universität wien

DISSERTATION

Titel der Dissertation

The *Drosophila eyes absent* Polycomb / Trithorax Response Element Fine Tunes Enhancer – Promoter Interactions During Eye Development and Contains an Optic Lobe Specific Enhancer

1 von 1

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Wien, 2012

Studienkennzahl Studienblatt:	lt.	A 091 490
Dissertationsgebiet Studienblatt:	lt.	Dr.-Studium der Naturwissenschaften Molekulare Biologie UniStG
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ABSTRACT

Polycomb / Trithorax binding sites have been identified at several hundred genes in both flies and mammals whereof many may represent Polycomb / Trithorax Response Elements (PRE / TREs). These elements have been best characterized at Hox genes where they are required to maintain the initial transcriptional state over multiple cell generations even after the initiating transcription factors have ceased. In doing so, the Polycomb group (PcG) proteins maintain the silent state of gene expression whereas the Trithorax group proteins maintain the active state. Thus, Hox gene PRE / TREs can be described as static elements. In addition to Hox genes, multiple dynamically regulated genes are targets of PcG suggesting that their corresponding PRE / TREs function fundamentally different from *Hox* gene PRE / TREs.

This study focuses on the characterization of the PRE / TRE of the *eyes absent* (*eya*) gene in *Drosophila melanogaster*. The *eya* gene is part of a gene regulatory network that governs the development of the eye. Most of these retinal determination genes contain PRE / TREs and are dynamically regulated. Here I present novel findings about endogenous *eya* expression and the function of the *eya* PRE / TRE. *eya* is expressed as two different isoforms derived from two alternative promoters. I detected a change in the ratio of the two endogenous isoforms during eye developmental progression using double *in situ* hybridization. Furthermore, I found that a reporter gene construct based on the *eya* regulatory sequence also displayed a change in ratio of the two reporter genes *gfp* and *miniwhite* during eye developmental progression. Strikingly, the differential regulation of the reporter genes requires the presence of the PRE / TRE.

Using previously characterized PRE / TREs from other *Drosophila melanogaster* genes or the orthologous sequences in *Drosophila pseudoobscura* I analyzed whether the *eya* PRE / TRE is an exchangeable element. My results agree with previous conclusions that PRE / TREs are interchangeable elements. However, my data also suggests that PRE / TREs are profoundly adapted to their corresponding gene. In case of the *eya* PRE /

TRE it is its ability to control differential regulation of the reporter genes, the transcriptional activity of two alternative promoters and to adjust to very high and very low expression levels at different stages of eye developmental progression. In contrast, replacement PRE / TREs were able to only rescue parts of the *eya* PRE / TRE properties. Furthermore, the *bxd* PRE / TRE (a HOX gene PRE / TRE) of both species resulted in variegation and an opposing sensitivity to the antero-posterior localization of the retinal cells thereby emphasizing the individual properties of these elements.

Finally, I could show that the *eya* PRE / TRE contains a conserved optic lobe specific enhancer that is localized within the entire 1,5 kb region of the PRE / TRE.

The results presented in this thesis have implications on *eya* regulation and function. Moreover, they broaden our understanding of PRE / TRE function in dynamically differentiating tissues.

ZUSAMMENFASSUNG

In Fliegen und Säugetieren wurden zahlreiche Bindestellen für Polycomb und Trithorax Proteine nachgewiesen. Bei vielen dieser Bindestellen handelt es sich möglicherweise um Polycomb / Trithorax Response Elements (PRE /TREs). Diese Elemente sind insbesondere bei Hox Genen charakterisiert worden wo sie dazu dienen den Initialen Transkriptionsstatus – auch in Abwesenheit der initiierenden Transkriptionsfaktoren - aufrecht zu erhalten. Dabei halten Polycomb Proteine den transkriptionell inaktiven Status aufrecht und Trithorax Proteine den aktiven Status. Daher können Hox PRE / TREs als statische Elemente bezeichnet werden. Da über die Hox Gene hinaus viele dynamisch regulierte Gene das ziel von Polycomb und Trithorax Proteinen sind, ist es anzunehmen, dass die entsprechenden PRE / TREs sich fundamental von Hox PRE / TREs unterscheiden.

Der Schwerpunkt dieser Arbeit liegt in der Charakterisierung des PRE / TREs des *eyes absent* (*eya*) Gens in *Drosophila melanogaster*. Als Teil eines genregulatorischen Netzwerkes, ist *eya* an der Augenentwicklung beteiligt. Die meisten dieser Gene besitzen PRE/ TREs und unterliegen einer dynamischen Regulierung.

In dieser Arbeit zeige ich neue Erkenntnisse über die endogene *eya* Expression und Funktion des *eya* PRE / TREs. *eya* wird als zwei Isoformen, basierend auf zwei alternativen Promotoren, exprimiert. Ich konnte durch doppel *in situ* Hybridisierungen zeigen, dass sich das Verhältnis der beiden endogenen Isoformen während der Augenentwicklung verändert. Des weiteren konnte ich diesen Wechsel im Verhältnis auch mit einem Reportergen Konstrukt feststellen welches aus *eya* regulatorischer Sequenz und den Reportern *gfp* und *miniwhite* besteht. Auffallender Weise wird das PRE / TRE für die unterschiedliche Regulation benötigt.

Zusätzlich habe ich mich mit der Frage auseinandergesetzt ob PRE / TREs austauschbare Elemente sind. Dazu habe ich die Auswirkungen von unterschiedlichen PRE / TREs von *Drosophila melanogaster* und *Drosophila pseudoobscura* auf die Regulierung der Reportergene *gfp* und *miniwhite*

untersucht. Meine Ergebnisse stimmen mit bereits veröffentlichten Studien überein, dass PRE / TREs austauschbare Elemente sind. Ich zeige jedoch auch, dass PRE / TREs tiefgreifend an ihre entsprechenden Gene angepasst sind. Im Falle des *eya* PRE / TRE ist es dessen Eigenschaft die Transkriptionsrate der zwei zugehörigen Promotoren unterschiedlich zu regulieren und sehr hohe und sehr geringe Expressionsraten - entsprechend dem Fortschritt der Augendifferenzierung - zu vermitteln. Der Austausch mit fremden PRE / TREs führt zu einer nur teilweisen Wiederherstellung dieser Funktionen. Darüber hinaus zeigte das *bxd* PRE / TRE (ein HOX PRE / TRE) beider Spezies starke Abweichungen in der Regulation der beiden Reportergene da es einerseits zu Variegierung führte und andererseits eine entgegengesetzte Sensitivität zur anteroposterioren Lage innerhalb des Auges zeigte. Dies unterstreicht die individuellen Eigenschaften dieser Elemente.

Abschließend konnte ich zeigen, dass das *eya* PRE / TRE einen konservierten Enhancer des optischen Lappens, innerhalb der 1.5kb Region des PRE / TREs besitzt.

Die Ergebnisse, dieser Arbeit tragen zum Verständnis der Regulation des *eya* Gens bei. Darüber hinaus erweitern sie das Verständnis von PRE / TREs in Genen die in dynamisch differenzierenden Geweben exprimiert werden.

I. INTRODUCTION

1. Polycomb and Trithorax Group Proteins

1.1. The Polycomb group (PcG) and Trithorax group (TrxG) proteins provide a system that mediates epigenetic memory

All somatic cell types in an organism are equally composed in terms of DNA sequence. Individual cell identities are based on different readouts of the genome that can be maintained over many cell generations. This kind of inheritance is referred to as epigenetic memory, which is - based on the classical definition of the word epigenetic - "… a change in expression of a gene which does not involve a mutation, but is nevertheless inherited in the absence of the factor that initiated the change" (Ptashne, 2007). The Polycomb and Trithorax group (PcG and TrxG) of proteins fulfill these requirements, as they are able to maintain the transcriptional state of a gene once the initiating factors have disappeared (Cavalli and Paro, 1999). Hereby, the PcG proteins maintain the silenced state of gene expression while the TrxG proteins maintain the active state of gene expression. PcG and TrxG mediated regulation has been best characterized in the regulation of homeotic genes that are expressed early in development under the control of specific transcription factors. Later in development, when those initial transcription factors have disappeared, the PcG and TrxG proteins maintain the initial state of gene expression over many cell generations (Kennison, 1995, Ringrose and Paro, 2004). Many aspects of PcG and TrxG mediated regulation are not completely understood to date. Nevertheless, a magnitude of functional details of these two antagonizing groups of proteins is well characterized and helps to approximate the underlying concept.

1.2. Polycomb and Trithorax group proteins act as complexes

Polycomb group (PcG) proteins

Given their important role in maintenance of different cell types it is not surprising that most null mutations of PcG proteins have been found to be

embryonic lethal (Kennison, 1995). Historically, the first *PcG* genes were identified by phenotypic analysis. PcG proteins belong to a group of genes with a similar mutant phenotype. A mutation in the founding member *Polycomb* (*Pc*) or the gene *extra sex combs* (*esc*) causes sex combs on the second and third legs of male flies instead of only the first leg as in wildtypes (Ringrose and Paro, 2004). This phenotype is caused by lack of *Hox* gene repression leading to the transformation of posterior body segments into more anterior ones (Lewis and Cowan, 1988, Jones and Gelbart, 1990). The list of *PcG* genes has been extended constantly since their discovery in the 1940s and to date, many of its members are functionally characterized and we are beginning to understand the biological mechanisms through which they act. Polycomb group proteins act within large core complexes that are highly conserved between flies and mammals (Brunk, et al., 1991, van Lohuizen, et al., 1991, Müller, et al., 1995, Gunster, et al., 1997, Brown, et al., 1998, Atchison, et al., 2003, Ringrose and Paro, 2004): The best characterized principal complexes are the Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) (Franke, et al., 1992, Gunster, et al., 1997, Satijn, et al., 1997, Jones, et al., 1998a, Tie, et al., 1998, Shao, et al., 1999, Tie, et al., 2001, Kuzmichev, et al., 2002, Tie, et al., 2003, Francis, et al., 2004, Morey and Helin, 2010) and the PhoRC complex (Klymenko, et al., 2006).

PRC1 consists of the PcG proteins encoded by *Polycomb* (*Pc*), *Posterior sex combs* (*Psc*), *polyhomeotic* (*ph*) and *dRING* (Saurin, et al., 2001). Its characteristic feature is the binding specificity to trimethylated Lysine 27 on histone H3 (H3K27me3) which is achieved over the chromodomain of PC (Fischle, et al., 2003). In addition, the RING domain of dRING catalyzes ubiquitinylation of Lysine 119 on histone H2A (Wang, et al., 2004a). *ph* is functionally less well characterized and known to be required for the maintenance of transcriptionally repressed states over its zinc finger domain (Bloyer, et al., 2003). *Psc* is able to bind DNA, compacts chromatin and inhibits chromatin remodeling, a general function of the PRC1 (Shao, et al., 1999, Lo, et al., 2009). One possible explanation for this inhibition is that the

PCC complex (a variant of PRC1) is able to compact chromatin thereby reducing the accessibility for nucleosome remodelers (Francis, et al., 2004).

The PRC2 consists of the proteins encoded by *Su(z)12*, *Enhancer of zeste* (*E(z)*), and *extra sexcombs* (*esc*). Its characteristic features are the H3K27 methyltransferase activity catalyzed by E(Z) (Czermin, et al., 2002, Kuzmichev, et al., 2002, Müller, et al., 2002) and the H3K27me3 binding specificity of the ESC subunit (Tie, et al., 2001).

The PhoRC complex is the most recently identified PcG complex and consists of the PcG proteins encoded by *Pleiohomeotic* (*Pho*) and *dSfmbt*. PHO possesses binding ability to a specific DNA motif (Brown, et al., 1998) and dSfmbt selectively binds to mono- and dimethylated but not trimethylated Lysine residues on Histones H3 and H4 (Klymenko, et al., 2006). In addition to interacting with dSfmbt, Pho has been reported to interact with the PcG proteins E(Z), PH and PC. Therefore, PHO is thought to play an important role in recruiting PcG complexes to target sites (Mohd-Sarip, et al., 2002, Wang, et al., 2004b).

All the PcG proteins so far characterized are functionally conserved in mammals, reflecting their importance. Genetic analyses in flies and mammals have identified the biological role of *PcG* genes in processes as early embryogenesis (Kennison, 1995), maintenance of differentiated states (Hennig and Derkacheva, 2009), segmental identity (Jones and Gelbart, 1990), gastrulation (Faust, et al., 1995, O'Carroll, et al., 2001) and differentiation. Complementary to the findings in the fly, null mutations of the PRC2 members in the mouse are embryonic lethal reflecting the essential requirement of these genes in the development of complex organisms (Leeb and Wutz, 2007, Pasini, et al., 2007).

The composition of PcG complexes can be modulated within different cell types and at different developmental stages: The chromatin associated silencing complex for homeotics (CHRASCH) was extracted from *Drosophila* Schneider cells. It is a derivate of the PRC1 complex that contains Pipsqueak (Psq) and is associated with the histone deacetylase 1 (HDAC1) (Huang and Chang, 2004, Breiling, et al., 2007).

The PcG protein Polycomblike (Pcl) is a component of the larval Pcl-PRC2 complex and has been suggested to anchor the complex to its targets (Savla, et al., 2008). Furthermore, it was shown that Pcl-PRC2 is required for high levels of H3K27 trimethylation (Schwartz and Pirrotta, 2007, Cao, et al., 2008). The examples of the CHRASH and Pcl-PRC2 complexes reflect an important attribute of the PcG system: The versatile exertion of PcG mediated regulation is based on the modular composition of PcG complexes.
The actual list of PcG proteins includes more genes than the ones presented above. Nevertheless, the presented genes give an overview on characteristic features that lead to PcG mediated silencing: Binding to specific DNA motifs, mediation of characteristic histone modifications, removal of histone modifications associated with active genes, binding to specific histone modifications and inhibition of nucleosome remodelers.

Trithorax Group (TrxG) proteins

Whereas PcG proteins maintain the silenced state of gene expression, TrxG proteins act antagonistically and maintain the active state of gene expression. *trxG* genes have been identified as suppressors of PcG mutations, as mutations that mimic mutations of HOX-genes in flies or as mutations that genetically interact with classic *trxG* genes (Ringrose and Paro, 2004). At a molecular level TrxG proteins act as heteromeric complexes that maintain active transcription via distinct characteristic properties of their subunits. Interestingly, they use the same levels of regulatory control as the PcG proteins:

Nucleosome Remodeling

Trithorax group proteins include components of nucleosome remodeling complexes such as the Brahma and NURF complexes that facilitate the binding of transcription factors and the basal transcription machinery over their ATPase subunits encoded by *brahma* (*brm*) and ISWI, respectively (Tamkun, et al., 1992, Tsukiyama and Wu, 1995, Orlando, et al., 1998).

Mediation of chromatin modifications that are associated with the active state of gene expression

Another class of TrxG proteins consists of the SET domain containing factors encoded by *trithorax* (*trx*), *absent, small or homeotic discs 1* (*ash1*), and the vertebrate MLL proteins which act in individual complexes with their associated proteins and mediate the methylation of histone H3 lysine 4 (H3K4) that is associated with the active state of gene expression. Moreover, the TAC1 complex consisting of the subunits TRX, Sbf1 and dCBP and the Ash1 / dCBP complex are able to acetylate H3K27 via the histone acetyl transferase activity of dCBP (Bantignies, et al., 2000, Bantignies F, 2000, Petruk, et al., 2001, Schuettengruber, et al., 2007).

Aside from SET domain factors histone modifications can be mediated by TrxG proteins consisting of further catalytic centers: The gene product of the *trxG* gene *grappa* (*gpp*) methylates histone 3 at arginine 79 (Shanower, et al., 2005).

Removal of chromatin modifications that are associated with silenced genes

Over the recent years it has become clear that the hallmark of PcG mediated silencing - H3K27 trimethylation - is reversible. Although the circumstances which lead to reactivation of silenced genes are currently poorly understood the *trxG* gene *dUTX* has been identified as an important factor. It codes for a H3K27 demethylase that mediates demethylation via its JmnjC domain (Lan, et al., 2007).

One surprising TrxG protein is encoded by *little imaginal discs* (*lid*) which demethylates trimethylated lysine 4 on histone 3, a hallmark of actively transcribed genes. The responsible feature for the classification as *trxG* gene has been shown to be its inhibiting binding ability to the histone deacetylase RPD3, an antagonist of TrxG function. LID was found in a complex with Ash2 (absent, small or homeotic discs 2) during eye development, implying a role at this specific developmental stage (Secombe, et al., 2007, Lee, et al., 2009).

