleles.

Overexpression of SOS^{M98} produces dominant negative phenotypes

In order to explore further the function of the SOS$^{EY2\text{-}3}$ mutant protein compared to wildtype SOS, transgenic flies carrying full length Sos^{wt} and Sos^{M98} cDNA under the control of the UAS promoter were used to misexpress SOS in the wing and eye. Among the EY2-3 alleles, Sos^{M98} was chosen for overexpression because it is the strongest enhancer of $sev\text{-}Yan^{act}$. Moreover the residue affected by this mutation is conserved not only between SOS homologs but also in DH domains such as those found in Trio and Tiam1 (Figure 4B), suggesting that this mutation may affect a conserved aspect of DH domain function. Both immunofluorescence and western blot analyses indicated no difference in the subcellular localization, expression level or size of Sos^{M98} compared to Sos^{wt} (Figure 7).

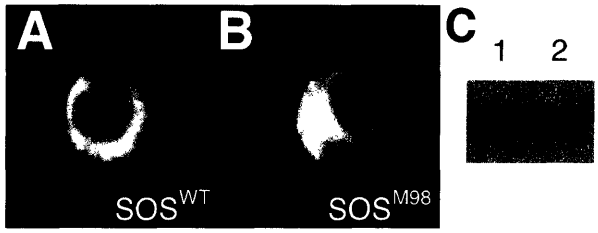
To examine the effect of overexpressing SOS in the wing, we used a GAL4 driver highly activated in the wing disc, *dpp-GAL4*, to induce expression of the two transgenes. Overexpression of Sos^{wt} leads to a thickening of the L3 wing vein (Figure 8B, 8E, compare with wildtype in 8A), consistent with increased RTK signaling leading to ectopic wing vein tissue

Figure 7:

SOS^{M98} is localized normally and expressed at similar levels to wildtype SOS

Antibody staining of SOS in transfected S2 cells shows cytoplasmic localization for wildtype SOS (A) and SOS^{M98} (B). Analysis by western blot (C) shows that wildtype and mutant protein are the same size and expressed at similar levels.

Figure 7



(Diaz-Benjumea and Hafen, 1994). Overexpression of Sos^{M98} yields a range of small, blistered wings (Figure 8C, 8D), some with ectopic wing margin bristles, shown at higher magnification in Figure 8F. The small size of these wings may be indicative of a growth defect, a phenotype often associated with loss of RTK signaling (Schweitzer and Shilo, 1997).

We used *sev-GAL4*, which is activated in a subset of photoreceptor cells (R3, 4 and 7) and in cone cells, to drive expression of Sos^{wt} and Sos^{M98} in the eye. Overexpression of Sos^{wt} has no observable effects on eye development, and does not affect the number of photoreceptors specified (Figure 8G), indicating tight controls on the activity of SOS protein. Strikingly, overexpression of Sos^{M98} leads to disorganization of the ommatidial structure, missing ommatidia, and loss of photoreceptors, particularly of R7, which is missing in about half the ommatidia (Figure 8H, compare to driver alone in Figure 8F). This loss of photoreceptor phenotype is consistent with defects in RAS pathway activation and with the size and patterning defects observed upon overexpression in the wing.

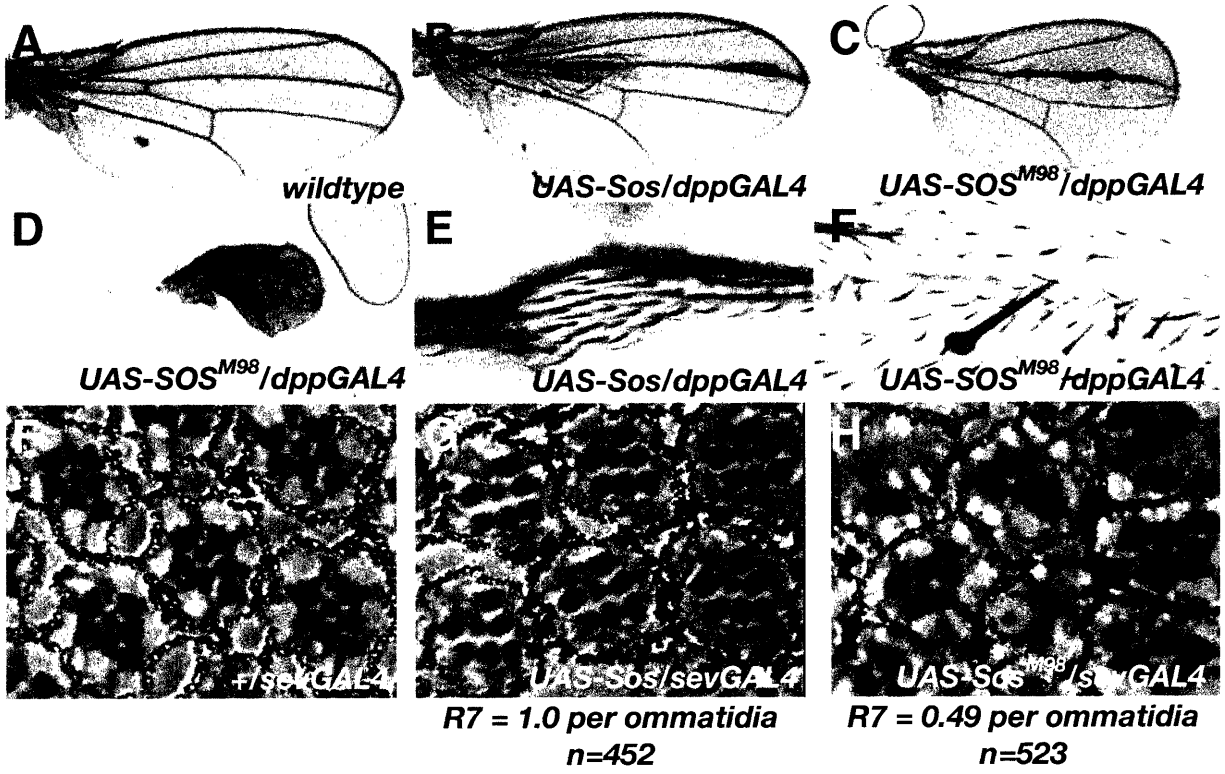
This leads us to propose that SOS^{M98} may function as a dominant negative when overexpressed, perhaps by sequestering fully active SOS into less active complexes, thus reducing the available pool of active SOS. Although Sos^{M98} behaves in certain respects as a hypomorphic allele, it also exhibits some dominant negative characteristics. For example, while Sos^{M98}/Sos^{M98} flies die at late embryonic or early larval stages, Sos^{M98}/Deficiency animals survive to adulthood. Similarly, if Sos^{M98} were simply a hypomorph, one would expect it to display more deleterious phenotypes in trans to a deficiency than in trans to a hypomorphic allele, whereas in fact comparable defects are observed (Figure 5 and data not shown).

