Distinct Biochemical Activities of Eyes absent During Drosophila Eye Development

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Eyes absent (Eya) is a highly conserved transcriptional coactivator and protein phosphatase that plays vital roles in multiple developmental processes from Drosophila to humans. Eya proteins contain a PST (Proline-Serine-Threonine)-rich transactivation domain, a threonine phosphatase motif (TPM), and a tyrosine protein phosphatase domain. Using a genomic rescue system, we find that the PST domain is essential for Eya activity and Dac expression, and the TPM is required for full Eya function. We also find that the threonine phosphatase activity plays only a minor role during Drosophila eye development and the primary function of the PST and TPM domains is transactivation that can be largely substituted by the heterologous activation domain VP16. Along with our previous results that the tyrosine phosphatase activity of Eya is dispensable for normal Eya function in eye formation, we demonstrate that a primary function of Eya during Drosophila eye development is as a transcriptional coactivator. Moreover, the PST/TPM and the threonine phosphatase activity are not required for in vitro interaction between retinal determination factors. Finally, this work is the first report of an Eya-Ey physical interaction. These findings are particularly important because they highlight the need for an in vivo approach that accurately dissects protein function.
TPM, the PST domain alone, and the TPM alone, respectively. Eya and So bind to each other through the ED of Eya and the Six domain of So to form a transcriptional activator complex. In addition, a series of *Drosophila* S2 cell-based transcriptional activation assays defined the PST/TPM domain as essential for Eya/So-mediated transactivation of a reporter. UAS-eya transgenes that lack both the PST-rich region and the TPM have drastically reduced ectopic eye-inducing capacity, with induction efficiency dropping from 98% to 1.5%.

In addition to regulating transcription, Eya has predicted tyrosine and threonine phosphatase activities in the ED and TPM, respectively. In *Drosophila*, tyrosine-phosphatase-dead mutations lead to strongly reduced activities in ectopic eye induction and in vivo genetic rescue using the GAL4-UAS system. In contrast to these studies, our previous findings revealed that eya genomic rescue (GR) constructs carrying mutations in two key tyrosine phosphatase active-site residues fully restore viability as well as eye formation and function in an eya null mutant background. In mouse and *Drosophila*, the threonine phosphatase activity has been suggested to play an important role in the innate immune system and a recent study using the GAL4-UAS system reported that Eya threonine phosphatase activity is not required for normal *Drosophila* eye development.

Although previous cell culture and in vivo GAL4-UAS based expression studies have suggested specific functions for conserved Eya domains, we have shown that such assays may not always be reliable. In particular, we have developed a genomic rescue (GR) system that provides an accurate method for assessing the functional significance of individual protein domains in vivo. In this study, we have used the GR strategy to conduct functional studies of Eya domains during *Drosophila* eye development. Interestingly, we found that a major function of Eya is transcriptional coactivation, while the threonine phosphates activity plays only a minor role during *Drosophila* development.

**Results**

**The threonine phosphatase activity of Eya plays only a minor role in normal *Drosophila* development.** To study eya function in vivo, we introduced a series of eya genomic rescue constructs (eyaGR) via site-specific transgenesis to investigate the transcriptional activation and threonine phosphatase activity of Eya. A wild-type eya genomic rescue construct (eyaGR) is known to fully rescue viability and eye formation in an eya null mutant background, therefore serving as a positive control throughout our studies. The eyaTPMGR construct has tyrosine-to-alanine substitutions for four key tyrosine residues known to be required for threonine phosphatase activity. The eyaTPM/GR construct has the entire TPM deleted but leaves the PST domain intact. Surprisingly, a single copy of each construct is able to substantially rescue eyaGR or eyaMutIDD mutant phenotypes, restoring viability and rescuing eye size to ~90% (Y4) or ~60% (ΔTPM) of wild-type, albeit with some mild disorganization (Fig. 1d,e and Fig. S1). While there appears to be a largely normal complement and arrangement of rhabdomeres in ommatidia of eya2; eyaY4GR/+ flies (Fig. 2e), eye discs from late third instar larvae (Fig. 2h) and 24 hrs after puparium formation (Fig. 2j) show defects in the number of cone cells and/or ommatidial fusion. Larval eye discs from eya14GR and eyaATPMGR rescued animals are smaller and show a reduction in Eya and So staining anterior to and within the MF while expression levels are normal posteriorly (Fig. 2b,c,e,f), suggesting that the threonine phosphatase activity does play a role during *Drosophila* eye development but this role is relatively minor as the eyaGR construct can restore up to 90% of the eye size. The expression of the core RD genes Dachshund (Dac) and Eyeless (Ey) appear similar in eye discs of positive control and eya14GR-rescued larvae (Figs 2a–c and 3a,b). In addition, we found no difference in photoreceptor axon projections between wild-type and eya2; eyaY4GR/+ flies (Fig. S2c), which show a regular pattern of projections in the lamina of the optic lobe.