Although *lid* specific demethylation was shown to be dispensable for development this function could play important roles in fine-tuning gene expression of target genes by dynamic demethylation (Li, et al., 2010).

Binding to chromatin

In order to mediate nucleosome remodeling and histone modifications the TrxG complexes need to be able to interact with chromatin. The ATPase subunit BRM of the above mentioned BRM-complexes is able to bind acetylated chromatin via its Bromo-domain (Mohrmann and Verrijzer, 2005). Furthermore, the H3K4 histone methyl transferases TRX and ASH1 are able to interact with modified chromatin over their Bromo domains (Mujtaba, et al., 2007).

The H3K4me3 demethylase activity of the above mentioned *trxG* gene *lid* is dispensable for development. Interestingly, mutations in the PHD finger domain responsible for interactions of H3K4me2 / me3 are lethal (Li, et al., 2010).

In summary, PcG and TrxG proteins act antagonistically. It is interesting to note that all the presented characteristics (nucleosome remodeling, mediation, removal and binding to chromatin marks) are utilized by the two groups of proteins reflecting the mutual antagonism at lower scale. Furthermore, the ability of removing chromatin modifications typical for the antagonistic protein group coincides very well with the finding that regulation mediated by PcG and TrxG is reversible.

1.3. DNA binding proteins involved in PcG and TrxG mediated regulation

DNA binding factors that interact with PcG and TrxG proteins are especially interesting as they have the potential to recruit PcG and TrxG complexes to target sites. Some of the DNA binding factors presented in this section have been classified as members of PcG or TrxG although the phenotypes of mutants classify for both groups. One DNA binding protein, PSQ, has been classified as enhancer of PcG mutations indicating a role in PcG mediated regulation. Yet another factor, CtBP, has been shown to play an important role in PcG mediated repression without categorization to one of the groups.

female sterile (1) homeotic (fs(1)h)

The *trxG* gene *female sterile (1) homeotic* (*fs(1)h*) codes for two gene products. One of them, FSH-S, is localized in the nucleus and displays a homeotic phenotype when mutated (Chang, et al., 2007). FSH-S binds to the same binding motif as Zeste, another TrxG protein that binds to DNA via the YGAGYG motif (Hur, et al., 2002, Man-Wook Hur, 2002). Aside from targeting a specific sequence FSH-S exhibits kinase activity that is hypothesized to modify interaction partners at target loci (Hur, et al., 2002, Chang, et al., 2007).

zeste

zeste has been classified as *trxG* member due to its ability to activate transcription. As mentioned above, Zeste and FSH-S bind to the same YGAGYG motif (Chang, et al., 2007) which has been shown to be of central importance for TrxG mediated maintenance of active gene expression (Déjardin and Cavalli, 2004). As *zeste* mutations are dispensable for viability and the *fs(1)h* mutations are lethal it has been hypothesized that *zeste* only plays a minor role in TrxG mediated maintenance of active gene expression while the main interactor with *zeste*-sites may be FSH-S (Chang, et al., 2007). Nevertheless, Zeste plays a central role in a characteristic property of PcG

function. Zeste is involved in two phenomena termed “transvection” (Pirrotta, 1991) and “pairing sensitive silencing” (PSS) which are both based on the ability of a regulatory region to interact in trans with the homologous counterpart on the sister chromatid. Although similar mechanisms may be involved, the experimental approaches to detect transvection and PSS differ: Transvection is the ability of a regulatory element to activate the homologous target promoter on the sister chromatid. These interactions in trans are lost in *zeste* mutant background (Pirrotta, 1991). The regulatory region of the gene *eyes absent* (*eya*) which is the central subject in this thesis also requires *zeste* function for transvection which is required for proper eye development in 3rd instar (Leiserson, et al., 1994).

PSS has been frequently observed in transgenes carrying reporter gene constructs that contain a PcG target region. It is characterized by a silencing effect that is stronger in homozygotes compared to heterozygotes although the number of reporter gene copies has doubled (Kassis, 2002, Okulski, et al., 2011). Strikingly, PSS has been shown to depend on *zeste* as well (Hagstrom, et al., 1997).

trithorax-like (trl)

The DNA binding TrxG protein GAGA-factor (GAF) is encoded by the gene *trithorax-like* (*trl*) which binds to the (GA)n motif. *trl* has been defined as a *trxG* member because mutations enhance the phenotype of the homeotic gene *Ultrabithorax* (*Ubx*) (Biggin and Tjian, 1988, Horard, et al., 2000). Nonetheless, other studies found that *trl* mutations exhibit characteristics for a classification as a *PcG* gene indicating that it is able to interact with both groups of proteins (Strutt, et al., 1997, Horard, et al., 2000, Bejarano and Busturia, 2004). The cooperation of GAF and PHO binding motifs (see below) in PRE / TRE cores has been reported to be indispensable for silencing (Kozma, et al., 2008).

pipsqueak (psq)

The (GA)n motif is further bound by the DNA binding factor encoded by *pipsqueak* (*psq*), which can also physically interact with GAF. *psq* was phenotypically characterized as an enhancer of PcG mutations indicating a function in PcG mediated silencing (Huang, et al., 2002, Schwendemann and Lehmann, 2002).

pleiohomeotic (pho)

The DNA binding factor Pleiohomeotic (PHO), a subunit of the above mentioned PhoRC complex has been classified as a PcG protein due to its role in the maintenance of repression of homeotic genes. Its binding motif is defined by the core sequence GCCAT (Brown, et al., 1998). PHO binding motifs have been shown to play a critical function in PRE / TREs as its binding motif has been shown to be indispensable for PRE / TRE function (Kozma, et al., 2008). Accordingly its motif plays a critical role for *in silico* prediction of PRE / TREs (Ringrose, et al., 2003). The closely related PcG protein Pho-like (PHOL) shares 80% sequence identity and its function is predominantly redundant with Pho (Brown, et al., 2003).

***grainy head* (*grh*)**

The DNA binding factor Grainyhead (GRH) binds to T-rich motifs (core motif TGTTTT) and cooperatively interacts with Pho (Blastyák, et al., 2006). Therefore, the authors hypothesized that motif composition of PcG target regions may specify the composition of PcG complexes at distinct target site.

***Dorsal switch protein* (*Dsp1*)**

The dorsal switch protein (DSP1) is a corepressor that converts its interaction partners - transcriptional activators - into repressors (Lehming, et al., 1994). Mutations in *Dsp1* display a homeotic phenotype characteristic of *PcG* and *trxG* mutants (Decoville, et al., 2001). DSP1 binds to the GAAAA motif which has been shown to play a decisive role in PcG mediated silencing of the Ab-Fab fragment, a PcG target region (Déjardin, et al., 2005). Interestingly, DSP1 is able to interact with DNA in two modes: specifically, over the GAAAA motif

and in unspecific manner via high mobility group (HMG) boxes providing the potential of recruitment to DNA via protein-protein interactions (Thomas and Travers, 2001). It is tempting to speculate about the functions of DSP1 in PcG / TrxG mediated regulation. As DSP1 is a corepressor, its presence or absence at a gene may decide about PcG mediated silencing or TrxG mediated gene expression. Additionally, it may be involved in transforming a locus from the silenced into a permissive state.

C-terminal Binding Protein (CtBP)

The last protein introduced in this section is not a DNA binding protein nor is it classified as PcG or TrxG protein. However, it displays crucial functions in PcG mediated regulation and is recruited to DNA by its interaction with transcriptional factors. The Carboxy-terminal binding protein (CtBP) acts as corepressor for developmental regulators as *knirps* and *krüppel* (Nibu and Levine, 2001). Furthermore, CtBP interacts with some PcG proteins and is required for recruitment of PHO and binding of PcG complexes to target sites (Sewalt, et al., 1999). Interestingly, reduction of CtBP levels results in loss of PcG binding and reduced trimethylation of H3K27 at PcG target sites (Srinivasan and Atchison, 2004).

1.4. PcG and TrxG complexes act via the same DNA elements in Drosophila: Polycomb / Trithorax Response Elements (PRE / TREs)

PcG and TrxG proteins target *cis*-regulatory DNA elements to maintain gene expression. These regulatory elements have been termed in various ways: Polycomb / Trithorax response elements (PRE / TREs) (Tillib, et al., 1999, Ringrose, et al., 2003), Polycomb response elements (PREs) (Chan, et al., 1994), cellular memory modules CMM (Cavalli and Paro, 1998) and maintenance elements (ME) (Rank, et al., 2002, Maeda and Karch, 2006). Throughout this thesis I will use the term Polycomb / Trithorax Response Element (PRE / TRE).

The involvement of PRE / TREs in the maintenance of gene expression states is well characterized with PRE / TREs of the Bithorax Complex (BX-C) a large regulatory domain containing three homeobox (HOX) genes. The *Hox* genes of the BX-C *Ultrabithorax* (*Ubx*), *Abdominal-B* (*Abd-B*) and *Abdominal-A* (*Abd-A*) are initiated by the maternal, gap and pair-rule genes in early embryogenesis (White and Lehmann, 1986, Irish, et al., 1989, Simon, et al., 1993, Shimell, et al., 1994, Casares and Sánchez-Herrero, 1995). These initial morphogenetic factors are expressed in individual patterns along the embryo resulting in unique combinations of morphogenetic factors at any given location along the anteroposterior dimensions that ultimately define distinct segments. Each of these regulatory environments acts on responsive DNA sequences that are then able to interact with promoters to drive segment specific expression. Regulatory elements that are not responsive to a combination of morphogens remain inactive and cannot interact with the promoter. For example, the *Abd*-B gene expression is regulated over specialized regulatory regions with inherent segment specificity (i.e. iab-7 and iab-8) that interact with the promoter in specific segments while they are not responsive in others (Casares and Sánchez-Herrero, 1995, Maeda and Karch, 2006). All regulatory DNA regions of the *Hox* genes characterized so far

contain PRE / TREs for the maintenance of the segment specific responses. The maintenance of the silent state is accomplished by PcG proteins while the active state is maintained by TrxG proteins (Moehrle and Paro, 1994).
Interestingly, the PRE / TREs of homeotic genes are targeted by PcG and TrxG proteins in the early embryo without playing a decisive role in the regulation of the target genes. This indicates that the collective presence of gap and pair-rule gene products and PcG and TrxG proteins may be important for the establishment of epigenetic memory (Orlando, et al., 1998). Later in development when the initial gap and pair-rule gene products have decayed, PRE / TREs are essential to maintain the initial expression patterns throughout developmental progression and the entire life of the fly (Struhl and Akam, 1985, Paro, 1990, Kennison, 1993, Simon, 1995, Pirrotta, 1997). At present it is not known whether enhancers interact directly with promoters in the presence of a PRE / TRE. The present model suggests that enhancers initiate the expression and the PcG / TrxG system takes over afterwards. However it is possible that the PcG / TrxG system acting over the PRE / TRE is already in control of the enhancer at the onset of gene expression acting as a mediator of enhancer activity. The answer to this question is difficult to address experimentally because enhancers may interact with promoters in absence of the PRE / TRE while the presence of a PRE / TRE may not interfere with the spatiotemporal activity of the enhancer and lead to same expression pattern. Interestingly, it has been shown by Perez and colleagues that wing disc specific *vestigial* specific expression in requires a PRE / TRE very early after or even synchronous with the initiation of vg enhancer activity in the wing blade cells. Thereby the PRE / TRE maintains the expression in cells that exit the highly proliferative wingblade center (Pérez, et al., 2011).

1.5. Hox gene PRE/TREs maintain the initial transcriptional state. Does this apply for PRE/TREs of other genes as well?

Most current concepts of PRE / TRE function are based on studies of the *Hox* PREs. These PRE / TREs maintain the transcriptional state of genes very early

in development and are not switched for the rest of a flies' life. Besides *Hox* genes several other PcG targets have been identified in genome wide studies (Ringrose, et al., 2003, Nègre, et al., 2006, Schwartz, et al., 2006, Tolhuis, et al., 2006, Schwartz, et al., 2010, Enderle, et al., 2011). Interestingly, the identified target genes are involved in dynamic processes as differentiation, proliferation and development which would require PRE / TREs that function differently from the *Hox* PRE / TREs that behave statically once the gene expression status is set up (reviewed in (Ringrose, 2007, Schwartz and Pirrotta, 2007). This establishes the possibility that *Hox* PRE / TREs may reflect a specialized subpopulation of PRE / TREs.

Interestingly, it has been shown that the memory which is maintained by a PRE / TRE can be switched upon induction of the regulated promoter (Zink and Paro, 1995, Cavalli and Paro, 1998, Cavalli and Paro, 1999), genetic removal of PcG and TrxG proteins using FLP-mediated recombination (Beuchle, et al., 2001, Klymenko and Müller, 2004) and transcription through the PRE / TRE of a reporter gene construct (Maurange and Paro, 2002, Rank, et al., 2002). The fact that PRE / TREs can be switched by forcing transcription through the promoter or the PRE / TRE itself and by altering the presence and absence of specific PcG and TrxG proteins establishes the possibility that these mechanisms of PRE / TRE switching may be used in general developmental transitions (Maurange and Paro, 2002, Buszczak and Spradling, 2006, Ringrose, 2006, Ringrose and Paro, 2007). Indeed it has recently been shown that both intergenic transcripts at PRE / TREs and levels of PcG proteins are regulated dynamically throughout development or differentiation:
Gene regulation in the BX-C is accompanied by transcription through cis-regulatory sequences and the expression of these transcripts is altered dynamically throughout development (Lempradl and Ringrose, 2008). As mentioned before, the CtBP corepressor that interacts with some PcG proteins has been shown to play an important role in recruitment of PcG complexes. Reduction of CtBP led to reduced levels of PcG binding and

reduced trimethylation of H3K27 (Srinivasan and Atchison, 2004). Interestingly, a recent study by the same laboratory showed that a reduction of CtBP levels led – in addition of the already observed effects - to elevated levels of intergenic transcription at the tested PREs (Basu and Atchison, 2010). As many PcG and trxG proteins bind to RNA it has been suggesdted that RNA interactions may be essential to target both groups of proteins to specific sites (Hekimoglu and Ringrose, 2009).

Examples of transcribed Polycomb target sites have been reported in the murine system (Hekimoglu-Balkan, et al., 2012). The so called transcribed intergenic polycomb (TIP) sites are dynamically transcribed throughout neuronal differentiation and can be distinguished into different categories that correlate positively and negatively with the transcriptional activity of their neighbouring – presumptive regulated - gene. At the scale of individual genes, noncoding transcription of TIP sites may impact the status of neighboring gene. The transcription at the TIP sites (silent / low / high) may have individual consequences for each neighboring gene and may depend on the simultaneous presence of transcription factors.

The levels of PcG proteins are altered in general differentiation processes: The PcG protein Ezh2 is down regulated in skeletal muscle cell specific differentiation which leads to the derepression of developmental regulators that are PcG targets in ES cells (Juan, et al., 2009). Furthermore, PcG proteins are removed from the nucleus into the nucleolus by interaction with the testis specific TBP interacting transcription factor (tTAF). The regional dilution of PcG proteins at target genes may allow a TrxG dependent activation of the testis specific differentiation factors (Chen, et al., 2005, Ringrose, 2006). Finally, it has been shown that the activation of the JNK signaling pathway causes the downregulation of some *PcG* genes indicating that this regulatory interaction may have applications in JNK dependent developmental processes (Lee, et al., 2005).

1.6. What makes a PRE / TRE?

At present, it is still not completely clear what constitutes a PRE / TRE. In contrast to enhancers that show a high degree of sequence conservation across genes, it has not been possible to define PRE / TREs by this feature (Ringrose, et al., 2003). Nevertheless, several sequence attributes of these elements are known. For example, it has been shown that the DNA binding motif of the PcG protein PHO and its closely related paralog PHOL plays a substantial role in PcG mediated silencing (Brown, et al., 1998, Mihaly, et al., 1998). The same holds true for the DNA binding motifs of GAF / PSQ, and ZESTE / FSH-S (Strutt, et al., 1997, Man-Wook Hur, 2002). However, these three motifs alone have not been sufficient for PcG recruitment to PRE / TREs indicating that further sequence attributes are needed to obtain functionality (Déjardin, et al., 2005). Analyses of a minimal *Fab-7* element that regulates the *Abd-B* gene revealed that a further motif plays a decisive role in the mediation of PcG mediated silencing at least within some PRE / TREs. The GAAAA motif which is targeted by the DSP1 Protein has been shown to be crucial for the silencing capacity of the *Ab-Fab* PRE / TRE. In addition, the DSP1 motif combined with the PHO / PHOL and GAF motifs constituted silencing capacity in an artificially constructed PRE / TRE. In absence of the DSP1 motif resulted in disability to mediate silencing in a reporter gene assay (Déjardin, et al., 2005). However, Kozma and colleagues reported that the combination of PHO and GAF motifs are indispensable for silencing function of the *bxd* PRE / TRE while the DSP1 motif is dispensable showing that PRE / TRE are diversely composed while still performing as such elements in transgenic assays (Kozma, et al., 2008).