Figure 8:

Overexpression of SOS^{M98} disrupts RTK signaling

Ectopic expression of wildtype SOS in the wing leads to thickening of wing veins (B and higher magnification, E, relative to wild type, A). In contrast, overexpression of SOS^{M98} is associated with smaller wings, disruption of wing veins and induction of ectopic margin bristles (C, D, and higher magnification in F). In the eye, overexpression of wildtype SOS has no effect (F, G), while overexpression of SOS^{M98} causes disorganization of ommatidia and loss of photoreceptors, particularly R7 (H).

Figure 8


A
wildtype
B
UAS-Sos/dppGAL4
C
UAS-SOS^{M98}/dppGAL4
D
UAS-SOS^{M98}/dppGAL4
E
UAS-Sos/dppGAL4
F
UAS-SOS^{M98}/dppGAL4
G
+/sevGAL4
H
UAS-Sos/sevGAL4
UAS-Sos^{M98}/sevGAL4
R7 = 1.0 per ommatidia
n=452
R7 = 0.49 per ommatidia
n=523

Concluding Remarks

Our screen for enhancers of *yan*^{act} has isolated a collection of new alleles in the RasGEF SOS affecting multiple conserved domains in this complex multidomain protein. These alleles impair but do not abolish SOS activity and thus may provide unique and useful means for dissecting the complexities of SOS function and regulation. Of particular interest is SOS^{M98}, which as discussed above contains a single amino acid change in the DH domain and may function as a dominant negative. This allele may prove an important tool for dissecting the dual roles of SOS in the activation of RAS and RAC, particularly in understanding the mechanism by which these activities of SOS are coordinated.

Acknowledgements

We thank U. Banerjee for *sE-Sos* plasmid, R. Latek for help with bioinformatics, and T. Tootle and A. Brown for helpful comments on the manuscript. We thank all Rebay Lab members for their valuable advice on this project. Scanning electron microscopy and confocal imaging were performed in the W. M. Keck Biological Imaging Facility. S.J.S. is a Howard Hughes Medical Institute Predoctoral Fellow. I.R. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences and is a Rita Allen Foundation Scholar. This work was supported by National Institutes of Health Grant RO1 EY-12549 to I.R.