eya plays an important role in the developmental events associated with morphogenetic furrow movement. Specifically, clonal analysis has shown that eya is required for the initiation and propagation of the MF and for regulation of the cell cycle. Since loss of threonine phosphatase activity leads to a reduction of Eya expression anterior to and within the MF, we analyzed the effects of eyaGR on both G1 arrest and induction of the proneural gene atonal (ato). We used the cell cycle marker Cyclin B to monitor G1 arrest. Normally, Cyclin B is exclusively expressed in the cells in the G2 and M phases. Immunohistochemistry shows eya14GR rescued animals have largely normal Cyclin B and Ato expression patterns (Fig. 3c–f), implying that the threonine phosphatase-inactive mutations do not adversely affect G1 arrest and initiation of retinal differentiation. This is not surprising since the loss of retinal cells in flies rescued with a single copy of eya14GR is relatively mild; therefore, strong alterations in the expression of markers of cell cycle progression or photoreceptor differentiation are not expected.

**The Eya threonine phosphatase-inactive mutation does not abolish interaction of Eya with Ey, So, or Dac.** Phosphorylation is well known in other systems to regulate protein complex formation and protein stability via ubiquitin-mediated degradation. Accordingly, we hypothesized that one or more of the RD proteins are direct substrates for Eya threonine phosphatase and that loss of this activity either disrupts the formation of RD protein complexes and/or destabilizes the RD proteins themselves. Furthermore, this effect may be specific to complexes involving Eyeless (Ey), thereby limiting effects anterior to the MF where Ey is expressed. We tested this hypothesis by doing co-immunoprecipitation (co-IP) in S2 cultured cells transiently transfected with epitope-tagged Eya, Ey, So, and Dac expression constructs. Similar amounts of RD proteins are expressed in transfected cells with or without Eya threonine phosphatase activity, and the Y4 mutation or the TPM deletion do not affect Eya protein expression levels in S2 cells (data not shown). As shown in Fig. 4, Eya14 and EyaATPM co-IP with Ey, So, and Dac without obviously altered efficiency as wild-type Eya. Notably, this is the first report that Eya can bind to Ey. Previous studies also found that both Eya and Ey proteins interact with So in S2 cultured cells transiently transfected with episomal p35, suggesting Ey, Eya, and So may form a complex to mediate *Drosophila* eye development. Taken together, these observations suggest that the threonine phosphatase activity of Eya is not essential for interactions with other RD proteins.

**The threonine phosphatase motif of Eya has transcriptional activation function.** In addition to threonine phosphatase activity, previous cell culture transactivation reporter assays showed that the TPM
has transcriptional activation function. To test the hypothesis that this function is biologically relevant in vivo, we replaced the TPM only with VP16, a well-known heterologous transcriptional activation domain (Chasman et al., 1989). The resulting construct, \( \text{eya}^{\Delta \text{TPM} + \text{VP16GR}} \), was tested for rescue activity. Remarkably, while \( \text{eya}^{\Delta \text{TPM}} \) can restore about 60% of eye size, VP16 is able to largely complement loss of the TPM and restore eye development to approximately 90% of wild-type, both in \( \text{eya}^2 \) (Fig. 1a,e,f) and \( \text{eya}^{\Delta \text{ID}} \) mutant backgrounds (Fig. S3a). The external eye morphology of \( \text{eya}^{\Delta \text{TPM} + \text{VP16GR}} \) rescued eyes shows only minor disorganization compared to \( \text{eya}^{\Delta \text{TPMGR}} \).