Using only the sequence motifs for GAF / PSQ, PHO / PHOL and Zeste / FSH-S Ringrose and Rehmsmeier developed an algorithm to predict such elements in the fly genome. The subsequent successful validation of many of the predicted PRE / TREs in a transgenic reporter assay proved that the accumulation of these motifs in a given sequence are a good predictor for PRE / TRE function (Ringrose, et al., 2003).

1.7. The role of Polycomb and Trithorax Group proteins - theoretical considerations

Over the past few years it has become clear that the *Hox* based model of PcG and TrxG mediated regulation (referred to as the "static PcG / TrxG model") may apply to *Hox* PRE / TREs but not to all the other targets in the genome. As mentioned above many PcG targets are dynamically expressed throughout development. It is difficult to combine this fact with the static PcG / TrxG model where the initial settings are maintained irreversibly. Nevertheless, a dynamic PcG and TrxG model would require just one additional attribute: Reversibility. As mentioned above, reversibility of silenced states is possible even for Hox PRE / TREs, for example by downregulation of PcG proteins, transcription through promoter and transcription through PRE / TREs. The requirements to a PRE / TRE of a dynamically expressed gene would therefore be the ability to unlock the silenced state upon exposure to a specific regulatory environment. As each of the two protein groups is able to remove histone marks that are characteristic of the antagonizing group it seems that reversibility is a inherent property of the PcG and TrxG system (Huang and Chang, 2004, Breiling, et al., 2007, Lan, et al., 2007, Secombe and Eisenman, 2007, Lee, et al., 2009, Li, et al., 2010).

The general perception of the PcG / trxG system as a maintenance system that is able to maintain the active or silenced state of gene expression even in absence of the initiating transcription factors is well established (Ringrose and Paro, 2004, Reik, 2007, Mohn and Schübeler, 2009). At a global scale, this has important implications for the regulation of the entire genome and the evolution of the many different specialized cell types of higher organisms.

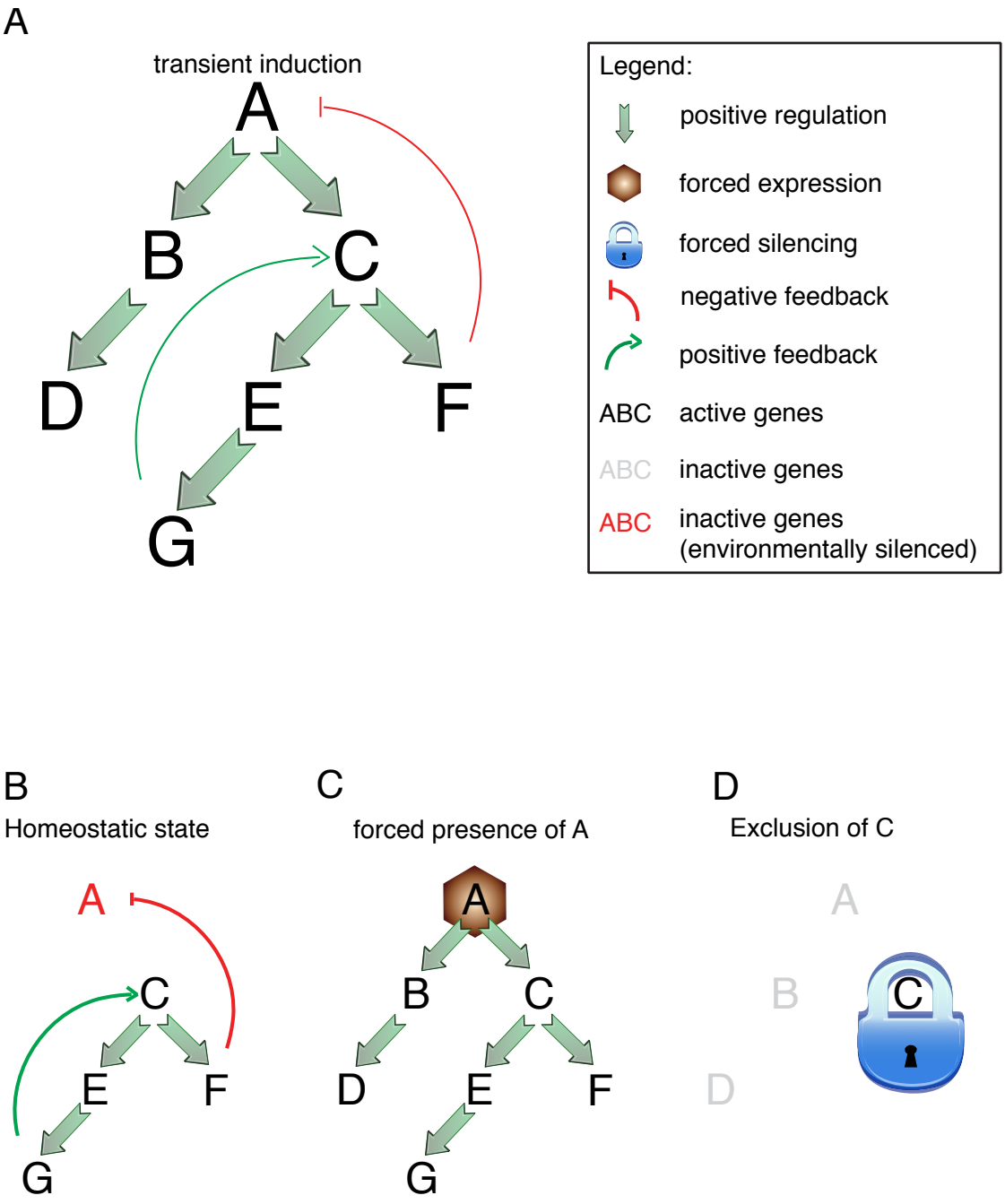


Figure 1: A maintenance system increases the variety of stable states.

(**A**) The temporary induction of gene A activates the genes B, C, D, E, F. (**B**) Over time the effects of this transient event are stabilized via feedback mechanisms. The gene products A, B and D diminish over time due to lack of A which was a) only transiently expressed and b) is being negatively regulated by F. On the other hand the expression of the genes C, E, F and G are maintained by positive feedback from G to C. This constellation displays the native homeostatic state without a maintenance system. (**C**) The forced presence of gene product A is ensured by a

maintenance system. As a consequence the feedback mechanisms play subordinate roles. (**D**) The exclusion of C is achieved by a maintenance system that maintains the silenced state of gene expression. This leads to a complete absence of the genes A, B, C, D, E, F and G as the positive feedback from G to C does not existent. The expression of the genes A, B and D is not maintained because the presence of A was temporary. Nevertheless, their expression could be induced at any time. (not shown) A further stable state is achieved by the combination of “forced presence of A” and “exclusion of C”. This would lead to the expression of the genes A, B and D. The presented regulatory network consisting of the genes A, B, C, D, E, F and G could be integrated into a much larger regulatory system. Depending on a high or low position within the hierarchy of the larger system the downstream effects of each stable state would be of higher or lower impact, respectively. In terms of cells a maintenance system could define different cell types on one hand or variations of one cell type with subtle differences on the other.

A maintenance system as the PcG / TrxG system is able to stabilize an otherwise unstable genome readout as it is able to force expression in absence of transcription factors or to silence gene expression in the presence of activating transcription factors. Furthermore, the PcG / TrxG system is also characterized by its flexibility and reversibility, thereby adding multiple possibilities to regulate the genome and stabilize the output. These features expand the number of stable genome readouts and are likely the foundation of the many different cell types that emerged during the evolution of higher organisms. The drastic flexibility that is provided by a maintenance system as the PcG / TrxG system on a minimal a gene network is illustrated in Figure 1. In terms of evolution it is more probable that a simple maintenance system evolves than multiple independent regulatory networks that will provide the regulation into multiple different cell types. Once established, a maintenance system enables for much quicker adaption to evolutionary pressure.

Another important feature of a maintenance system is that it provides protection from fluctuations of the regulatory environment of a cell during

developmental transitions (Bird, 2007). A maintenance system would protect target genes that are not supposed to change their activity. On the other hand, a maintenance system must be flexible enough to allow for reactivation of silenced genes and silencing of active genes. The requirement for the DNA sequences used by the maintenance system to interact with target genes (in our case PRE / TREs) would be the potential to reset the mode of maintenance under specific regulatory conditions.

1.8. PcG and TrxG proteins are involved in eye development

Aside from the homeotic genes that are regulated statically by PcG and TrxG proteins an increasing number of PRE / TREs has been identified at genes that display dynamic behaviour (Fauvarque and Dura, 1993, Kassis, 1994, Maurange and Paro, 2002, Bloyer, et al., 2003, Lee, et al., 2005, Martinez, et al., 2006). Many potential PRE / TREs have been identified using genome wide computational and experimental approaches (Ringrose, et al., 2003, Nègre, et al., 2006, Schwartz, et al., 2006, Schwartz, et al., 2010, Enderle, et al., 2011). The bulk of these subsequently identified PcG targets are known to be dynamically regulated as they participate in processes that involve proliferation, differentiation and signaling. Furthermore, many of them form part of regulatory networks and are subject to cross-regulation (Ringrose, 2007). One especially interesting set of PcG target genes are the members of the retinal determination (RD) gene network. RD genes are subject to cross- and auto-regulation (reviewed in (Kumar, 2010)). During eye development in 3rd instar the RDGN genes are dynamically expressed. Interestingly, PcG and TrxG proteins have been shown to play a central role during this dynamic process. Janody and colleagues have shown that the *PcG* genes *Pc* and *E(z)* are involved in expressing the correct levels of the RDGN members *eyeless* (*ey*), *teashirt* (*tsh*), *eyes absent* (*eya*) and *dachshund* (*dac*) directly or indirectly (Janody, et al., 2004). As mutations in *PcG* and *trxG* genes may have dramatic effects on the genome readout of cells it is difficult to address

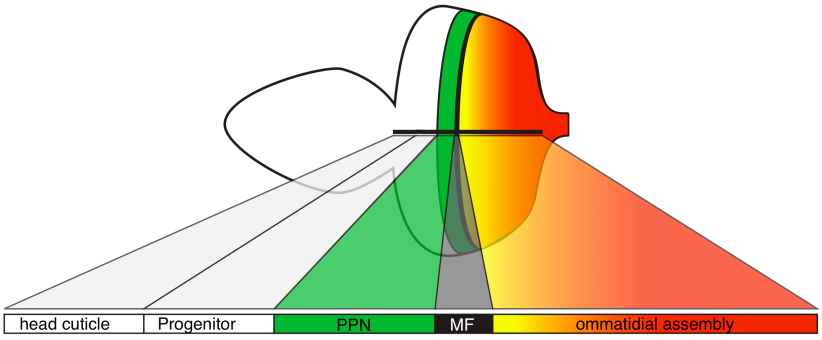
whether the RD genes are direct targets of PcG and TrxG proteins during eye development.

2. Eye development in *Drosophila*

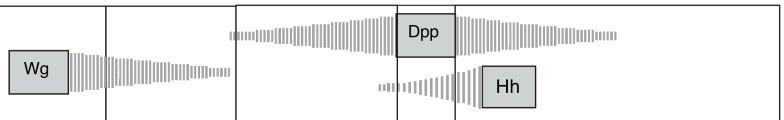
2.1. The Retinal Determination (RD) genes

Retinal determination (RD) genes are defined by their ability to generate ectopic eyes in non-retinal tissue individually or in combination with other RD genes (Halder, et al., 1995, Bonini, et al., 1997, Pignoni, et al., 1997, Shen and Mardon, 1997, Pan and Rubin, 1998). Furthermore, mutations in RD genes lead to the disruption of eye development. RD genes are not solely involved in eye development but in many other developmental processes where they are expressed in multiple context specific combinations. Not surprisingly, null mutations of RD genes are generally lethal. The identification of heteromorphic mutations displaying the eponymous “no eye” phenotype corresponds to mutations eliminating eye specific enhancers of the respective genes. Examples are *eyeless* (*ey*), *twin of eyeless* (*toy*), *eyegone* (*eyg*) / *twin of eyegone* (*toe*), *sine oculis* (*so*), *eyes absent* (*eya*) (Quiring, et al., 1994, Niimi, et al., 1999, Bui, et al., 2000, Pauli, et al., 2005, Wang, et al., 2008). To date, 14 RD genes have been identified, many of them being paralogs with partially redundant functions and often overlapping expression patterns. RD genes are involved in processes such as tissue specification and differentiation (Kumar, 2010, Baker and Firth, 2011), control of proliferation / growth (Bessa, et al., 2002, Jang, et al., 2003, Lopes and Casares, 2010) and cell survival by inhibition of apoptosis (Bonini, et al., 1993, Clark, et al., 2002, Mirkovic, et al., 2002, Peng, et al., 2009). The activation of the RD genes follows a stringent sequential order whereby the gene *toy* is on top of the chronological order (Czerny, et al., 1999). Nevertheless, the RD genes are not organized in a simple hierarchical manner. Once expressed, the RD gene products are involved in cross- and auto-regulation thereby generating a regulatory network (Kumar, 2010). Moreover, most RD gene products are able to interact with each other forming multiple complexes that alter their functions. For example at different stages of eye development, EY has been shown to act in a complex together with Teashirt (TSH) and in a complex with both Homothorax (HTH) and TSH. Interestingly, the two complexes have opposed

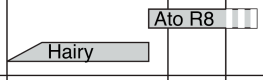
A



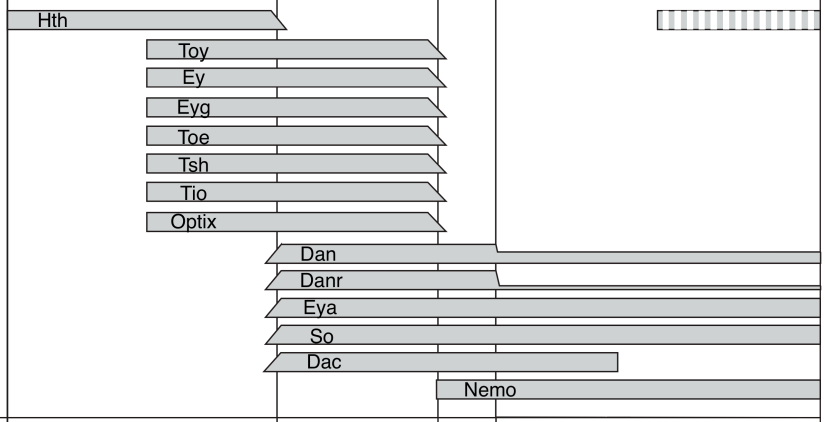
B signaling



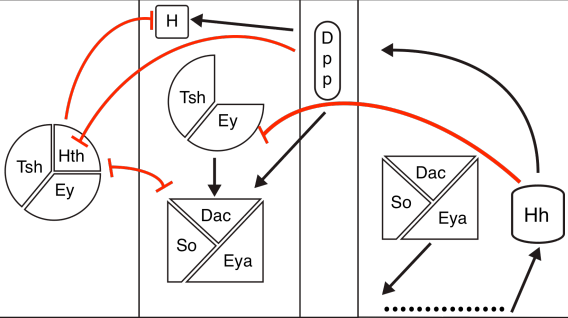
C neuronal differentiation



D RD genes



E main regulatory interactions



F

		proliferation	transition	MF	differentiation
eyFLP *trx* mutant clones	Protein				
	Hth	normal	normal		overexpression at post. margins
	Ey	LOSS	LOSS		normal, not expressed
	Tsh	LOSS	LOSS		normal, not expressed
	Eya		delay, reduction		reduction
	Dac		delay		normal
	H		reduction		
	Dpp			n.d.	
eyFLP *kto/skd* mutant clones	Protein				
	Hth	reduction	normal		normal
	Ey	normal	increase		inappropiate maintenance
	Tsh	reduction	reduction		normal, not expressed
	Eya		reduction		normal (exception large clone behind MF)
	Dac		normal		inappropiate maintenance
	H		reduction		
	Dpp			n.d.	
eyFLP *Pc / E(z)* mutant clones	Protein				
	Hth	normal	misexpression		misexpression
	Ey	reduction	reduction		normal
	Tsh	normal	normal		inappropiate maintenance, high levels
	Eya		reduction		normal
	Dac		LOSS		normal
	H		reduction		
	Dpp			LOSS*	

Figure 2: Summary of eye development during third larval instar (disc proper tissue).