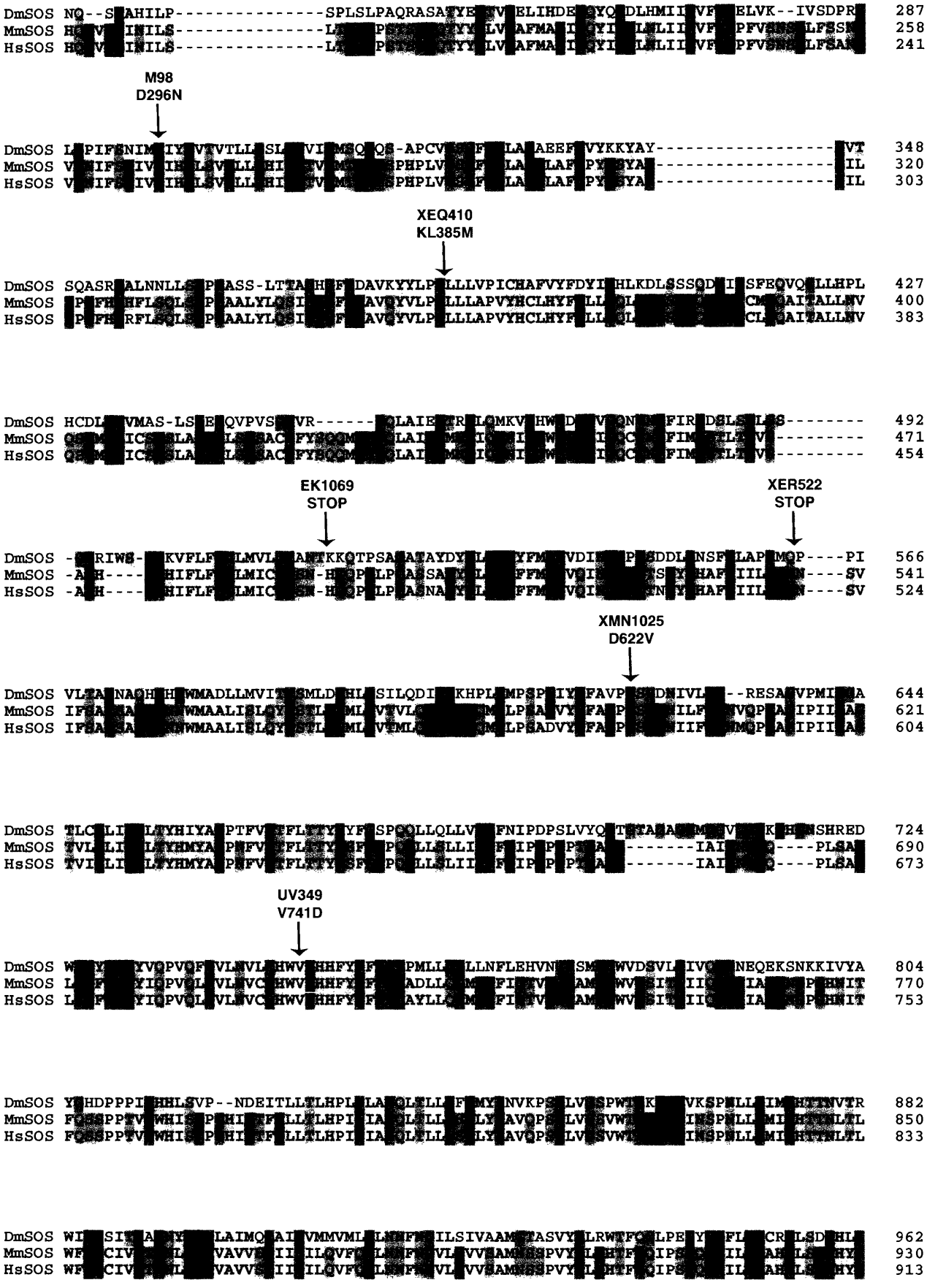
Supplementary Figure

Supplementary Figure 1:

***Sos*$^{EY2\text{-}3}$ alleles disrupt conserved residues in SOS**

Alignment of *Drosophila,* mouse, and human SOS proteins shows that all *Sos*$^{EY2\text{-}3}$ alleles cause molecular changes in conserved residues (each allele shown by arrow). Analysis of the C-terminus of SOS reveals 5 putative MAPK phosphorylation sites (defined as P-X-S/T-P) in *Drosophila* SOS (indicated by magenta stars) and 6 putative MAPK phosphorylation sites in mammalian SOS (indicated by green stars), one of which is conserved from *Drosophila* to human (indicated by a red star).

Supplementary Figure 1



Materials and Methods

Molecular Biology and Genetics

The *Sos* coding region was subcloned from the plasmid *sE-Sos* kindly provided by U. Banerjee (Karlovich et al., 1995). Stratagene's Quick-Change mutagenesis scheme was used to alter the affected residue in Sos^{M98}, using primer pairs: M98S 5'-ccgatattctccaacataatgaacatttacgaggtgacggtc-3' and M98 A 5'-gaccgtcacctcgtaaatgttcattatgttggagaatatcgg-3'. *Sos* constructs were shuttled into pUAST (Brand and Perrimon, 1993), and transgenic lines were generated as previously described (Rebay et al., 1993). All overexpression data was confirmed by use of at least three independent transgenes. In all cases, the data were consistent with the example shown.

For sequencing, Sos^{M98} and $Sos^{XMN1025}$ were isolated in trans to a deficiency uncovering the *Sos* locus, genomic DNA isolated and sequenced. The remainder of the $Sos^{EY2\text{-}3}$ alleles were balanced over CyOact-GFP, embryos were collected, allowed to hatch, and then homozygous mutant larvae identified by lack of GFP expression. Genomic DNA was purified and sequenced using an ABI 373 DNA Sequencer.

To remove any unlinked secondary lethals, *Sos* alleles were recombined onto the multiply marked second chromosome *b pr cu pl s*. The markers were then removed by recombination with a wild-type chromosome. All experiments described here were performed with "cleaned" alleles.