As shown in Fig. 2f, loss of the TPM causes abnormal ommatidial morphology in adult compound eyes. Flies rescued by one copy of \( \text{eya}^{\Delta \text{PST/TPMGR}} \) have a reduced number and unusual arrangement of rhabdomeres compared with the normal trapezoidal array of photoreceptors in wild-type animals. Tangential sections of \( \text{eya}^2; \text{eya}^{\Delta \text{TPM} + \text{VP16GR/+}} \) adult eyes reveal ommatidia with the correct number and largely normal arrangement of rhabdomeres (Fig. S3b). Moreover, in contrast to wild-type (Fig. S2a) and \( \text{eya}^{\Delta \text{ID}} \) mutant backgrounds (Fig. S2e) flies, axon terminations in the lamina plexus have irregular gaps and breaks (yellow arrows) in \( \text{eya}^{\Delta \text{PST/TPMGR}} \) rescued flies, reminiscent of the photoreceptor axon defects in \( \text{eya} \) loss-of-function mutants. These observations suggest that a major role of the TPM during Drosophila eye development is to provide transactivation function, that this activity is required for normal ommatidial development and photoreceptor axon projections, and that this function can be largely substituted by the VP16 domain.

**The entire PST/TPM domain of Eya is critical for transcriptional activation during eye development.** The PST/TPM domain of Eya is critical for transactivation in cell culture reporter assays. In order to characterize the Drosophila Eya transcriptional activity in its native context in vivo, we generated four genomic rescue constructs: \( \text{eya}^{\text{PST/TPMGR}} \) (deletion of the PST/TPM domain), \( \text{eya}^{\text{PSTGR}} \) (deletion of the PST domain alone), \( \text{eya}^{\text{PST/TPM + VP16GR}} \) (substitution of both the PST and TPM domains with the VP16 activation domain) and \( \text{eya}^{\text{PST + VP16GR}} \) (substitution of the PST domain alone with the VP16 activation domain) (Fig. 1a). We found that \( \text{eya}^{\text{PST/TPMGR}} \) completely fails to rescue \( \text{eya}^2 \) or \( \text{eya}^{\Delta \text{ID}} \) mutant phenotypes, even when the transgene is
present in two copies (Fig. 1h and data not shown). We can readily detect the predicted, truncated eyaΔPST/TPM transcript and protein (Fig. 5a–f) in late second instar eye discs prior to MF initiation, suggesting that although the transgene is expressed, at least initially, the EyaΔPST/TPM protein is non-functional. While the eyaΔPST/TPMGR construct completely fails to rescue eya2 mutant animals, the eyaΔPSTGR retains slightly more function and can rescue about 5% of normal eye size (Fig. 1j). Previous S2 cell culture studies have suggested that both the PST and TPM domains contribute transcription activation function10 and our GR data are consistent with these results. In addition to eyaΔPST/TPM+VP16GR, our other VP16 substitution genomic rescue results also confirm these findings. Specifically, the eyaΔPST/TPM+VP16GR is sufficient to rescue about 5% of eye size in an eya2 background (Fig. 1i), similar to that of the eyaΔPSTGR construct alone. eyaΔPST+VP16GR is able to restore eye development to ~30% of wild-type (Fig. 1k). Two copies of eyaΔPST/TPM+VP16GR or eyaΔPST+VP16GR consistently rescue eya2 eye size better than one copy (Fig. S4). Moreover, eyaΔPST/TPM+VP16GR, eyaΔPSTGR, and eyaΔPST+VP16GR fail to rescue eyaclIID mutants. These functional dissection studies reveal that the transactivation domain PST/TPM is essential for eye formation and viability in Drosophila. In addition, the PST domain is likely playing a more significant role than