(**A**) Schematic representation of the eye antenna disc during 3rd instar, anterior to the left. Different zones can be distinguished from anterior to posterior (left to right): (1) progenitors that will give rise to head cuticle, (2) progenitors that will give rise to retinal tissue, (3, green) pre-proneural cells within the pre-proneural zone (PPZ), (4, black) cells within the morphogenetic furrow and (5, yellow / red) cells undergoing retinal differentiation and ommatidial assembly. (**B**) Signaling events during eye development (simplified). (**C**) Expression domains of genes involved in neuronal differentiation. (**D**) Expression domains of RD genes. (**E**) The main regulatory interactions between signaling molecules and / or RD genes are illustrated as follows: black arrowheads indicate activation of target genes and red, blunt ends indicate inactivation of target genes. The geometric forms indicate proteins or protein complexes but also target genes when arrows or blunt ends point to them. (**F**) effects of *eyeless*-GAL4 induced UAS-*PcG* / *trxG* mutant clones on the relative concentration of RD gene products compared to adjacent wt tissue (summarized from (Janody, et al., 2004)). The respective mutations and the effects on the expression of RD genes are indicated on the first two columns starting from the left: Effects of *PcG* / *trxG* mutant clones are categorized into 3 groups marked by the colors grey (no change compared to adjacent tissue), yellow (moderate effects) and orange (strong effects). The columns correspond to the above zones (see Figure 2A: eye progenitors, PPZ, morphogenetic furrow and ommatidial assembly). “n.d.” stands for “no data” available.
The eye disc is subdivided into different zones: the most anterior zone will give rise to head cuticle and is characterized by expression of the signaling gene *Wingless* (*Wg*) and the RD gene *homothorax* (*hth*). Adjacent cells are progenitors ready to undergo eye differentiation. Eye progenitor cells are characterized by expression of the RD genes *hth*, *Optix*, the paralogs *twin of eyeless* (*toy*) and *eyeless* (*ey*) and *eyegone* (*eyg*), *twin of eyegone* (*toe*), *teashirt* (*tsh*) and *tio*. Cells undergoing differentiation are named pre-proneural cells because they express the transcription factor *hairy*, a suppressor of the proneural gene *atonal* (*ato*). These cells are located within the pre-proneural zone (PPN) and are characterized by mitotic arrest, the absence of hth and expression of the RD genes *eyes absent* (*eya*), *sine oculis* (*so*), *dachshund* (*dac*) and the paralogs *distal antenna* (*dan*) and *distal antenna related* (*danr*). Cells within the morphogenetic furrow express the morphogen *Decapentaplegic* (*Dpp*) as a result of *hedgehog* (*hh*) expression in more differentiated cells that undergo formation of ommatidia (ommatidial assembly, marked in yellow-red). Cells just prior to the morphogenetic furrow express the proneural gene *atonal* (*ato*) as a consequence of short range acting HH thereby inducing photoreceptor differentiation. *ato* expression becomes restricted to R8 retinula cells upon further differentiation and is indirectly involved in establishing *hh* expression thereby generating a delayed feedback mechanism. Another delayed *hh* feedback mechanism is acting in parallel: SO (acting in complex with EYA) is induced by DPP (HH→*Dpp*). In turn *Hh* becomes activated by SO and downstream factors of ATO thereby combining two feedback loops for the propagation of the HH signal from posterior to anterior. HH also controls the initiation of photoreceptor differentiation. At long range, it activates *hairy*, a negative regulator of *ato* over HH→ *Dpp*; DPP→*hairy*. At short range, HH inhibits *hairy* and promotes *ato* expression thereby controlling the initiation of neuronal differentiation of the photoreceptor neurons. Altogether, the propagation of the morphogenetic furrow from posterior to anterior is a natural consequence of this HH based delayed feedback mechanism.

Cells entering the morphogenetic furrow are characterized by inactivation of the RD genes *toy*, *ey*, *eyg*, *toe*, *tsh*, *tio*, *Optix* and the activation of *nemo*. Cells behind the morphogenetic furrow are characterized by the reduction and a change of ratio of *dan* and *danr*. Furthermore, *dac* becomes repressed halfway in the zone of ommatidial assembly. HTH, that acts in complex with EY and TSH is repressed by the long range acting DPP morphogen. EY and TSH activate *eya* and *so* in absence of HTH. Behind the morphogenetic furrow, *hth* becomes reactivated in a subset of cells (future bristle cells) in a region where the DPP concentration have fallen below a critical level to mediate *hth* repression. *eya* is target of multiple signaling pathways. These are underrepresented in this illustration. See text for further detail.

RD gene	Ref. [1]	Ref. [2]	Ref. [3]
twin of eyeless (toy)		*	
eyeless (ey)	*	*	
eyegone (eyg)	*		
twin of eyegone (toe)	*		
homothorax (hth)	*		*
teashirt (tsh)		*	
tiptop (tio)		*	
sine oculis (so)	*	*	
Optix	*	*	
eyes absent (eya)		*	*
dachshund (dac)	*	*	
Nemo (nmo)			
distal antenna (dan)			
distal antenna related (danr)			

Table 1: Most RD genes are targets of PcG.

Asterisks represent positive identification of the RD genes as PcG targets in genome-wide profiling studies [1], [2] or genome-wide computational prediction of PRE / TREs followed by experimental validation in a transgenic reporter assay [3]. [1] Schwartz et al. 2006. [2] Tolhuis, 2006, [3] Ringrose et al. 2003.

influence on the regulation of the RD genes *so*, *eya* and *dachshund* (*dac*) (Bessa, et al., 2002, Kumar, 2010). Consequently, eye development can be described as the successive activation of the RD network that is accompanied by a modulation of the regulatory relationships between the involved RD genes (see Figure 2).

2.2. Most of the RD genes have been identified as targets of PcG proteins

Most of the RD genes have been identified as targets of PcG in independent genome wide computational and experimental studies indicating PRE / TREs at these genes (Table 1) (Ringrose, et al., 2003, Schwartz, et al., 2006, Tolhuis, et al., 2006). One important question arising from this observation is why the RD gene network which is characterized by cross- and autoregulation by its own members requires the PcG mediated regulation and contains PRE / TREs. Is it that these genes require to be silenced until specific spatiotemporal conditions are attained? If so, the PRE / TRE elements of these genes must contain sub-elements that are adapted to respond to these specific conditions in order to release the gene from the silenced state and allow tissue specific enhancers to become activated. Do the PRE / TREs of the RD genes regulate the transcriptional output of their target genes? Given that so many RD genes are targeted by PcG proteins it may be that central regulation by one system may be the most efficient way to achieve a balanced regulation of RD gene expression. However, this could also be achieved by tissue specific enhancers.

2.3. The early RD genes specify and promote the growth of the eye imaginal disc while signaling organizes axis formation.

The *Drosophila* eye-antennal imaginal disc is derived from a small group of approximately 20 epidermal cells (Cohen, 1993) and specified by the expression of a subset of the retinal determination (RD) genes in the late *Drosophila* embryo. These are the four Pax genes *toy*, *ey, eye gone* (*eyg*) and *twin of eyegone* (*toe*) (Quiring, et al., 1994, Jones, et al., 1998b, Czerny, et al., 1999, Aldaz, et al., 2003). The eye imaginal disc is maintained through the 1st larval instar (L1), enlarged by proliferation during the 2nd larval stage (L2) and differentiated while steady proliferating and growing in size during the 3rd larval instar (L3). The genetic program of the eye imaginal disc is additively altered with the expression of RD genes *homothorax* (*hth*) during L1 and *teashirt* (*tsh*) during L2 (Pichaud and Casares, 2000, Bessa, et al., 2002). Both *hth* and *tsh* have been shown to play important roles in proliferation and survival of eye progenitor cells (Bessa, et al., 2002, Peng, et al., 2009, Lopes and Casares, 2010) (see Figure 2). Additionally, the L2 eye imaginal disc is asymmetrically patterned defining the dorso-ventral and anteroposterior polarity. This process involves multiple signaling pathways including the Hedgehog (HH), Decapentaplegic (DPP), Wingless (WG), Notch and EGF receptor (Egfr / Ras) signaling pathways in different context specific manners (Cho, et al., 2000, Atkins and Mardon, 2009). In one context, these three signaling pathways define the dorso-ventral midline where *Notch* becomes activated and contributes to regulate the growth of the eye imaginal disc. In another context, after the dorso-ventral midline has been established in L2, *hh* and *dpp* signaling are involved in the induction of the eye primordium marked by the expression of *eya, so* and *dac* and accompanied by mitotic arrest at the posterior margin of the eye imaginal disc. At the same time *wg* signaling from the anterior of the eye imaginal disc counteracts differentiation thereby establishing the anteroposterior polarity of the eye imaginal disc that ensures the posterior induction of differentiation. Consistently, ectopic expression of *wg* blocks eye differentiation (Pappu and Mardon, 2004,

Domínguez and Casares, 2005). Consequently, the posterior induction of differentiation is a result of the asymmetric activity of the differentiation inhibiting (Wg) and differentiation activating (HH, Dpp) signaling pathways.

2.4. The eye imaginal disc consists of different cell types organized in two apposed layers

During L2 the eye imaginal disc undergoes morphological changes that lead to the formation of a lumen separating the eye imaginal disc into two layers of cells. The disc proper cells with tall, narrow and cubical cell morphology, will give rise to the adult eye and the antenna. The flat, broad and squamous cells from the peripodial epithelium are apposed to the disc proper thereby forming a cover and defining the distal limits of the eye imaginal disc. During metamorphosis in the pupal stage the peripodial epithelium tissue splits thereby ensuring the eversion of the eye and antenna. Accordingly, the eye and antenna come to be located at the outer surface of the fly and the peripodial epithelium tissue gives rise to adult head capsule. Historically, peripodial epithelium cells were thought to be required solely in eversion and disc fusion during metamorphosis (Fristrom, 1993). Nevertheless, findings over the past decade indicate that peripodial epithelium cells play fundamental roles during eye development (Atkins and Mardon, 2009):

(1) The peripodial epithelium tissue is the exclusive source of the asymmetrically expressed signaling molecules WG, HH, and DPP during early eye development (Cho, et al., 2000). The expression patterns change dynamically throughout developmental progression and in turn organize the patterning of the dorso-ventral midline within the disc proper tissue during L1 and early L2 (L1/2) and the antero-posterior polarity during late L2 /early L3 (L2/3). By L1/2, *wg* expression is restricted to cells in the dorsal domain and by L2/3 in the anterior dorsal domain of the eye imaginal disc. *hh* is expressed in the ventral domain during L1/2 and at high levels at the posterior margin during late L2/3. *dpp* is expressed in the lateral and posterior margins of the eye imaginal disc throughout L1/2 and L2/3. The Notch expressing

dorso-ventral midline in the disc proper tissue is established at the boundary of the Notch ligands Delta and Serrate which are exclusively expressed within the dorsal or ventral domain of the disc proper, respectively. Strikingly, HH signaling is sufficient to induce *ser* in the ventral domain as it has been shown that ectopic expression of *hh* induces *ser* expression when misexpressed in disc proper tissue (Cho, et al., 2000). Consistent with the finding that Notch signaling is required for disc growth by activating *eyg* and *toe* (Wang, et al., 2008) it was shown that the loss of peripodial epithelium derived HH disrupts disc growth (Cho, et al., 2000, Atkins and Mardon, 2009). Consequently, the peripodial epithelium tissue is indispensable for growth and patterning of the eye imaginal disc.

(2) Before the formation of the lumen that separates the two apposed layers, peripodial epithelium cells contribute to the disc proper by cell migration / equatorial cell division. These cells will give rise to the bristle cells located between ommatidia (Pallavi and Shashidhara, 2003, Lim and Choi, 2004, McClure and Schubiger, 2005).

(3) Peripodial epithelium derived cells have been shown to be required for the induction of the morphogenetic furrow. At the margin of peripodial epithelium and disc proper tissues a sub-type of peripodial epithelium cells – cuboidal cells – are involved in signaling as well. At the posterior part of the eye imaginal disc, these cells co-express the three *odd skipped* family members *odd skipped* (*odd*), *drumstick* (*drm*) and *brother of odd with entrails limited* (*bowl*). Strikingly during early L3 the expression of *odd*, *drm* and *bowl* in cuboidal cells is required for the initiation of the morphogenetic furrow in the adjacent disc proper tissue. disc proper tissue is not able to induce the morphogenetic furrow if the adjacent cuboidal cells clones are mutant for bowl. The absence of bowl in cuboidal cells is accompanied with a loss of *hh* expression that is required for the initiation and propagation of the morphogenetic furrow within the disc proper. Furthermore, misexpression of *odd* and *drm* in pre-proneural cells of the disc proper induces ectopic furrows

and premature differentiation (Pallavi and Shashidhara, 2003, McClure and Schubiger, 2005, Bras-Pereira, et al., 2006).

(4) Peripodial epithelium cells signal to disc proper cells over the lumen (Cho, et al., 2000, Gibson and Schubiger, 2000). Strikingly, this is achieved by microtubule based, funnel shaped translumenal cell extensions that range through the lumen and terminate at the disc proper tissue. Generally, one translumenal extension is formed per cell. Peripodial epithelium cell specific genetic disruption of the microtubule motor subunit Glued led to an arrested furrow phenotype. Furthermore, genetic ablation of peripodial epithelium cells during late L3 disrupted ommatidial formation and led to a significant decrease in eye size (Gibson, et al., 2002).
Many of the RD genes are expressed in both the peripodial epithelium and disc proper tissues (*ey, eyg*, *Optix*, *so, eya*). Interestingly, *dac* is not. This may be a reason why peripodial epithelium tissue does not differentiate into retinal tissue but head cuticle instead (Bessa, et al., 2002, Atkins and Mardon, 2009). Taken together, these findings demonstrate that the three-dimensional design of the eye imaginal disc serves fundamental developmental processes during eye development. Besides their role during metamorphosis, peripodial epithelium cells determine the patterning of the Notch expressing dorso-ventral midline of the eye imaginal disc, play a central role in the initiation of the morphogenetic furrow and are involved in the propagation of the furrow via translumenal signaling.

2.5. Photoreceptor differentiation is characterized by initiation of the RD genes *eya*, *so*, *dac*, the pre-proneural gene *hairy* and mitotic arrest

Eye differentiation takes place during L3 where a wave of differentiation initiates at the posterior margin of the eye imaginal disc and traverses it leaving rudimentary ommatidial clusters behind (Figure 2). This wave of differentiation is visually represented by an indentation – the morphogenetic

furrow – that forms in the wake of cells undergoing the first steps of differentiation. The complete traversion of the morphogenetic furrow requires 2 days leaving behind a compound eye that consists of approximately 750 ommatidial clusters that were assembled row by row. The high degree of coordination required for ommatidial assembly may be based on the synchronization of the cell cycle in retinal progenitors. Approaching the morphogenetic furrow, these progenitors undergo rapid and unsynchronized mitotic cycles (first mitotic wave) until entering mitotic arrest where they pause in G1 phase. This zone spans over 15-20 rows of cells anterior to the furrow (Ready, et al., 1976, Tomlinson and Ready, 1987, Wolff and Ready, 1991). The mitotic arrest is achieved by the downregulation of *hth* that has been shown to promote cell proliferation (Bessa and Casares, 2005, Peng, et al., 2009).

hth repression is achieved by a DPP signaling gradient originating from cells within the morphogenetic furrow. The same DPP gradient is also responsible for the induction of the RD genes *eya, so, dac* and the transcription factor hairy (Greenwood and Struhl, 1999, Bessa, et al., 2002, Firth and Baker, 2009). These cells stop proliferating and are referred to as “pre-proneural” because they express the transcription factor hairy, a repressor of the proneural gene *atonal* (*ato*). Loss of hairy leads to the premature expression of *ato* and precocious neurogenesis (Brown, et al., 1995, Greenwood and Struhl, 1999). Pre-proneural cells are locked in to differentiation and mark a band of 15-20 cells located anterior to the morphogenetic furrow. The expression of the proneural gene *ato* is induced right prior to the morphogenetic furrow under control of the short range acting molecule HH. Interestingly, it is HH that activates *dpp* within the morphogenetic furrow (Cho, et al., 2000, Curtiss and Mlodzik, 2000, Atkins and Mardon, 2009). As DPP acts at long range to activate *hairy*, HH is indirectly involved in both repression of *atonal* at long range and its activation at short range. Expression of *ato* at the morphogenetic furrow induces photoreceptor neurogenesis (Greenwood and Struhl, 1999)(Figure 2).