For the examination of clones in adult eyes, *Sos FRT40A/CyO* males were crossed to w^{1118} *eyeless-FLP; FRT40A P{w⁺}/CyO*. Mutant tissue was recognizable by the absence of red eye pigment. For larval eye disc clones *Sos FRT40A/CyO* was crossed to w^{1118} *eyeless-FLP; P{Ubi-GFP}, FRT40A*. Mutant clones were recognizable by the absence of green fluorescent protein

fluorescence.

Fly stocks used: dpp-GAL4 (57A1); sev-GAL4 (K24); sev-Ras^{V12} (CR2); Df(2L)b87e25/CyO (Deficiency uncovering the *Sos* locus).

Immunohistochemistry

Mouse polyclonal antibody to SOS was raised against a GST-SOS antigen consisting of amino acids 1722-2863, purified as described (Rebay and Fehon, 2000). Fixation and staining of S2 cells and embryos were performed as previously described (Fehon et al., 1991; Fehon et al., 1990). S2 cells staining was performed using mouse polyclonal antibody to SOS 1:1000, with CY3-conjugated goat anti-mouse secondary (1:10000). Embryo stainings were performed using mouse anti-ENGRAILED (monoclonal antibody 4D9, Developmental Studies Hybridoma Bank (DSHB)), followed by CY3 conjugated goat anti-mouse (1:1000). Fixation and antibody staining of larval eye discs were performed essentially as described (Wolff, 2000). Larval eye discs were stained with mouse anti-ELAV (9F8A9, 1:100; DSHB), followed by CY3 conjugated goat anti-mouse (1:1000, all secondary antibodies from Jackson ImmunoResearch, West Grove, PA)

Histology

Adult flies were prepared for scanning electron microscopy as described in (Tootle et al., 2003). Fixation and tangential sections of adult eyes was performed as previously described (Tomlinson et al., 1987).

Bioinformatics

All alignments were performed using ClustalX (Thompson et al., 1997). SOS protein sequences from Drosophila (DmSOS, gi: 24584199), mouse (MmSOS, gi:6678062), human (HsSOS), gi:15529995), were aligned with each other and with the DH domains of Human TRIO (HsTRIO, gi:3522969), and Human TIAM1 (HsTIAM-1, gi:4507500).

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Appendix B

Generation of Mouse and Guinea Pig antisera for SINE OCULIS

Serena J. Silver, Ishara Mills, and Ilaria Rebay

I.M. made the GST-SO fusion protein construct

In order to study the RD gene network member SINE OCULIS more effectively, we generated a set of polyclonal antibodies raised against the full length SO protein fused to GST. GST-SO was grown in BL21 E. coli and, as it is highly insoluble, we used the protocol for insoluble GST fusion proteins using Urea following by dialysis (Rebay and Fehon, 2000). This produced a protein yield of approximately 1mg/ml, which was then used as antigen for antibody production in mice and guinea pigs by Curagen. Three mice were used and two guinea pigs, with 50ug of antigen per injection for each mouse and 100ug of antigen per injection for each guinea pig. Each animal underwent five rounds of injections with an injection schedule as previously described (Rebay and Fehon, 2000).

Antibody from the first two bleeds was not useful, but serum from the third bleed until exsanguinations produced good quality antibody from both guinea pigs and mice, although the GP antibody is somewhat better.

These antibodies can be used for tissue and western blots (Figure 1), and nicely recapitulate the mRNA expression pattern of SO previously reported (Seimiya and Gehring, 2000). In embryos, use the guinea pig antibody 1:10000, and the mouse antibody 1:2000-5000. In eye discs, use the guinea pig antibody 1:5000, and on western blots use the guinea pig antibody 1:10000, and the mouse antibody 1:5000. It is particularly useful to have antibodies from two different species for SO when performing immunoprecipitation experiments, as the size of SO is similar to heavy chain. Thus for clear results, immunoblotting should be performed with antibody from an organism different from that used for immunoprecipitation.

Perhaps most interestingly, the SO antibody recognizes a triplet of protein via western blotting (Figure 1), similar to the triplet observed upon western blot analysis of human SIX1, which is hyperphosphorylated in mitosis (Ford et al., 2000). It remains to be determined whether

the SO triplet corresponds to a similar phosphorylation event and how this phosphorylation might affect SO function.