Figure 2. Threonine phosphatase activity is required for normal anterior expression of eya and cone cell development. Expression of Ey (a–c), Eya (a’–c’), and So (a”–c”) proteins are shown in eya+GR, eyaY4GR, and eyaΔTPMGR rescued animals. (d–f) Adult plastic sections in flies rescued with one copy of eya+GR, eyaY4GR and eyaΔTPMGR, respectively. (g,h) Third instar eye discs by Cut staining. (i,j) Eye discs prepared from 48 hrs after puparium formation and stained with Dlg.
the TPM during Drosophila development since eyaΔTPMGR rescues 60% of the eye size compared to 5% of the eye size rescued by eyaΔPSTGR and eyaΔTPMGR is able to restores viability to eyacliIID null mutants.

The PST/TPM domain regulates retinal determination gene expression. Eya can act as a transcriptional coactivator and physically interact with other RD proteins to regulate multiple developmental processes. Therefore, we were interested in understanding the role of PST/TPM in RD gene regulation since it is critical for Eya function. Since eyaΔPST/TPMGR fails to rescue the eye phenotype of eya2 animals and little Eya expression is detected at late third instar (data not shown), we used second instar larvae to assess the function of the PST/TPM when EyaΔPST/TPM protein is still expressed (Fig. 5f).

eya2 flies rescued with two copies of eyaΔPST/TPMGR show slightly lower Eya expression compared to wild-type or eya GR-rescued animals at 68 hrs after egg laying (AEL) (Fig. 5c–f). We also found that Eya expression in eya2; eyaΔPST/TPMGR eye discs is lower than that of wild-type discs at 56 hrs AEL (Fig. 5g–j). Similar reductions are observed for the expression of the retinal determination protein Dac, a known downstream target of Eya (Fig. 6a–h). In addition, in eyaΔPST/TPM clones (eyacliIID null clones rescued by a single copy of eyaΔPST/TPMGR) at 72 hrs AEL, Dac expression is reduced while the expression of EyaΔPST/TPM is normal (Fig. 6i–l, yellow arrows). Taken together, these data imply that the PST/TPM domain of Eya is required for normal Dac expression.

Moreover, ey-Gal4 induced So expression in eya2 animals rescued by one copy of eyaΔPST/TPMGR partially restores Dac expression (Fig. 7a–d), but has no effect on expression of Eya (Fig. 7e–h). These observations suggest that the PST/TPM positively regulates expression of Dac through the Eya binding partner So.

To test if the PST/TPM deletion affects Ey regulation and photoreceptor differentiation, we assayed Ey and Elav expression in eyaΔPST/TPM rescued eya null mutant clones. We found that eyaΔPST/TPM rescued eya null mutant clones show a complete loss of Elav expression, a marker of photoreceptor differentiation, posterior to the MF (Fig. 8a–d). In eyaΔPST/TPM clones posterior to the furrow, we found strong Ey expression (Fig. 8a’–d’), suggesting the PST/TPM domain of Eya is required for Ey suppression. Additionally, eyaΔPST/TPM clones result in the loss of photoreceptor development and black overgrowths in adults (Fig. S5d).

Deletion of the PST/TPM does not abolish interactions between Eya and Ey, So, or Dac. So and Dac are known binding partners of Eya7,8,11. Since eyaΔPST/TPMGR rescues flies have no eyes, similar to the loss-of-function phenotypes of the core RD genes (cy, so, and dac), we hypothesized that the PST/TPM domain may mediate specific, essential interactions between Eya and Ey, So, or Dac. To test this hypothesis, we carried out co-immunoprecipitation (co-IP) experiments. As shown in Figs 4 and 9, both wild-type and EyaΔPST/TPM...
can co-IP with Ey, So, and Dac, suggesting that deletion of the PST/TPM does not abolish the interactions between Eya and these three RD proteins. These observations are consistent with previous findings that Eya-So and Eya-Dac interaction is mediated via the ED of Eya. The Eya domain that mediates Eya-Ey physical interaction remains to be determined.