2.6. The progression of the morphogenetic furrow is based on dual delayed autoregulatory feedback loop of *hh* signaling

The reason for the spreading of the morphogenetic furrow is partially resolved at present. One attractive model proposes that the expression of the secreted signaling molecule HH, which is expressed predominantly in the ommatidial retinula cells R2 and R5 behind the morphogenetic furrow, spreads by indirectly inducing its own expression in anterior cells (Roignant and Treisman, 2009). HH signaling induces *ato* expression right prior to the morphogenetic furrow. In turn, *ato* promotes R8 specification. R8 cells express the proteases *Rho* and *Ru* that activate *spitz*. Activated Spitz induces *pointed* expression in R2 and R5 cells. The eye specific enhancer of *hh* requires the combined activity of Pointed and the RD protein SO. Therefore, *hh* can only be induced if DPP mediated activation of *so* (*eya, dac* and *hairy*) has been accomplished. Consequently, the propagation of the HH signal combines two indirect autoregualtory feedback loops that lead to its self-propagation. The establishment of the above processes is not immediate but requires time. The propagation of the morphogenetic furrow is a natural consequence of this HH based feedback loop (Roignant and Treisman, 2009). Consistent with these findings ectopic expression of *hh* anterior of the morphogenetic furrow induces ectopic furrows (Heberlein, et al., 1995). In summary, the propagation of the morphogenetic furrow is based on a dual delayed autoregulatory feedback loop:

1. HH activates *ato* at the morphogenetic furrow. ATO activates *spitz* in R8 cells. Spitz activates *pointed* in R2 / R5 cells.
2. HH activates *dpp* in the morphogenetic furrow. DPP activates *so* in pre-proneural cells.
3. SO and Pointed are both required to induce *hh* expression. This combination of transcription factors coincides posterior of the morphogenetic furrow where R2 and R5 cells are specified

2.7. RD gene expression requires activity of multiple signaling pathways

The activation or inactivation of RD genes requires signaling input from multiple signaling pathways (Firth and Baker, 2009). Nevertheless, activation of RD genes requires also the activity of RD genes. For example, *eya* activation requires DPP activity but also the RD members EY and TSH (Bessa, et al., 2002). During L3 eye development *hth* becomes repressed both by DPP and Ras signaling but does not respond to HH (Firth and Baker, 2009). In contrast, the RD genes *ey* and *tsh* require the combined input of DPP and HH for repression. This is reflected in the distinct expression patterns of these genes. *hth* becomes repressed in the pre-proneural zone where DPP acts at long range (Figure 2). Consistently, DPP or Ras unreceptive progenitors maintain inadequate *hth* expression within the pre-proneural zone. In contrast, *ey* and *tsh* remain being expressed until the morphogenetic furrow where the influence of HH results in their repression. Indeed, HH / DPP double unreceptive clones were unable to repress *ey* and *tsh* expression behind the morphogenetic furrow while single unreceptive clones only delayed the repression (Firth and Baker, 2009). The three RD genes *eya, so* and *dac* have been experimentally identified as direct targets of DPP and HH signaling (Chang, et al., 2001). DPP acts on *dac* over an eye specific enhancer located within the eighth intron (Pappu, et al., 2005). Moreover, HH or DPP unreceptive clones display reduced expression levels of *eya*, *so* and *dac* compared to wt tissue (Curtiss and Mlodzik, 2000, Firth and Baker, 2009) (Figure2). Interestingly, *eya* has been shown to be a direct target of HH, DPP but also Ras and Notch signaling (Firth and Baker, 2009). Additionally, it is negatively regulated by WG signaling (Treisman and Rubin, 1995, Baonza and Freeman, 2002, Legent and Treisman, 2008, Salzer and Kumar, 2008).

In summary, the diffusible signaling molecules HH, DPP and WG act upstream in the process that defines the dorso-ventral dimensions and the

anteroposterior polarity of the eye imaginal disc and regulate its growth. and thereby the dorso-ventral midline that is required for disc growth. Additionally, HH organizes a cascade of events that lead to the establishment of a delayed autoregulatory feedback loop to mediate progression of eye differentiation from posterior to anterior. The pre-proneural cells lock-in to retinal differentiation, become arrested in G1 and are defined by the co-expression of the DPP and HH target *eya, so* and *dac* and the pre-proneural gene hairy. Activation of these four genes requires repression of *hth* that is achieved by DPP and Ras signaling. With exception of *hth*, the genes *eya, so* and *dac* are coexpressed with *ey* and *tsh* within the pre-proneural zone. Although *ey, tsh, eya, so* and *dac* cross-regulate each other it is HH and DPP signaling that determine the repression of *ey* and *tsh* at the morphogenetic furrow (see Figure 2).

2.8. The RD genes cross-regulate each other

HTH, EY and TSH form a complex that negatively regulates the expression of *eya* and *so.* In the absence of HTH *eya* and *so* are positively regulated by EY and TSH (Bessa, et al., 2002, Pappu, et al., 2003, Ostrin, et al., 2006). EYA forms a bipartite transcription factor with SO that targets the RD genes *ey, so* and *dac (Halder, et al., 1998, Pappu, 2002)*. In turn, DAC positively regulates *eya* expression thereby forming a regulatory circuit that is able to maintain its expression in absence of TSH and EY behind the morphogenetic furrow (Punzo, et al., 2002, Pappu, et al., 2005). EYA and DAC have been reported to act in a complex whereby EYA transactivates DAC (turning it from a corepressor into an activator) while DAC provides target specificity (Chen, et al., 1997).

2.9. RD genes form multiple complexes involving cofactors associated with chromatin interactions.

Optix is able to interact with multiple cofactors during eye development, including the corepressor Groucho and the gene product of *Optix-binding protein* (*Obp*) that acts in cell proliferation (Kenyon, et al., 2005a, Kenyon, et

al., 2005b)(see Figure 2). The *dan* and *danr* gene products form multiple distinct complexes including the interaction partners EY, DAC and CtBP (Curtiss, et al., 2007, Hoang, et al., 2010). Given its association to chromatin remodeling, the interaction with CtBP links the RD factors to interactions with chromatin. Furthermore, CtBP has been reported to be required for the recruitment of PHO and the binding of PcG complexes (Sewalt, et al., 1999). Therefore, it is possible that PcG interactions are a regular phenomenon in the regulation of RD genes during eye development, especially as EY is associated with negative regulation of *eya* and *so* in eye progenitors and DAC acts as corepressor. Indeed, a subset of both *PcG* and *trxG* genes has been shown to play important roles in retinal differentiation. These include the *trxG* genes *trx*, osa, *brm*, *skd* and *kto* and the *PcG* genes *Pc* and *E(z)* (Janody, et al., 2004) (see Figure 2).

Behind the morphogenetic furrow, *dac* expression becomes repressed halfway during ommatidial assembly (Figure 2). The SO-Groucho complex has been reported to be required for *dac* repression in the retinal tissue. Strikingly, *groucho* mutant clones were not able to repress *dac* expression in retinal tissue (Salzer and Kumar, 2008). This raises the question how this could mechanistically be achieved: As *groucho* is ubiquitously expressed and *dac* was co-expressed together with *so* without being repressed beforehand another factor must coincide with the repression of *dac* (Figure 2). Interestingly, the advent of the *Sine oculis-binding protein* (*Sbp*) has been shown to approximately correlate with the downregulation of *dac* in retinal tissue (Kenyon, et al., 2005b). Similar to Groucho, it can physically interact with SO thereby possibly changing its target specificity. Although little is known about its function *sbp* may be a good candidate for *dac* repression in retinal tissue (see Figure 2).

2.10. The relative concentration of RD genes determines which complexes will predominantly form

Interestingly, Danr is required for high levels of *eya* expression indicating that the regulation of *eya* levels plays a functional role during eye development. Furthermore, both dan and danr are asymmetrically downregulated behind the morphogenetic furrow leading to a change in ratio of these antagonistic factors (Figure 2) (Curtiss and Mlodzik, 2000, Curtiss, et al., 2007). Based on their findings, Curtis and colleagues made a thoughtful proposition that can be applied to the entire RD network and its cofactors: the relative concentration and availability of cofactors may have deep impact on which protein-protein complexes predominantly form thus playing a decisive role on the regulatory output of the genome. Consequently, RD gene expression levels need to be tightly regulated (Curtiss and Mlodzik, 2000, Curtiss, et al., 2007). Indeed, it has been shown that the RD gene *nemo*, which is initiated directly at the morphogenetic furrow and maintained in retinal tissue may titrate the relative concentrations of RD factors (Braid and Verheyen, 2008). Nemo is a Serine / Threonine kinase that genetically interacts with *ey, eya* and *dac*. Interestingly, *ey* and *eya* mutant alleles with a mild rough eye phenotype retaining some expression were rescued upon loss of *nemo*. This indicates that reduction of Nemo protein can modulate the activity of RD complexes restoring developmental integrity (Mirkovic, et al., 2002, Fiehler and Wolff, 2008).

In summary, cofactors interacting with RD genes may change the stoichiomertry for the formation of any given complex thereby having a deep impact on which complexes will predominantly form. Consequently, the relative levels of RD genes are required to be strictly controled in order to specify regulatory outcomes. The interaction DAN and DANR with cofactors such as CtBP potentially link RD gene regulation with the PcG / TrxG system.

3. *eyes absent (eya)*

The main focus of this thesis is the regulation of *eya* and especially the function of the *eya* PRE / TRE during eye development. The *eya* locus contains a characterized eye specific enhancer (Bui, et al., 2000) and a characterized PRE / TRE (Ringrose, et al., 2003). Aside from eye development, *eya* plays roles during embryogenesis and ocellar development (Bonini, et al., 1993). Furthermore, it is expressed within lamina cells of the optic lobe, individual cells within the brain, the ventral nerve chord and the ovary (Bonini, et al., 1998).

3.1. Biological and molecular properties of EYA

During eye development, *eya* is required to prevent apoptosis, to initiate and propagate the morphogenetic furrow and to acquire neuronal development and axon targeting of retinal neurons into the optic lobe (Bonini, et al., 1993, Pignoni, et al., 1997, Xiong, et al., 2009).

EYA acts as transcriptional coactivator (Ohto, et al., 1999, Silver, et al., 2003) and as tyrosine phosphatase (Li, et al., 2003, Rayapureddi, et al., 2003, Tootle, et al., 2003). It is the founding member of the EYA phosphatase family and one of the first examples of a transcription factor with enzymatic activity (reviewed in (Rebay, et al., 2005). EYA is not able to bind DNA itself and regulates transcription via transactivation of cofactors such as SO and DAC (Chen, et al., 1997, Pignoni, et al., 1997, Niimi, et al., 1999, Tavsanli, et al., 2004). Transactivation is mediated over the *eyes absent* domain (ED) that has been reported to interact with the six domain of SO. Phosphorylation of EYA by the Egfr / Ras / mitogen activated protein kinase (MAPK) results in an increase of the transactivation potential (Hsiao, et al., 2001, Silver, et al., 2003). Furthermore, EYA serves as substrate for the gene product of the *abelson tyrosine kinase* (*abl*). ABL mediated phosphorylation of EYA results in its export to the cytoplasm (Xiong, et al., 2009). Finally, EYA is able to interact with itself, especially when phosphorylated and displays autocatalytic

dephosphorylation resulting in nuclear reimport (Silver, et al., 2003, Tootle, et al., 2003).

3.2. *eya* expression requires EY, TSH and DAC. Interactions with DAN and DANR could recruit the PcG complexes

Induction of *eya* and *so* within the pre-proneural zone has been shown to require activation by EY and TSH in the absence of HTH (Halder, et al., 1998, Bessa, et al., 2002, Michaut, et al., 2003, Ostrin, et al., 2006). Targets of SO-EYA include *hh*, *ey*, *so* and *dac* reflecting feedback (*hh* and *ey*) and autoregulatory feedback (*so*) loops that define the network nature of the RD genes (Halder, et al., 1998, Pappu, 2002). In turn, DAC being the only of the three not being activated by EY-TSH feeds back to *eya* and *so* thereby generating a feedback loop that is able to maintain the expression of *eya, so* and *dac* behind the morphogenetic furrow where *ey* and *tsh* expression has ceased (Punzo, et al., 2002, Pappu, et al., 2005). The feedback to *eya* is probably achieved over the *eya* eye specific enhancer that has been shown to be responsive to EY and DAC (Bui, et al., 2000). Interestingly, the RD Proteins DAN and DANR have been shown to undergo complex formation with CtBP but also with EY and DAC (Curtiss, et al., 2007, Hoang, et al., 2010). This makes it possible that CtBP, a PcG interactor is directed to target genes, including *eya*.

3.3. *eya* is target of several signaling pathways involved in eye development

As previously mentioned, the induction of the pre-proneural zone requires activity of the signaling pathways DPP and HH in order to induce *eya, so* and *dac* and the signaling pathways DPP and Ras to repress *hth* (Pappu, et al., 2003, Firth and Baker, 2009, Baker and Firth, 2011). Interestingly, *eya* is target of additional signaling pathways: It is activated by Notch and Ras signaling and negatively regulated by *wg* signaling. Therefore, *eya* adopts a

unique position within the RD network members integrating the main signaling pathways involved in eye development: HH, DPP, RAS, Notch and WG. The ability to detect the activity of these signaling pathways makes *eya* a central player in eye development (Firth and Baker, 2009, Salzer, et al., 2010, Baker and Firth, 2011). The EGFR receptor pathway (including Ras) has been shown to play a critical role in regulating *eya* expression levels. The effector of EGFR signaling is the MAPK and its activity is regulated by the antagonists Yak / *anterior open* (*aop*) and *pointed* (*ptd*). Strikingly, *aop* mutant clones display increased *eya* levels while *ptd* mutant clones display decreased expression levels of *eya* (Salzer, et al., 2010). This is especially intriguing as the transactivation potential of EYA depends on phosphorylation by MAPK (Hsiao, et al., 2001, Silver, et al., 2003). In turn, EYA may act on its own expression by modifying the activity of the corepressor DAC that feeds back to *eya* (Chen, et al., 1997, Punzo, et al., 2002, Tavsanli, et al., 2004, Pappu, et al., 2005). Interestingly, *aop* is not expressed anterior of the morphogenetic furrow but at high levels directly behind the morphogenetic furrow with decreasing levels further to the posterior of the eye imaginal disc. This expression pattern reflects the activity of *aop* that was found to repress premature neuronal specification. In the context of *eya,* these findings indicate that EGFR / RAS signaling regulates *eya* expression levels during normal eye development (Salzer, et al., 2010).

Considering that the *eya* gene contains a PRE / TRE and is not expressed in eye progenitors, it is possible that *eya* becomes “unlocked” prior or simultaneous with its activation in response to signaling events.

3.4. The *eya* locus is a target of Zeste during eye development

The *eya* mutant allele eya^2 lacks the eye specific enhancer resulting in eyeless flies when homozygous. Biologically, eya^2 mutant cells undergo apoptosis in the pre-proneural zone indicating that EYA has anti-apoptotic functions

(Bonini, et al., 1993, Zimmerman, et al., 2000). Interestingly, the combination of the *eya*^{2} allele with the likewise eyeless *eya*^{4} allele resulted in complementation with a rescue of the mutant phenotype marked by eyes ¾ the size of wt eyes. This complementation was disrupted in a *zeste* mutant background leading to a reduction in eye size and showing that the *eya* locus is a direct target of Zeste during eye development (Leiserson, et al., 1994). As *zeste* is linked to PcG and TrxG mediated regulation, the above observation makes it likely that the *eya* locus is a target of PcG and TrxG proteins during eye development.