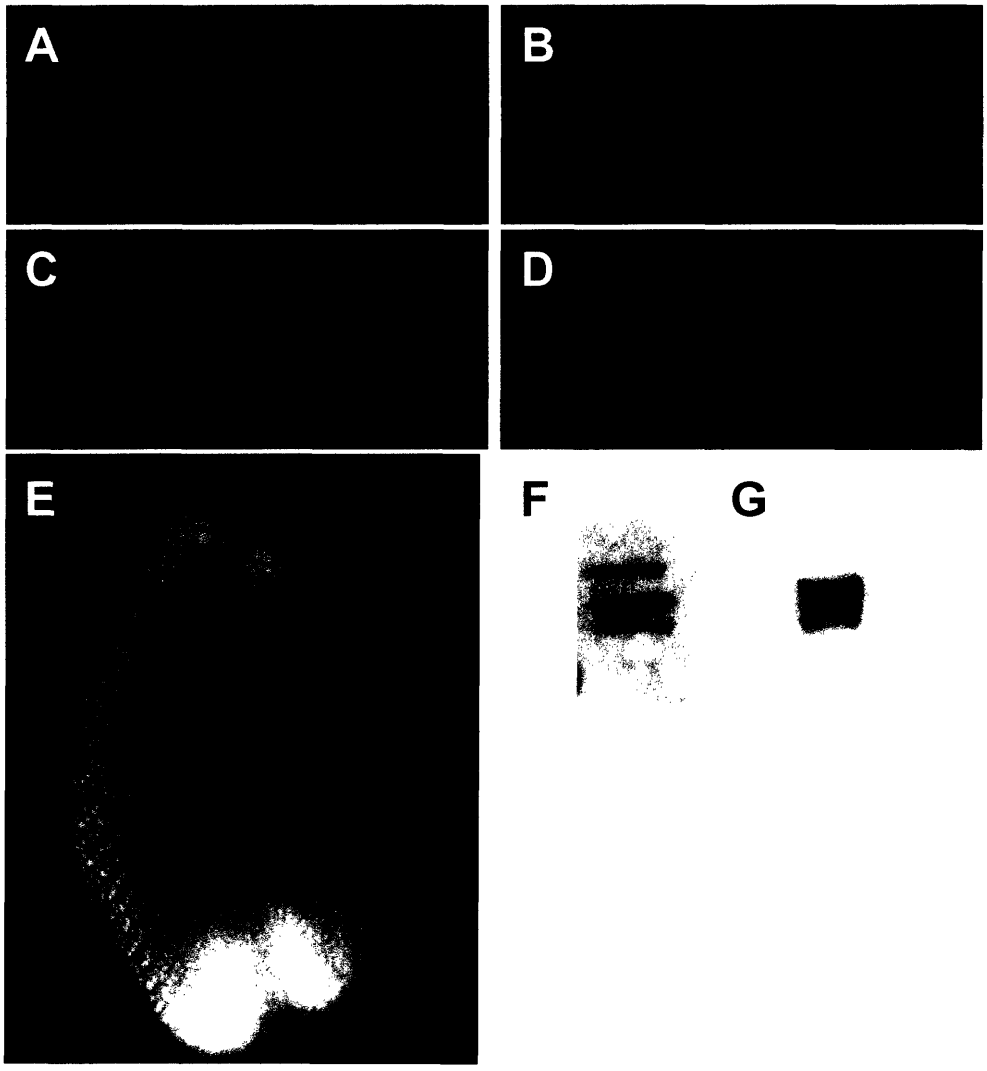

Figure 1: The SO antibody works well in tissue and on western blots. Wildtype embyros at early and late stages stained with GP anti-SO antibody, a-d; third instar larval eye disc stained with GP anti-SO antibody, protein is also visible in MF but out of focus, e; western blot showing the SO protein runs as a triplet, as visualized using GP anti-SO, f, and Mouse anti-SO, g (note: gels run for different lengths of time).

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Appendix C

Analysis of phosphatase activity of human BOR and fly loss of function EYA domain mutations

Serena J. Silver, Tina Tootle, Mousumi Mutsuddi,
Ben Chaffee, Justin Cassidy and Ilaria Rebay

M.M., B.C., and J.C. recapitulated the BOR and fly ED mutants in Mouse EYA3 EYA DOMAIN GST fusion expression constructs. S.J.S. and T.T. purified GST fusion proteins and performed phosphatase assays.

Results

We and others have recently identified a novel function for the EYA DOMAIN of EYES ABSENT as a protein tyrosine phosphatase (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). As several identified mutations in *Drosophila* EYA (Bui et al., 2000), and more intriguingly, in human patients suffering from Branchio-oto-renal syndrome (BOR) or Ocular Defects (OD) (Azuma et al., 2000), map to the EYA DOMAIN (Table 1), we asked whether any of those molecular lesions affect the phosphatase activity of EYA.

Phosphatase assays were performed to analyze the ability of MmEYA3 EYA DOMAIN containing the mutations listed on Table 1 to dephosphorylate the peptide I(pY)GEF as previously described (Tootle et al., 2003). We found that most ED were not able to dephosphorylate peptide, except for the fly allele, T250M, and the complex BOR/OD allele G344S , and one of the OD alleles K465G (Table 2). Thus at least some aspect of the BOR phenotype may be associated with loss of phosphatase activity.