Discussion

In this paper we report that loss of threonine phosphatase activity has little effect on Drosophila eye development, since eye development in eyaY4GR rescued flies proceeds relatively normally. On the other hand, the essential function of the PST and the threonine phosphatase motif (TPM) is transcriptional activation that can be largely complemented by the heterologous activation domain VP16. Together with our findings that the PST and TPM are required for normal Drosophila eye development, we conclude that a major function of Eya during Drosophila eye development is as a transcriptional coactivator. Although the tyrosine phosphatase activity of the Eya Domain (ED) is dispensable for Eya function, the specific role the ED plays in vivo has not been reported.

The retinal determination (RD) network is a small group of highly conserved transcriptional regulators that are both necessary for eye development and sufficient to trigger ectopic eye formation when overexpressed...
As a vital member of the RD network, a unique feature of the Eya proteins is that they have several distinct biochemical activities. In *Drosophila*, previous cell culture reporter assays and cDNA-based Gal4-UAS genetic rescue studies suggested that the PST-rich region is a transactivation domain and plays a role in ectopic eye induction, while the TPM and ED possess threonine and tyrosine phosphatase activity, respectively. Intriguingly, our results using genomic rescue constructs are consistent with previous studies of the PST/TPM transactivation domain, but are contrary to previous reports that the tyrosine phosphatase domain, but not the threonine phosphatase domain, governs *Drosophila* eye development.

In our work, we have found that both the TPM and PST contribute transcriptional activation for normal eye development. Substituting the heterologous activation domain VP16 for the TPM and PST domain substantially restores Eya function. Two reasons could account for the failure of complete rescue by VP16. First, the TPM or PST have other, distinct functions. Although we have excluded the possibility that the TPM and PST are required for Eya binding with Ey, So, or Dac in this report, we cannot rule out other possibilities. For example, previous findings identified the PST/TPM domain of Eya as the primary target of Nmo and Abl-mediated phosphorylation in kinase assays. Second, there may be insufficient activation function provided by VP16 - perhaps due to an inability to make specific contacts with other proteins, or that the fusion proteins do not have the proper conformation to interact properly via other domains.

The transcriptional role of Eya has been studied in *Drosophila* through genetic and/or biochemical interaction with the transcription factors So and Dac. In this paper, we further indicate that the PST/TPM domain positively regulates Dac expression and this regulation may be mediated via So. Moreover, the PST/TPM is required to suppress Ey expression posterior to the furrow. These observations are consistent with previous reports that dac expression requires both so and eya, and both Eya and So are necessary to mediate Ey repression posterior to the MF.

Our studies localize these functions of Eya to the PST/TPM domain.

Although genetic interactions between Eya and Ey have been widely reported, physical interactions between these two RD proteins have not. In this paper, we report that Eya physically interacts with Ey for the first time. Previous studies also found physical interactions between Eya-So and Ey-So, suggesting that Ey-Eya-So may form a ternary complex. In addition, previous findings show that ectopic eye induction by Ey requires the presence of Eya and So, and the expression patterns of all three genes overlap extensively and are nearly identical anterior to the MF. Moreover, misexpression of Eya and So induces the formation of ectopic eyes; however, this effect is lost in an eya mutant background. Finally, ey is a direct target of Eya and So, and vice versa - eya and so are direct targets of Ey. Since Groucho is a repressor of the Eya-So complex, Ey may act as an activator of...
Figure 6. PST/TPM is required for normal Dac expression. (a–d) Dac expression in Canton-S (a), eya2 (b), eya2; eya+GR (c) and eya2; eyaPST/TPMGR (d) eye imaginal discs from 68 hrs AEL. (e–h) Immunostaining of Dac on 56 hrs AEL eye discs. (i–k) Dac, Eya and GFP expression in eyaPST/TPM rescued eyaΔnull clones. Yellow arrow indicates one of the larger, more posterior clones in which Dac expression is reduced. (l) Merge of channels.