3.5. EYA is expressed as two alternative isoforms that differ at the N-terminus

The *eya* gene architecture of provides two splice variants that differ from each other only within their first exon each deriving from an alternative promoter. Both isoforms are expressed during regular eye development and have been shown to rescue the mutant phenotype of *eya*^{2} and *eya*^{4} mutants when induced during L3 under control of a heat shock promoter (Leiserson, et al., 1998). The coding sequences of the alternative first exons 1.1 and 1.2 code for 19 and 23 amino acids, respectively. The amino acid sequence is well conserved and codon integrity has been under selective pressure during the evolution of both species suggesting an important function for each of the N-terminal ends. These findings are summarized in Figure 3.

While EYA is well characterized in terms of enzymatic function and coactivator activity very little is known about the functional attributes that distinguish both isoforms. Nevertheless besides conservation, one interesting observation hints to different requirements for both isoforms: During embryogenesis, only *eya* 1.2 is expressed at the onset of zygotic gene expression in a specific and dynamic expression pattern within head and segments including parts of the visual primordium and optic lobe (Bonini, et al., 1993, Leiserson, et al., 1998).

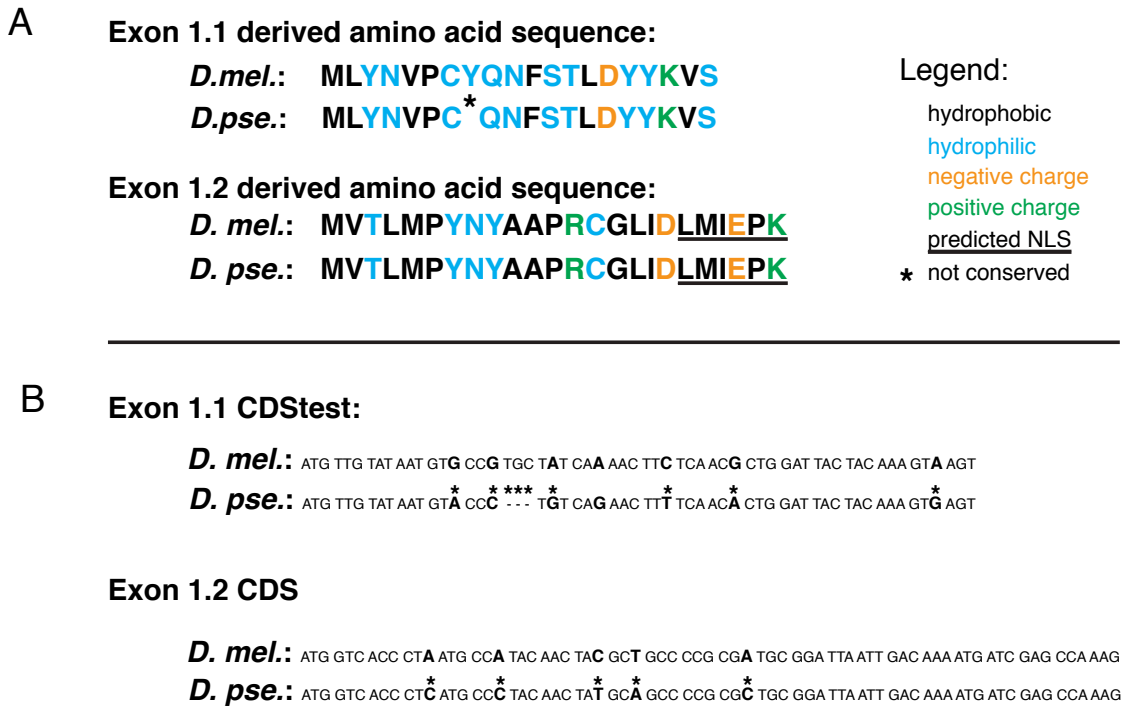


Figure 3: Conservation of the coding sequences of *eya* exon 1.1 and 1.2 at levels of amino acid and DNA sequence.
(**A**) The exon 1.1 derived peptide predominantly consists of hydrophilic amino acids while the exon 2.2 derived peptide is mainly composed of hydrophobic amino acids. Both peptides contain amino acids of positive and negative charge. Additionally, both peptides contain tyrosine residues that could be targets of phosphorylation. The exon 1.1 derived peptide of *D. pseudoobscura* lacks one tyrosine residue compared to the *D. melanogaster* sequence. Apart from that, the amino acid sequences are identical in both exon 1.1 and exon 1.2 derived peptides. (**B**) The conservation at level of DNA sequence is lower as indicated by asterisks that depict not conserved nucleotides. Together these findings suggest a functional requirement for both exon 1.1 and exon 1.2 derived N-terminal peptides.

3.6. The transactivation property of EYA is largely independent of its phosphatase function

As mentioned above EYA acts as tyrosine phosphatase (Li, et al., 2003, Rayapureddi, et al., 2003, Tootle, et al., 2003). Two lines of evidence suggest that the transactivation property of EYA acts largely independent of the

phosphatase activity although certain developmental aspects seem to require both functions: First, using eye specific *ey*-Gal4 mediated expression of UAS-*eya* in wildtype and phosphatase mutant form Rayapureddi and colleagues compared the degree of rescue obtained in eya^{2} mutant flies (these flies lack eye specific *eya* expression and have no eyes). Phosphatase mutant EYA^{D493N} showed a lower degree of rescue compared to wt but was still able to generate eyes approximately half the size of the wt rescue or wt flies (Rayapureddi, et al., 2003). Second, the reduction of EYA phosphatase activity does not alter the transcriptional output at global scale. Using heat-shock mediated transgenic overexpression of wildtype *eya* and phosphatase mutant *eya* Jemc and Reebay were able to show that the two transgenes activated largely the same target genes (80% overlap) (Jemc and Rebay, 2007a). Consequently, both results suggest that EYA can act as coactivator that is able to actuate gene expression largely independent of its phosphatase function while both functions are required for the full range of EYA activity.

Further insight into the role of the phosphatase activity comes from experiments carried out in murine cell culture. Cotransfection of the murine *Six1* and *Dach* genes silenced reporter gene expression from a SIX1 responsive promoter. In contrast, additional cotransfection with *Eya3* relieved *Dach1* mediated repression and activated reporter gene activity by 4-5 fold. Expression levels using a phosphatase mutant form of *Eya3* were comparable to expression of *Six1* and *Dach* alone. These results show that EYA3 switches DACH from a repressor to an activator. This process requires the EYA3 phosphatase function (Li, et al., 2003). Although these experiments were performed in a murine system a similar relationship between the *Drosophila* homologs EYA and DAC seem likely as genetic synergy between the genes is well characterized (Bonini, et al., 1993, Cheyette, et al., 1994, Mardon, et al., 1994). According with this hypothesis is the finding that phosphatase mutant *eya* was not able to entirely mimic target gene activation of wildtype *eya* when overexpressed (80%) (See above, (Jemc and Rebay, 2007a)). Therefore in *Drosophila*, EYA may require its phosphatase function for a subset of

regulatory events within the nucleus (involving DAC), while the bulk of EYA mediated regulation does not require phosphatase activity and involves the EYA-SO complex.

3.7. The cytoplasmic localization of EYA is important for eye development

In addition to its role as nuclear cofactor EYA has been shown to be required in the cytoplasm (Xiong, et al., 2009). In murine cell culture, EYA2 localizes to the nucleus when SIX family proteins are absent (in *Drosophila*, SO is a SIX family protein) (Ohto, et al., 1999, Zhang, et al., 2004). Cytosolic EYA2 has been shown to interact with several Gαi proteins that localize it to the plasma membrane and prevent interaction with SIX family members (Embry, et al., 2004). G proteins are components of cellular communication pathways that mediate the transmission of extracellular signals from cell surface receptors to a wide range of intracellular effectors (Gilman, 1987, Neer, 1995). In *Drosophila*, Xiong and colleages were able to show that EYA is a substrate of the ABL tyrosine kinase that mediates phosphorylation of EYA resulting in its nuclear export (Xiong, et al., 2009). Taken together, these results indicate a model in which EYA exists in a nuclear and cytosolic pool. The abundance of EYA in each pool can be regulated by the activity of six family (EYA nuclear pool), Gαi proteins (cytosolic pool, retention at the plasma membrane) and ABL activity (export into the cytosol). Considering the autocatalytic activity of EYA (Tootle, et al., 2003) it seems possible that nuclear relocalization of EYA may be achieved upon EYA mediated self-dephosphorylation that counteracts ABL activity (Xiong, et al., 2009). Regarding the function of EYA in the cytoplasm, it has been shown that cytoplasmic EYA is required to rescue the *eya*^{2} mutant phenotype. Nuclear restricted NLS-EYA resulted in a severely reduced capacity to rescue the *eya*^{2} mutant phenotype. In contrast, EYA^{wt} resulted in 100 % rescue when expressed under control of ey-Gal4. Similar results were obtained using the Dpp-Gal4 driver resulting in the

formation of ectopic eyes in non-retinal tissues. Nuclear restricted NLS-EYA displayed a lower efficiency in the generation of ectopic eyes compared to misexpressed EYA^{wt}. Furthermore, coexpression of the nuclear restricted NLS-EYA and the cytosol restricted Myc-EYA was able to adapt to the efficiency of ectopic eye formation to EYA^{wt} levels. Strikingly, this was not the case with the phosphatase dead version of Myc-EYA^{mut} showing that the phosphatase function of EYA is required in the cytoplasm independent of nuclear – cytoplasmic circulation (Xiong, et al., 2009).

3.8. *eya* expression levels play an important role in axon guidance and targeting to the optic lobe

eya mutations have been shown to genetically interact with *abl* mutations. During embryogenesis, double mutants display a defect in axon pathfinding that leads to malformation of the central nervous system (Xiong, et al., 2009). During L3 eye development the manipulation of *eya* levels in postmitotic photoreceptors perturbs axon pathfinding into the optic lobe. Both GMR-Gal4 driven *eya* RNAi and GMR-Gal4 driven *eya* overexpression caused photoreceptor axons to mistarget the lamina of the optic lobe and to form bundles of axons. Strikingly, only overexpression of cytosol retained MYC-EYA but not phosphatase dead MYC-EYA generated a mistargeting phenotype. These results show that EYA phosphatase activity in the cytosol is required for axon targeting into the optic lobe (Xiong, et al., 2009). Furthermore, over- and underexpression of *eya* leads to mistargeting of axons indicating that *eya* levels need to be tightly regulated. As the phenotype observed in over- and under-expression experiments mimics the abl phenotype both in mistargeting and in axon bundle formation it seems likely that EYA is involved in the same processes as ABL. *abl* has been reported to regulate axon outgrowth, to influence the actin cytosceleton, and to link axon guidance receptors to the cytoskeleton (Liebl, et al., 2000, Grevengoed, et al., 2001). Additional data supporting the role of EYA in axon pathfinding comes from a study that identified both EYA and DAC as players in the

establishment of the neuropeptide FMRFamide related (Fmrf). Fmrf is a neuropeptide expressed in a subset of neurons of the central nerve chord and required for axon pathfinding. Within these neurons *eya* directs axon pathfinding and regulates BMP signaling. BMP signaling in turn regulates axon pathfinding of adjacent neuronal axons as an extrinsic effector (Miguel-Aliaga, et al., 2004).

While there are indications that the nuclear and cytoplasmic pools of EYA may be regulated by shifting parameters such as six protein expression, Gαi protein activity and abl activity, the total amount of EYA protein may play a central role in this equation. It has been hypothesized that optimal levels of RD genes and cofactors are a decisive factor that determines which of the complexes are predominantly formed and take leadership on regulation (Curtiss and Mlodzik, 2000, Curtiss, et al., 2007). Necessity for optimal an optimal level of *eya* expression has also been demonstrated for the cytosol, where it is required for proper axon targeting into the lamina of the optic lobe (Xiong, et al., 2009). Finally, It has been shown that *eya* levels can be regulated during eye development. The RD gene *danr* is required for high levels of *eya* expression (Curtiss, et al., 2007). Interestingly, *danr* directly interacts with CtBP and therefore makes it possible that the regulation of *eya* levels may require chromatin-associated machineries.

3.9. *eya* is misexpressed in *PcG* and *trxG* mutant clones during eye development

eya has been shown to be misregulated in eye specific *PcG* and *trxG* mutant clones (Janody, et al., 2004) (see Figure 2). *trx* mutant clones displayed a reduction in *eya* expression levels and a delay of its induction compared to wt tissue. *skd* and *kto* mutant clones were characterized by a reduction of *eya* levels. *Pc* and *E(z)* mutant clones displayed a reduction in *eya* levels as well. One plausible explanation for these effects on *eya* may be the regulation of *ey*, *tsh* or *hth* in either of the mutant clones: *trx* mutant clones have a complete lack of *ey* and *tsh* expression (see Figure 2). *skd* and *kto* mutant

clones have a reduction of *tsh* and an increase of *ey* expression accompanied with its inadequate maintenance. *Pc* and *E(z)* mutant clones have a reduction in *ey* levels, and misexpression of *tsh* and *hth* in posterior clones. Possibly, any of these changes may lead to a misregulation of *eya*. However, direct effects of PcG and TrxG proteins on the *eya* gene cannot be ruled out.

3.10. Open questions regarding the two *eya* isoforms

The above findings make it likely that *eya* levels are transcriptionally tightly regulated to achieve an optimal total number of EYA protein in order to safeguard transcriptional regulation and axon pathfinding. To date, only the redundant functions of the *eya* isoforms have been characterized. Both isoforms can induce ectopic eye formation and rescue *eya* mutant phenotypes (Leiserson, et al., 1998). However, it is possible that the two isoforms may not be entirely redundant as indicated by the differential regulation during embryogenesis (Bonini, et al., 1998, Leiserson, et al., 1998). Future experiments will address the function of the distinct N-termini and answer the question whether the difference is relevant at all or has a functional role in the nucleus, the cytoplasm or in both cellular compartments.

3.11. *eya* summary

In summary, *eya* is expressed in form of two splice variants that differ at their N-terminal ends. Only one of them *eya* 1.2 is expressed during embryogenesis while both, *eya* 1.1 and *eya* 1.2 are expressed during eye development. At present, it is not known whether both isoforms have distinct functions or are fully redundant. Neither is it known whether the two isoforms are regulated individually or as unit during eye development. The molecular function of EYA has been determined as coactivator and tyrosine phosphatase. Both functions act largely independent from each other. The coactivator function is required in the nucleus while the phosphatase function is mainly required in the cytoplasm and with secondary importance in the nucleus. The cytoplasmic activity of EYA is important for axon pathfinding but

also required for complete retinal development. *eya* is targeted by the RD proteins EY / TSH and DAC and positively regulated by the signaling pathways HH, Dpp, Notch, Egfr / Ras and negatively regulated by Wg. The *eya* locus is a target of Zeste and *eya* is misregulated in *PcG* and *trxG* mutant clones. EYA undergoes complex formation with the DNA binding factors SO and DAC. Among others, SO-EYA regulates *Hh*, *ey*, *so* and *dac* while EYA-DAC regulates *eya*. Multiple lines of evidence suggest that a tight regulation of *eya* expression levels is required to achieve optimal eye development with the corresponding axon targeting into the optic lobe. As *eya* is not expressed until retinal differentiation is initiated although EY and TSH are present it is possible that the *eya* gene is actively maintained silenced in other tissues and at earlier time points. While the presence of HTH turns EY and TSH into repressors, it is still possible that silencing of *eya* depends on PcG proteins. Accordingly, interactions between EY and CtBP have been reported.

In addition to eye specific expression, *eya* expression has been reported within the lamina of the optic lobe. However, to date it is not known where the optic lobe specific enhancer is located within *eya* regulatory sequence.

4. Structure, Function and Development of the Optic Lobe

During eye development, the axons of the photoreceptor neurons innervate into different layers of the optic lobe. Interestingly, *eya* is transiently expressed in the lamina precursor cells, the uppermost layer of the optic lobe (Bonini, et al., 1998).