Table 1 – EYA amino acid substitutions

Dm EYA	Mm Eya3	Hs Eya1	Source of mutation
T497M	T250M	T332M	Fly
E528K	E281K	E363K	OD
G594S	G344S	G426S	Complex type with OD & BOR
T643I	T393I	T475I	Fly
S655P	L405P	S487P	BOR
L673R	L423R	L505R	BOR
R715G	K465G	R547G	OD
G723E	G473E	G555E	Fly- in conserved HAD motif

Table 2: Phosphatase activity of EYA DOMAIN mutations

Mm Eya3	Results
T250M	normal ptpase activity - km= 220uM, kcat =.0008
E281K	no ptpase activity
G344S	good ptpase activity - km = 871uM, kcat = 4.7 * 10^-7
T393I	no ptpase activity
L405P	no ptpase activity
L423R	no ptpase activity
K465G	Some ptpase activity
G473E	no ptpase activity

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Appendix C

EYA protein protocols

Purification of EYA DOMAIN GST fusion proteins with French Press

1. Grow two overnight cultures (100 ml) of BL21 E. coli containing an IPTG inducible GST construct of interest

2. Dilute cultures 50ml in 500ml of LB in the morning, grow to an OD_{600} of 1.0 (2 liters of LB is considered one prep, and all volumes below assume 2 Liters). Once you dilute the cultures, make sure the water bath shaker in the cold room is set to 16-18 degrees.

3. Move flasks to shaker in the cold room and let equilibrate to 18 degrees (about 10-15 minutes)

4. Add 500ul .8M IPTG to each flask and induce for 2.5 - 3 hours

5. Centrifuge at 3500rpm in Beckman, and resuspend in 7.5ml of Tris buffer for French press (in cold room - 50mM Tris buffer pH 8.0, 1mM EDTA and 100mM NaCl plus protease inhibitor cocktail (1 tablet per 10 mls, Roche)).

6. KEEP EVERYTHING ON ICE! Put resuspended stuff into Sorvall centrifuge tube. The bacteria from 2 Liters will fit into one tube (~40mls)

7. Take your ice bucket with your prep and a small beaker over to the Baker lab to use the cold room French press (dress warmly and cover your ears!! also, bring at least two pairs of gloves)

8. Lyse by three passes through the French press at 1000psi.

9. Clarify lysate by spinning at 16K in sorvall SS-34 rotor for 15-20 minutes.

10. Transfer supernatant to a fresh tube (50ml Falcon tube works well) and add 1ml of 50:50 glutathione-agarose slurry

11. Incubate overnight, rocking at 4 degrees

12. Use the Beckman to spin down beads gently (1000rpm for 30 seconds), and wash 5 times with 50mM HEPES, 300mM NaCl. Leave in a 1:1 slurry of beads and buffer, and transfer to an eppendorf tube. (Make sure that the small centrifuge is cold!)

13. Wash once with elution buffer (50mM Tris pH 7.0, 150mM NaCl) – spin eppendorfs at 3500 for 1 minute to gently pellet beads.

14. Elute in 10mM reduced glutathione in elution buffer (1ml per elution) 15 minutes is generally good for the 1st elution. Three elutions will get most of the protein off of the beads. If the protein is being troublesome sometimes a higher pH (between 8.0-9.0) will work better. GLUTATHIONE can go bad over time!!! If your elutions aren't working and your bottle is old, order a new bottle.

15. Determine the protein concentration by microplate Bradford Assay, using a BSA standard curve (see Biorad protocol). You should get between 0.4 – 1.0 mg/ml protein from a 2 Liter prep.

16. Protein can be stored at 4 degrees in a covered ice bucket, but is best used fresh for phosphatase assays.

Protocol for EYA DOMAIN phosphatase assays

Phosphatase assays are performed with fresh GST-ED fusion protein.

Assays with pNPP

1. Make a 2x buffer mix: 400mM PIPES pH 7.0, 5mM EDTA, and 20mM $MgCl_2$ – use this 2x buffer to dissolve pNPP.
2. Make a 200 mM stock solution of pNPP – I find it easiest to weigh out approximately the right amount of pNPP and then add the appropriate volume of liquid for 200mM – use the molecular weight printed on the bottle – it is sold in anhydrous and regular forms – don't use the tablets, as they don't dissolve in our buffer.
3. Don't forget to set up buffer alone controls!! Perform each timepoint in duplicate or triplicate. Do 6 timepoints and 6 substrate concentrations if possible.
4. Dilute enzyme in ddH_2O such that you are adding about 5ug of enzyme per reaction in a volume of 40 ul; total reaction volume 80ul. Final buffer conditions: 200mM PIPES pH 7.0, 5mM EDTA and 10mM $MgCl_2$
5. Allow reaction to procede at 30 degrees for different time intervals, quench reaction at each timepoint using 40ul of 10M NaOH.
6. Mix by inverting tube, spin down briefly in centrifuge and pipet into plate for Tecan GENios plate reader (be careful – solution is now VERY basic)
7. PNP anion is detected at 405 nm (extinction coefficient $\varepsilon_M = 1.78 \times 10^4$ /cm M). Data from platereader can be copied directly into Microsoft Excel. Use Excel to analyze results – subtract background, average replicates, and correct for Moles of Enzyme in

reaction and Moles of substrate in reaction compared to Moles of product formed (use appropriate conversion based on extinction coefficient)