Figure 7. Overexpression of So in eya2; eyaPST/TPMGR animals causes increased Dac expression. (a–d) Dac staining in third instar larvae after inducing so expression with ey-Gal4/UAS-so. Yellow arrows (d) indicate region of the disc in which Dac expression is increased. (e–h) Eya expression after ey-Gal4/UAS-so induction. eya2; eya+GR (a,e) and eya2 (b,f) are used as positive and negative controls.
Eya-So to increase transcriptional output of Dac. Consistent with this hypothesis, loss of ey, eya, or so function causes loss of Dac expression, suggesting that Ey, So, and Eya are primary regulators of Dac. Similar relationships have been observed with Pax6, Eya1/2, and Six3, mouse orthologs of ey, eya, and so, respectively. Specifically, mouse Pax6 mutants have reduced levels of Eya1 and eya2 in the optic vesicle and overlying ectoderm, and Pax6 induces expression of Six3 when ectopically expressed in mice. In addition, we used STRING, a database of known and predicted protein interactions, to predict protein-protein interactions for Ey, Eya, and So. As expected, we found equally high associations for all three pairs of complexes, providing further evidence of strong interactions among these RD proteins, which may act together in a ternary complex.

In addition, our genomic rescue assays show that the threonine phosphatase activity is largely but not entirely dispensable for Drosophila eye development. Our threonine-phosphatase inactive GRs can robustly rescue eye formation in eya null mutants, but the rescued eyes show disorganized external and internal morphology as compared to wild-type rescue controls. This result is in contrast to another report based on the GAL4-UAS system that finds the threonine phosphatase activity of Eya to be dispensable during eye development. The reason for this difference is that our GR system offers higher resolution thereby allowing detection of more subtle defects in morphology, while the GAL4-UAS system is a less accurate approach. In particular, Liu et al. did in fact observe a disorganized eye phenotype in eya flies rescued by UAS-eya. However, this phenotype appeared similar to the imperfect rescue achieved with the wild-type UAS-eya transgene. For this reason, they could not uncover the requirement for the threonine phosphatase activity during differentiation. This report highlights the need for careful interpretation of results based on the GAL4-UAS system and the superior sensitivity of the GR method.

Figure 8. The PST/TPM of Eya is necessary for ey repression posterior to the morphogenetic furrow. (a–a”)
Wild-type clones. (b–b”) eya null clones. (c–c”) eya GR rescued eya null clones. (d–d”) eyaGR rescued eya null clones. Grayscale images of Elav, Ey, and GFP expression are shown in grayscale (a–d”) and as red, blue, and green, respectively, in a”–d”; Elav marks differentiating photoreceptors and complete loss of GFP expression marks homozygous mutant clones.
Although the threonine phosphatase activity of Eya plays only a minor role during eye development, it has been reported to be involved in the innate immune response in both *Drosophila* and mouse. In summary, we have shown that both the transcriptional activation and threonine phosphatase activity of Eya are required for normal *Drosophila* eye development. However, a primary function of Eya during this process is transcriptional coactivation, while the phosphatase activity plays only a minor role. Our study provides an accurate approach to assess the functional significance of individual protein domains in vivo, highlighting the importance of the transactivation function of Eya during *Drosophila* development. As Eya is conserved and plays important roles in retinal development throughout the metazoa, the underlying mechanisms of Eya function are likely to be conserved in vertebrates as well.

### Methods

**Fly strains and maintenance.** All flies were maintained with standard corn meal and yeast extract medium at 25 °C. *Canton-S* was used as a wild-type control. Heat shocks were performed at 37 °C as described previously. To test the function of the mutant eyaGR during eye development, we crossed transgenes into the following mutant backgrounds: eya, which completely lack eyes due to a deletion of an enhancer required for eya expression during eye development, and eyaPST/TPM+, which is a null allele caused by a premature stop codon that causes recessive embryonic lethality. Wild-type clones and eyaΔPST/TPM clones were generated by crossing w/Y; FRT40A and w/Y; eyaΔPST/TPMGR with ywhs-flp; w+ubiGFP, FRT40A animals, respectively.