Each ommatidium contains the 8 retinula cells R1 – R8, photoreceptors that mediate visual information over their axons into the optic lobes of the fly brain (reviewed in (Fischbach and Hiesinger, 2008)). The retinula cells R1 – R6 surround the R7 and R8 cells that are located in the center of each ommatidium. R1 – R6 cells are required for spatial vision, while R7 and R8 cells mediate color vision. Axons from the retinula cells project into the optic lobe, a neuronal macrostructure that processes visual information and is organized in a highly specific and symmetric form. The optic lobe is composed of different ganglions, the lamina, medulla and lobula complex. The lamina and medulla are separate neuronal layers that are specialized to process visual information whereby the lamina is located distal to the medulla. Both layers serve as target for distinct retinal neurons. The lamina is targeted by the axons of R1 – R6, while R7 and R8 target into the deeper located medulla (Braitenberg, 1967). Axon targeting into the optic lobe is a highly controlled process and organized in such a way that axons from R-cells of 6 different ommatidia that see the same point in space innervate into the same lamina cartridge. Each cartridge is composed of three lamina monoplanar cells (L1 – L3) that are synaptically connected to R1 – R6 cells from 6 different ommatidia. Axons deriving from the lamina monoplanar (L1 – L3) cells innervate into different layers of the same medulla column. Additionally, R8 cells target the outer layer of the medulla while R7 cells innervate a deeper layer of the same medulla column. Therefore, each lamina cartridge unites visual input from one point of space via R1 – R6 cells from 6 different ommatidia, while the corresponding medulla cartridge unites the same information plus additional color vision information from a 7th

ommatidium (L1, L2, L3, R7 and R8). Hence, the medulla integrates spatial and color information. The process of wiring R-cells from distinct ommatidia takes place during the pupal stage. During axon outgrowth in L3, the R1 – R6 axons from a single ommatidium stay in close proximity and terminate at the lamina neuropil. Interestingly, mutant flies that do not develop retinal tissue also do not develop a lamina and have a severely reduced medulla and lobula. Similarly, mutations reducing eye size are characterized by a reduced lamina (Steller, et al., 1987). This means that the development of the optic ganglions is an inductive process fueled by innervation from retinal axons that induce the neurogenesis of lamina neurons and lamina glia (Selleck and Steller, 1991, Selleck, et al., 1992, Winberg, et al., 1992). Indeed, HH and the Egfr signaling ligand Spitz have been shown to be transported in retinal axons (Huang and Kunes, 1996). As lamina precursor cells express EGFR, lamina differentiation is induced by Spitz. R cell innervation to the lamina is temporally linked to eye differentiation. Accordingly, the lamina grows and differentiates from posterior to anterior. The molecular mechanism that is responsible for R1 – R6 cells to terminate at the yet undifferentiated lamina neuropil, while R7 and R8 cells terminate in the medulla is not resolved at present but it may involve connectivity to glial cells. A mutation in the gene *nonstop* which is specifically expressed in glia cells resulted in mistargeting of R1-R6 cells indicating that R1-R6 axons terminate by contacting lamina glia cells – they happen to surround the lamina - until the lamina has differentiated (Poeck, et al., 2001). Similarly, *medea* mutant clones that are characterized by the lack of glia cells resulted in R1-R6 mistargeting (Yoshida, et al., 2005)(reviewed in (Fischbach and Hiesinger, 2008)). On the other hand, overshooting phenotypes have also been reported in abl and *eya* mutant R-cells indicating that these genes are involved in processes that direct cell-cell recognition (Xiong, et al., 2009).

A shape resembling a horseshoe characterizes the lamina of the optic lobe. The innervation scheme of retinal axons into this structure follows a stringent pattern where dorsal (D) and ventral (V) retinal cells innervate to the extremities of the horseshoe (D’ and V’), while cells are located along the

midline (M) innervate into the center position of the horseshoe (M') to lamina cells reflecting the respective anteroposterior position. The Innervation scheme is illustrated in Figure 14 AA.

5. Connections between PcG / TrxG mediated regulation and RD gene regulation

5.1. Does the dynamic regulation of the RD genes require PRE / TREs that are different from Hox gene PRE / TREs?

The RD genes are known to undergo extensive cross- and auto-regulation. In addition, multiple signaling pathways organize their spatiotemporal expression. This raises the question about the role of PcG proteins in RD gene regulation. *Hox* PRE / TREs have been shown to be bi-stable switchable elements that are able to maintain active or silenced reporter gene expression over multiple mitotic divisions. Therefore, they have properties of epigenetic memory elements (Simon, et al., 1993, Chan, et al., 1994, Chiang, et al., 1995, Cavalli and Paro, 1998, Cavalli and Paro, 1999). In contrast, RD genes are dynamically expressed during eye development and they can maintain, activate or inactivate their expression via cross- and autoregulation and the input from signaling pathways. Therefore, the role of the PcG and TrxG system in RD gene regulation might be substantially different from regulation of the *Hox* genes where a clear distinction between initiation by transcription factors and the maintenance by PcG and TrxG proteins has been demonstrated (Ringrose, 2007). Nevertheless, similarities between *Hox* gene regulation and the RD gene *eya* regulation exist: in both cases, the factors responsible for initiation are expressed transiently. The transiently expressed factors responsible for *eya* activation are DPP, HH, EY, TSH and DAC. After these factors have decayed, *eya* expression is still maintained. Therefore, the main difference compared to *Hox* genes is that eye specific expression of *eya* is transcriptionally silent in eye precursor lineage until larval development.

5.2. The PcG / TrxG system may be involved in regulating transcriptional levels of target genes

Although multiple analyzed PRE / TREs display the basic attributes of maintaining activation and silencing one attribute may require more attention. The range of transcriptional activity goes from silencing to activation and therefore leaves place for regulation of transcriptional levels. Strikingly, Oktaba and colleagues found that PcG proteins regulate the expression levels of the PcG target and cell cycle regulator *Cyclin B*. Both *Psc-Su(z)2* and *ph* mutant clones displayed higher levels of *Cyclin B* expression (Oktaba, et al., 2008). Similarly, Martinez and colleagues identified *Cyclin A* as a target of PcG proteins (Martinez, et al., 2006). Furthermore, it was shown that the *ph-p* PRE / TREs are *cis* regulatory elements that modulate the transcriptional output rather than mediate silencing or activation. Consistent with this finding is the fact the *ph* gene is maternally and ubiquitously expressed leaving no room for complete silencing (Fauvarque, et al., 1995, Bloyer, et al., 2003). It is tempting to speculate that the promoter output of multiple PcG target genes could be regulated over the PcG / TrxG system thereby reinforcing or modulating the transcriptional output of each gene in the RD network (Ringrose, 2007). Given the indications that the regulation of RD genes requires precise adjustment of expression levels (Curtiss and Mlodzik, 2000, Curtiss, et al., 2007), one question springs to mind:

Is it possible that PcG and TrxG proteins are involved in adjusting specific *eya* gene expression levels in the developing eye and the optic lobe?

5.3. Is it possible to analyze the role of PcG and TrxG proteins in *eya* regulation without indirect effects resulting from *PcG* and *trxG* mutations?

It could be that PcG mediated regulation of the RD genes simply serves to prevent expression in inappropriate tissues or to prevent premature

expression. Nevertheless, such a role does not exclude that the PcG / TrxG system is directly involved in the dynamic regulation of RD genes during eye development. As mentioned above, it has been shown that eye specific *PcG* and *trxG* mutant clones fail to develop retinal tissue and exhibit misexpression of RD genes or that eye specific RNAi of *PcG* and *trxG* mutant genes disrupts eye development at distinct stages of retinal differentiation (Janody, et al., 2004). These findings show that the PcG and TrxG system indeed plays a substantial role in eye development but it is difficult to distinguish between a direct or indirect involvement in RD gene regulation during eye development. Given that the signaling genes *hh*, *dpp*, *Egfr* and *wg* have been identified as PcG targets (Schwartz, et al., 2006, Tolhuis, et al., 2006, Pérez, et al., 2011), inappropriate regulation of these genes would lead to a disruption of eye development without allowing conclusions about direct RD gene regulation by the PcG / TrxG system. In addition, the same argumentation applies to multiple other PcG targets. Given the high number of PcG and TrxG target genes that are involved in transcriptional regulation and signaling, the removal of any *PcG* or *trxG* gene during eye development could cause random derepression or repression of target genes thereby initiating a chaotic cascade of gene regulatory events. In order study the possible role of PcG / TrxG mediated regulation of the RD genes it is essential to circumvent the indirect effects that may be caused in *PcG* and *trxG* mutant backgrounds.

5.4. *eya* plays a central role in eye development and is a good choice to study PcG / TrxG mediated RD gene regulation in eye development

eya has been predicted and verified as a target of PcG making it good choice to study the role PcG / TrxG mediated RD gene regulation during eye development (Ringrose, et al., 2003). Furthermore, *eya* is target of multiple signaling pathways making it a central player among the RD gene network (Firth and Baker, 2009, Salzer, et al., 2010). Its correct expression levels have

been shown to play a decisive role in eye formation and axon targeting into the optic lobe (Braid and Verheyen, 2008, Xiong, et al., 2009). Strikingly, the RD members DAN and DANR regulate *eya* expression levels. These two RD genes interact with CtBP, a known PcG interactor. EY and DAC can also interact with CtBP thereby revealing a possible link for PcG mediated regulation of *eya* during eye development. A further possible link to both PcG and TrxG mediated regulation is the presence of Zeste at the *eya* locus during eye development (Leiserson, et al., 1994). All above attributes make *eya* a promising candidate gene to study the gene regulatory role of the PcG and TrxG system during eye development and to test whether its PRE / TRE is a interchangeable element or possesses individual adaptations to regulate the expression of the *eya* gene.

II. AIM OF THIS THESIS

The aim of this thesis is to study the role of the PcG / TrxG system during eye development using the example of the *eya* gene. To do so, I decided to work with a *gfp* and *miniwhite* expressing reporter gene construct that contains *eya* specific regulatory information including the eye specific enhancer and the *eya* PRE / TRE (Bui, et al., 2000, Ringrose, et al., 2003). In order to maintain the regulatory integrity, the reporter construct maintains the *eya* gene architecture as closely as possible. In order to address the role of the PcG and TrxG system in regulating *eya* sequence, multiple different variants of the reporter gene construct including complete and partial deletions of the *eya* PRE / TRE are required. Additionally, it is interesting to further dissect the *eya* PRE / TRE in order to characterize the function of its sub-elements. The purpose of this reporter gene based approach is to investigate whether the PcG / TrxG system targets the *eya* regulatory sequence during eye and optic lobe development while keeping both the PcG / TrxG system and the RD network intact. Using such a reporter system allows to test multiple questions regarding *eya* PRE / TRE function: (1) Is the *eya* PRE / TRE required to maintain the gene silent prior to differentiation? (2) Is the *eya* PRE / TRE involved in regulating transcription levels of adjacent promoters? (3) Are both promoters regulated in the same way?

PRE / TREs have been shown to be interchangeable elements (Sipos, et al., 2007, Kozma, et al., 2008, Pérez, et al., 2011). Does this also apply to the *eya* PRE / TRE? In order to investigate whether PRE / TREs from different genes can functionally replace the *eya* PRE / TRE during eye development I designed reporter gene constructs where the *eya* PRE / TRE is replaced with the well characterized *vg* and *bxd* PRE / TREs. To test whether the *eya* PRE / TRE function is functionally conserved in *D. melanogaster* and *D. pseudoobscura,* I designed a variant of the reporter construct replacing the *eya* PRE / TRE with the homologous sequence from *D. pseudoobscura*. To avoid genomic positioning effects, all transgenes were generated using site directed integration. To achieve a relative quantification of reporter gene expression during eye development I developed an approach combining

microscopy of *whole mount* fluorescent RNA double *in situ* hybridization followed by quantitative analysis using image analysis tools. To evaluate PRE / TRE activity, it is also useful to examine adult *mw* mediated pigmentation of the eye. Finally, in order to test whether the reporter gene construct is sensitive to *PcG* and *trxG* mutations the adult eye pigmentation was examined in a *PcG* mutant background.

In summary, the aim of this thesis is (1) to study PcG / TrxG mediated regulation of *eya* regulatory sequence during eye development, (2) to identify the critical components of the *eya* PRE / TRE during eye and optic lobe development, (3) to test whether these properties are functionally conserved and (4) to test whether PRE / TREs are functionally exchangeable elements in the context of *eya* regulation.

III. RESULTS

Contributions

The results sections 1-3 and 5-7 are taken from the results section of a manuscript written in collaboration with Leonie Ringrose. The results shown in Figure 4 and Table 2 were generated by Yael Rotkopf.

1. Initial experiments and experimental strategy

1.1. *PcG* and *trxG* genes are essential for eye development

The analysis of homozygous null mutant clones or eye specific overexpression of *PcG* and *trxG genes* in the *Drosophila* eye has revealed roles for several of these genes in eye development (Janody, et al., 2004, Ferres-Marco, et al., 2006, Classen, et al., 2009, Martinez, et al., 2009). Interestingly, for many of these genes, mutant clones gave different phenotypes in different regions of the disc, indicating distinct roles at different stages of disc development (Janody, et al., 2004) (Figure 2). In order to gain more insight into the roles of *PcG* and *trxG genes* at different stages of eye development, we performed tissue specific RNAi knockdown experiments. We selected 26 UAS-RNAi lines from available stocks (Dietzl, et al., 2007) covering 17 *PcG* and *trxG* genes (Table 2). To examine the requirement of each gene at different stages of eye development, each UAS-RNAi line was crossed to two GAL4 driver lines, *ey*-GAL4 and *GMR*-GAL4. *ey*-GAL4 is expressed under the control of an enhancer derived from the *eyeless* gene (Hauck, et al., 1999). GAL4 is expressed in all cells of the eye imaginal discs during early larval development when all cells are proliferating. In 3rd instar eye discs, expression remains in precursor and pre-proneural cells (Figure 2) anterior to the morphogenetic furrow. GMR GAL4 is expressed from a *glass* enhancer in 3rd instar eye discs, posterior to the morphogenetic furrow in developing photoreceptor cells (Moses and Rubin, 1991). To enhance the effects of RNAi, a UAS dicer2 transgene was used in all experiments (Dietzl, et al., 2007) (see Materials and Methods).

Surprisingly, very few of the RNAi lines gave a phenotype when driven with GMR GAL4 in the differentiated cells behind the morphogenetic furrow (Table 10). In contrast, the majority of RNAi lines (21/25) gave severe developmental defects when driven with *ey* GAL4 in the entire eye disc (Table 10, Table 2, Figure 4). This result confirms the efficacy of the RNAi knockdown lines and distinguishes a more stringent requirement for *PcG* and *trxG genes* in mitotic and undifferentiated cells than in differentiated eye tissue. Strikingly, the majority of phenotypes observed upon *ey*-GAL4 mediated knockdown did not fall into distinct classes typical of a given group of genes. Although some *PcG* genes gave similar phenotypes (for example *pho* and *Pc*, Figure 4 G,H), in other cases *PcG* and *trxG* genes gave similar phenotypes (for example, *brm* and *Su(z)12*, Figure 4 I,J; *Trl* and *ph*, Figure 4 K,L). The most extreme phenotypes were observed for *ph*, in which larval eye antenna discs were massively overgrown and no adults survived, consistent with previous observations (Classen, et al., 2009, Martinez, et al., 2009). Interestingly, *ph* was also one of the few genes for which *GMR*-GAL4 driven knockdown gave a phenotype (Figure 4 and Table 10). The diversity of phenotypes observed upon knockdown of different genes suggests that different target genes may be differentially regulated by PcG and TrxG proteins, as has been proposed previously (Janody, et al., 2004). Taken together, these results demonstrate that *PcG* and *trxG genes* are essential for specific stages of eye development and suggest that each may have specific effects on different target genes.