8. Plot on a Lineweaver Burke or Eadie-Hofstee graph to determine K_m and V_{max}.

Assays with Peptide substrate: I(pY)GEF

1. Reactions are performed in a 50ul volume with a final buffer concentration of 200mM HEPES pH7.0, 10mM MgCl2, 5mM EDTA. Make a 4x buffer mix of 800mM HEPES pH 7.0, 40mM MgCl2, 20mM EDTA – MAKE SURE TO USE PHOSPHATE FREE WATER FOR EVERYTHING.
2. Perform assays for 6 timepoints and 6 substrate concentrations if possible.
3. Make 4x dilutions of substrate using phosphate free water – good starting substrate concentrations are 70, 80, 90, 100, 110, 120 mM final concentration – mix equal parts 4x substrate and 4x buffer to 25ul
4. Dilute enzyme in phosphate free water so that by adding 25ul you add 5ug enzyme per tube.
5. Set up assays such that each timepoint is a separate rack – pipette appropriate substrate concentrations into appropriate tubes so that each timepoint has the full complement
6. Add enzyme to each rack, mixing after rack is complete and put at 25 degrees. Repeat for all timepoints
7. Make a standard curve using the free phosphate and instructions given with the BIOMOL green kit. Aliquot out BIOMOL green needed for the day's experiments using a tissue culture sterile pipette to avoid contamination of the entire bottle.

8. Quench reactions by addition of 100 ul BIOMOL green, which contains Malachite Green/Ammonium Molybdate dye, which will form a complex with free phosphate and color develops over 30 minutes.
9. Detect at 595 nm in the plate reader, copy to excel, and convert to moles of free phosphate using the standard curve.
10. Normalize to background, average points, make sure to account for Moles enzyme used in each reaction.

Note: as the reactions are performed in eppendorfs and transferred, we only read 100ul of the reaction on the plate reader, including the phosphatase standard, to avoid errors due to missed liquid.

Protocol for Immunoprecipitation of EYA from Eye discs and Embryos

EYE DISCS

1. Dissect 100 discs from 3rd instar larvae in S2 cell media – should wind up with 50-75ul of discs when they settle. Transfer with a wide mouth pipet tip into an eppendorf. Pipet off the extra media.

2. Add 100ul whole cell lysis buffer (100 mM NaCl; 50 mM Tris, pH7.5; 2 mM EDTA; 2 mM EGTA; 1% NP-40) and grind with blue pestle. Add 400ul more buffer and rock for 30 minutes. After rocking, grind again (you may want to remove some liquid and put it aside while grinding).

3. Spin at 14K for 1min at 4 degrees. Move supernatant to a fresh tube (KEEP OUT 40ul FOR PRE-IP, add 40ul 2X SDS buffer to PRE-IP), and add GP anti-EYA 1:1000

4. Rock at 4 degrees for 1½ hours. Meanwhile, wash Protein G sepharose beads in lysis buffer at least 3 times (one of these times rock for a while). After final wash add an equal volume of lysis buffer to beads to make 1:1 slurry.