**Recombineering-induced mutagenesis of eyaGR and Drosophila transgenesis.** A two-step recombineering method was used to create the Y4, ΔTPM, ΔTPM+VP16, ΔPST/TPM, ΔPST/TPM+VP16, ΔPST and ΔPST+VP16 mutations in the eyaGR construct as described previously. Recombineering products were verified by DNA sequencing and restriction enzyme fingerprint digestion prior to transgenesis. Constructs were inserted into the attP2 docking site on the third chromosome using PhiC31-mediated transgenesis and site-specific integration was confirmed by genomic PCR with attP/attB primers. Transgenic flies were confirmed by genomic DNA PCR sequencing. Primer sequences are available on request.

**Construction of cell culture expression plasmids.** We used the Q5 Site-Directed Mutagenesis Kit (NEB) to introduce a series of mutations in cell culture expression plasmids which were confirmed by DNA sequencing. These mutations include: pMT-Flag-EyaΔ4, pMT-Flag-EyaΔTPM, pMT-Flag-EyaΔPST/TPM and pMT-Flag-EyaΔPST/TPM. pMT-HA-Dac. pAHW-Ey was generated from destination vector pAHW and pUAST-Ey (a gift from Dr. Rui Chen, Houston, TX) according to the Gateway protocol provided by the *Drosophila* Genomics Resource Center. pMT-Flag-Eya, pMT-Myc-So, pMT-dac, and pAHW were kindly provided by Dr. Ilaria Rebay (Chicago, IL). Primers used in this report are listed in Table S1.

**S2 cell culture and transfection.** *Drosophila* S2 cells were cultured in Schneider's medium containing 10% heat-inactivated fetal bovine serum and antibiotics at 25 °C. Cells were transiently transfected in 6-well plates using the FuGENE HD Transfection Reagent (Promega) according to the manufacturer's protocol. 24 hrs after transfection, cells were induced by addition of 0.1 M CuSO4.
Co-IP and western blots. Transfected cells were lysed by rocking at 4 °C for 30 min in Pierce IP lysis buffer (Thermo Fisher Scientific) with a Roche Complete, Mini, EDTA-free protease inhibitor cocktail tablet. The lysates were subjected to immunoprecipitation with anti-Flag-conjugated agarose beads (Sigma) for 2 h at 4 °C. After washing three times with lysis buffer, immunoprecipitates were boiled in 4× NuPAGE LDS sample buffer (Novex), and western blotting was carried out according to the NuPAGE electrophoresis (Novex) protocol with rabbit anti-Flag (1:1000, Sigma), rabbit anti-MYC (1:100, Santa Cruz Biotechnology), and rabbit anti-HA (1:200, Santa Cruz Biotechnology) antibodies.

For tissue preparation, 68 hrs AEL eye discs (n = 40) were collected in cold RIPA lysis buffer (Thermo Fisher Scientific). After centrifuge at 20000 g for 10 min at 4 °C, the supernatant was transferred to a new tube and ready for western blot analysis.

Histology and immunohistochemistry. Staining of eye discs and imaging of the adult eye were conducted as described previously42. Immunohistochemistry on 48 hr pupal eye discs and tangential sections of adult eyes were generated as previously described55. For antibodies used, please reference Table S2.

RT-PCR. RNA was extracted from 56 hrs AEL eye discs using PureLink RNA Mini Kit (Ambion). Reverse transcription was performed according to the instructions of SuperScript One-Step RT-PCR kit (Invitrogen).

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Acknowledgements
We would like to thank past members of Mardon lab (2011–2015) for their kind support and help. We are grateful to Dr. Ming Fa and Dr. Baojun Wu for critical reading of manuscript, Xuan Zhu and Trevor Davis for technical help. We thank the Bloomington Stock Center for providing fly stocks, Dr. Hugo Bellen and Dr. Uwe Walldorf for antibodies and Dr. Justin Kumar, Dr. Rui Chen and Dr. Ilaria Rebay for plasmids.

Author Contributions
Conceived and designed the experiments: M.J. and G.M. Performed the experiments: M.J. Analyzed the data: M.J. Contributed reagents/materials/analysis tools: M.J. Wrote the paper: M.J. and G.M.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jin, M. and Mardon, G. Distinct Biochemical Activities of Eyes absent During Drosophila Eye Development. Sci. Rep. 6, 23228; doi: 10.1038/srep23228 (2016).