5. Add 30ul of Protein G slurry to lysate – rock for another 1 ½ hours

6. Wash beads 3x in lysis buffer (spin at 3000K 1 minute in between washes)

7. Add equal volume of 2X SDS buffer to the beads.

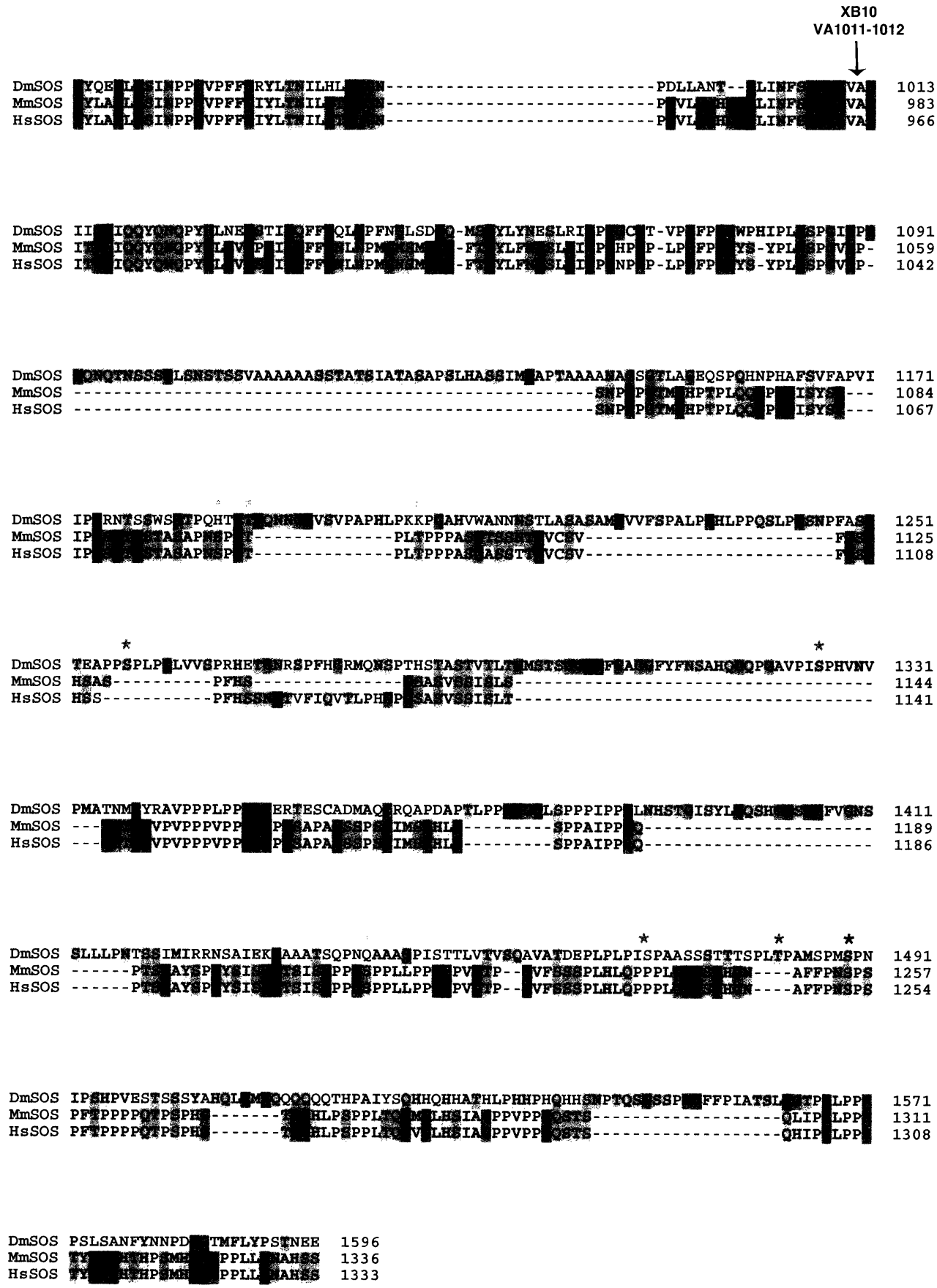
EMBRYOS

1. Collect wildtype embryos overnight in the stinky room

2. Dechorionate embryos.

3. Put embryos into an eppendorf and add some lysis buffer to grind. Add extra lysis buffer and rock at 4 degrees for 30 minutes. After rocking, grind again (you may want to remove some liquid and put it aside while grinding).

4. Follow above from step 3.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Dual functions of the Retinal Determination Gene Network member EYES ABSENT as a transcription factor and protein phosphatase

by

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SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Dual functions of the Retinal Determination Gene Network member EYES ABSENT as a transcription factor and protein phosphatase

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submitted to the Biology Department on June 10, 2004 in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in Biology

Proper development of cell types and tissues requires the integration of extracellular signals to provide context specific information that insures appropriate differentiation. The Drosophila eye is an excellent model for the study of this signal integration, as its development is orchestrated by the interactions of common signal transduction pathways in conjunction with organ specific gene expression. Signaling through these pathways sets the stage for appropriate deployment of the Retinal Determination (RD) gene network members to direct formation of the eye and other organs.

Our studies have focused on the RD gene network member EYES ABSENT as a point of signal integration necessary for the formation of the *Drosophila* eye. We have examined two functions for EYA, the first as a transcriptional co-activator, and the second, more novel function as a protein tyrosine phosphatase.

Previous work suggested that EYA functions as a transcriptional co-activator, particularly in a complex with the DNA binding domain containing RD network member SINE OCULIS (SO). In order to better understand RD network regulation, we performed a structure-function analysis of the EYA protein, which defined the P/S/T rich region of EYA as crucial for EYA transactivation potential. This region is also necessary for EYA mediated ectopic eye induction and rescue of the eya^2 mutant phenotype. We showed that RAS/MAPK signaling potentiates EYA transactivation, providing a mechanism for previously described *in vivo* activation of EYA by MAPK. We have also demonstrated roles for GROUCHO and DACHSHUND in negative and positive regulation of the EYA-SO transcription factor, respectively.

Recently we have begun to study a novel function of EYA suggested by the homology of the highly conserved EYA domain (ED) to the Haloacid dehalogenase (HAD) family. Using the substrate analog para-nitrophenyl phosphate (pNPP), we showed that recombinant ED possesses phosphatase activity, which is affected by tyrosine phosphatase inhibitors but not serine/threonine phosphatase inhibitors. To determine whether this activity is important for EYA function in vivo, mutants that reduce or abrogate phosphatase activity, as shown by lower specific activity or higher K_m in pNPP assays, were tested for their ability to induce ectopic eyes or rescue the EYA mutant phenotype. These mutants, which we refer to collectively as EYA^{HAD}, are unable to induce ectopic eyes or rescue the eya^2 phenotype to the degree of wildtype EYA. As the EYA^{HAD} mutants are all within the ED, which is known to bind to SO, we tested whether these mutants are competent transcriptional coactivators with SO, and found that they retain this activity. Thus the phosphatase and transactivation functions of EYA may represent two distinct essential functions of EYA.

As EYA represents one of the first transcription factors found to possess phosphatase activity, and modulation of phosphorylation state represents a common mode of transcriptional regulation, it will be of particular interest to elucidate the role of EYA phosphatase function *in vivo*, studies which will require identification of transcriptional targets and phosphatase substrates.

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