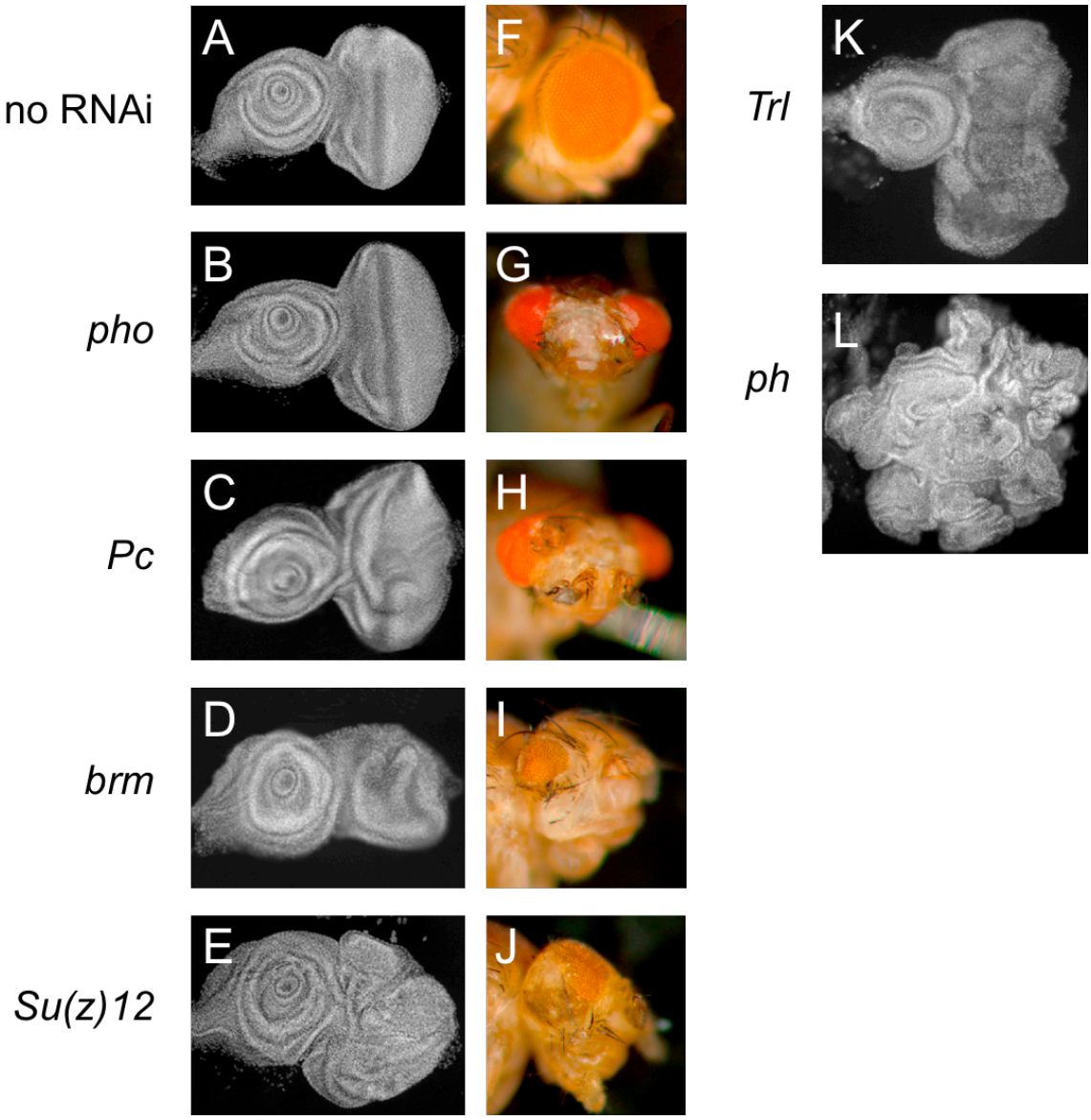


Figure 4: Phenotypes of RNAi knockdown of *PcG* and *trxG* genes in the developing eye.

The figure shows examples of phenotypes listed in Table 1. 3rd instar larval eye imaginal discs are shown in (**A-E**), anterior is to the left, posterior to the right. Adult eyes are shown in (**F-L**). Control flies lacking a UAS RNAi construct are shown (**A,F**). Phenotypes of the following RNAi lines are shown (for full list see Table 10): *pho* 39529 (**B,G**); *Pc* 2-1 (**C,H**); *brm* 37721 (**D,I**); *Su(z)12* 42422 (**E,J**); *Trl* 17198 (K); *ph-p* 10679 (**L**). *Trl* and *ph-p* RNAi gave no surviving adults.

	RNAi	larval eye disc phenotype	adult eye phenotype
trxG	*brm*	disrupted morphology; small eye disc	small or absent eye
	mor	disrupted morphology; small or absent eye disc	lethal; escapers with small or absent eye
	osa	disrupted morphology; small or absent eye antenna disc	lethal
	Trl	disrupted morphology; overgrown eye disc	lethal
	trx	normal	small or absent eye
PRC1	*ph-p*	disrupted morphology; massively overgrown eye antenna disc	lethal
	Sce	disrupted morphology; small eye disc	lethal
	Pc	normal or slightly disrupted morphology	protruding, rough eye (1)
PRC2	*E(z)*	disrupted morphology; small or absent eye disc	lethal (2)
	Su(z)12	disrupted morphology; overgrown eye disc	small, rough eye or absent eye(3)
DNA binding	*pho*	normal	protruding, rough eye

Table 2: Tissue specific RNAi knockdown of *PcG* and *trxG* genes disrupts eye development.

Flies carrying a UAS-RNAi construct directed against the gene of interest were crossed to flies carrying both an *ey*-GAl4 driver and UAS *dicer 2*. The Table lists the *PcG* and *trxG genes* for which for which a phenotype was observed upon *ey*-GAL4 mediated RNAi knockdown. Phenotypes observed in 3rd instar larval imaginal discs and in adult flies are given. Selected phenotypes are shown in Figure 4 ; full lists of RNAi lines and phenotypes are given in Table 10. Eye disc phenotype descriptions: “disrupted morphology”: tissue disrupted by folding, morphogenetic furrow not correctly formed, photoreceptor pattern not detectable. “Slightly disrupted morphology”: morphogenetic furrow recognizable, but with partially disturbed morphology behind furrow, small areas of missing photoreceptor pattern, folding or outgrowths. Antenna discs were normal unless stated otherwise. Notes: (1) *Pc* RNAi lines frequently showed missing antennae, antenna to leg or antenna to wing transformations. (2) A second RNAi line for *E(z)* was not lethal. (3) A second RNAi line for *Su(z)12* was lethal. See Table 10 for details.

1.2. The *eyes absent* gene is a target of PcG and TrxG regulation in the eye

Several genetic studies have identified target genes that are regulated directly or indirectly by *PcG* and *trxG genes* in the *Drosophila* eye (Janody, et al., 2004, Ferres-Marco, et al., 2006, Classen, et al., 2009, Martinez, et al., 2009), and many of these genes contain putative PRE / TREs, identified in genome wide bioinformatic or profiling studies (reviewed in (Ringrose, 2007). However, whether these PRE / TREs play a role in mediating the effects of PcG and TrxG regulation during eye development has not been addressed. In order to gain insight into the role of PRE / TREs in the dynamic regulation of eye specific genes, we chose the *eya* gene as a model (Figure 5A). The EYA protein is misregulated in *PcG* and *trxG* mutant clones (Janody, et al., 2004), and has been proposed on the basis of these genetic studies to be both a direct and an indirect target of specific *PcG* and *trxG genes*. The *eya* gene contains a PRE / TRE (black box, Figure 5 A) that was identified by bioinformatic prediction, and has been confirmed as a Polycomb binding site by ChIP and as a Polycomb responsive element in a transgenic reporter assay (Ringrose, et al., 2003). Furthermore, the *eya* locus has been identified in several independent genome wide profiling studies as a target of PcG and TrxG protein binding, thus it is a good candidate for direct regulation (Tolhuis, et al., 2006, Schuettengruber, et al., 2009) (http://cav-ouranos.igh.cnrs.fr/viewer-0.3_public/index.php). In addition to the PRE / TRE, the *eya* locus contains a well-characterized eye specific enhancer (yellow box, Figure 5 A) (Bui, et al., 2000, Zimmerman, et al., 2000).

The *eya* gene is expressed from two alternative promoters (Figure 5 A). Each of the two alternative first exons (1.1 and 1.2) is spliced to a common second exon (Bonini, et al., 1993). In order to investigate *PcG* and *trxG* mediated regulation of the two *eya* promoters in the developing eye, qPCR was performed to detect transcripts from each of the two alternative promoters in eye imaginal discs prepared from *PcG* and *trxG* mutant larvae. Primers were

designed to specifically amplify the spliced *eya* transcripts *eya* 1.1 (Figure 5 B) and *eya* 1.2 (Figure 5 C), and to amplify the third exon, detecting both spliced transcripts in addition to the nascent transcript (Figure 5 D). Transcript levels were normalized to *tbp,* and are expressed as % of wt levels in Figure 5B-D. All mutants were heterozygous over a balancer chromosome with the exception of ph^{410} which is homozygous viable (see materials and Methods for genotypes).

Remarkably, each promoter was misregulated in *PcG* and *trxG* mutants in a characteristic manner. For example, *eya* 1.1 was approximately twofold upregulated in all mutants, irrespective of whether the mutation affected a *PcG* or *trxG gene* (Figure 5 B). In contrast, the levels of *eya* 1.2 were reduced in all mutants except Pc^{XL5} (Figure 5 C). For exon 3, representing the sum of both spliced transcripts in addition to the nascent transcript, all mutants showed approximately two fold higher levels than wild type. These results are consistent with the previous observation that the EYA protein is misexpressed similarly in both *PcG* and *trxG* mutants (Janody, et al., 2004), and with our observation that several *PcG* and *trxG* RNAi knockdowns gave similar phenotypes (Figure 4). Taken together these results demonstrate that the *eya* gene is a target of *PcG* and *trxG* regulation in the eye, and suggest that the *PcG* and *trxG* regulate, either directly or indirectly, the choice between the two alternative *eya* promoters.

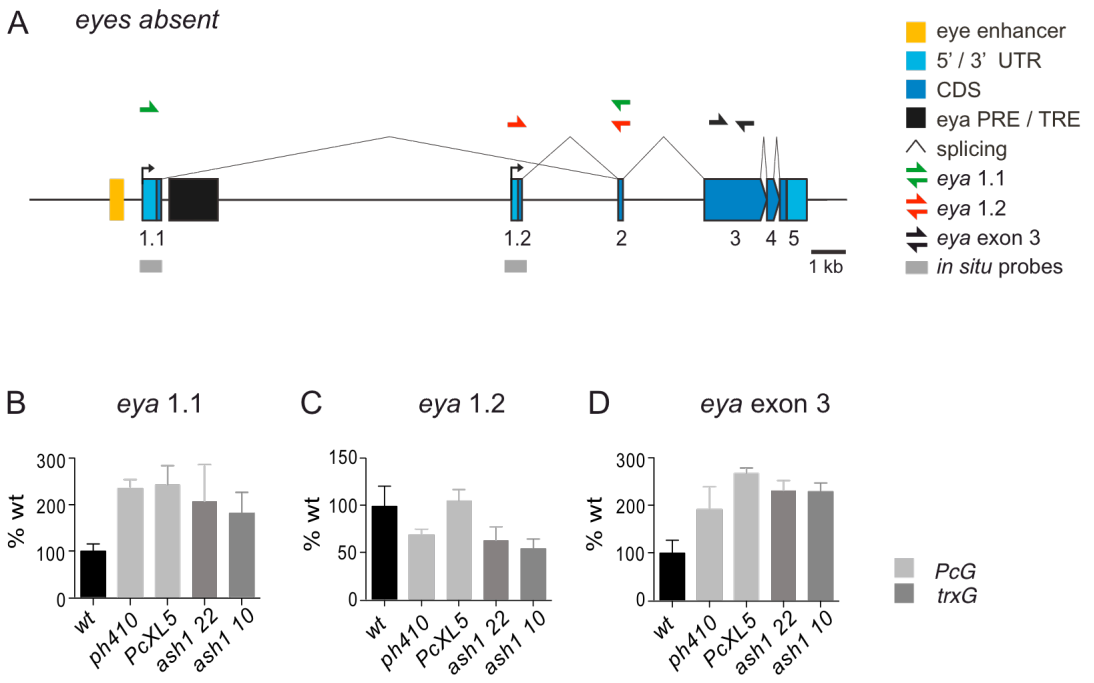


Figure 5: *eyes absent* is a target of *PcG* and *trxG* regulation in the eye.

(**A**) Diagram to scale of the eyes absent locus with regulatory elements (yellow and black boxes) exons (blue) and positions of qPCR primers used in **B,C,D** (arrows). Exon numbers are shown below the figure, the gene uses two alternative first exons 1.1 and 1.2, each of which is spliced to a common second exon. *In situ* probes used in Figure 8 A-C are shown as grey bars below exon 1.1 and 1.2. (**B-D**), qPCR analysis of *eya* transcript levels in 3rd instar larval eye imaginal discs of wildtype and *PcG* or *trxG* mutant larvae, as indicated. All mutants were heterozygous with the exception of ph^{410} which is homozygous viable (see materials and Methods for genotypes). (**B**) primers detecting exon 1.1 spliced to exon 2; (**C**) primers detecting exon 1.2 spliced to exon 2; (**D**) primers against exon 3, detecting both splice forms and the nascent transcript. Graphs show mean and standard deviation of two biological and two technical replicates.

1.3. The REGFP reporter construct reflects the architecture of the endogenous *eya* gene and reveals the regulatory roles of the enhancer and the PRE

To determine the roles of the *eya* PRE / TRE and the *eya* enhancer in regulating the two *eya* promoters, I designed a reporter construct that reflects

the architecture of the endogenous *eya* locus (Figure 6A). The construct is referred to as REGFP (Regulatory region of *eya* fused to *gfp*). The construct consists of a 4.2 kb genomic fragment containing the eye specific enhancer (Bui, et al., 2000, Zimmerman, et al., 2000), the first exon and the intronic PRE / TRE (Ringrose, et al., 2003). In addition, a 740 bp fragment containing the first three codons of the second *eya* exon and the intronic sequence with splice acceptor and branch site was placed adjacent to this fragment. The *turbogfp* (recommended for tracking promoter activity, hereafter referred to as *gfp*) coding sequence lacking only the ATG start codon was fused in frame to the second *eya* exon, so that an mRNA encoding functional GFP protein is produced by splicing. Thus GFP serves as a reporter for the use of the first promoter. Downstream of the PRE / TRE I placed the *miniwhite* (*mw*) gene, which serves both as a transformation marker and as a reporter for the use of the second promoter. In summary, the REGFP reporter recapitulates several essential features of the endogenous *eya* locus, namely the relative positioning of the upstream enhancer and the intronic PRE / TRE, flanked by two promoters (Figure 6 A). The use of *gfp* and *mw* as the two reporter genes enables a rapid readout of the activity of each promoter by fluorescent live imaging for GFP and adult eye pigment levels for *mw*. Furthermore both transcripts can be monitored simultaneously by double *in situ* hybridization on larval tissue.

To examine the roles of the enhancer and the PRE / TRE in regulating each of the two promoters in the REGFP construct I generated REGFP variants lacking the enhancer, the PRE / TRE, or both elements (Figure 6 C-F). To avoid genomic position effects on reporter activity, all constructs were integrated at an identical genomic location by ΦC31 site-specific integration (Groth, et al., 2004); (see Materials and Methods for details). To obtain an initial readout of the activity of each promoter in the four transgenic reporter lines I examined GFP by live imaging in eye imaginal discs for promoter 1 (Figure 6 G-J), and *mw* pigment levels in adult eyes for promoter 2 (Figure 6 L-O). The GFP pattern in eye imaginal discs of the REGFP reporter line was

essentially identical to that of the endogenous EYA protein (Bessa, et al., 2002), (Figure 6 G). The GFP expression domain extended from the pre-proneural zone immediately anterior to the morphogenetic furrow, to the posterior edge of the disc. In constructs lacking the enhancer (Figure 6 I), or lacking both the enhancer and the PRE (Figure 6 J), no GFP expression was detectable, confirming that the enhancer is required for expression from the first promoter in the REGFP reporter. Surprisingly, deletion of the PRE / TRE had no detectable effect on the pattern of GFP expression (Figure 6 H). This result suggests that this element either plays no role in regulation of the first promoter, or that its effects are quantitative rather than qualitative and are thus not detectable by live imaging of GFP.

To evaluate the output of the second REGFP promoter, I examined *mw* expression levels in adult eyes (Figure 6 L-O). This reporter has the advantage that quantitative changes are detectable as changes in eye pigmentation, and low levels of *mw* expression can be detected. Indeed, unlike the GFP result described above, constructs lacking the enhancer (Figure 6 N), or lacking both the enhancer and the PRE / TRE (Figure 6 O) showed low, detectable levels of *mw* expression suggesting that REGFPΔen and REGFPΔenΔPRE displayed *mw* expression as a result of *white* specific regulation on the *mw* promoter. Indeed, the transgenic line 16Amw that carries only the *mw* gene produces adult eyes with strong pigmentation. *mw* expression in these transgenes is obtained with the identical minimal promoter used in REGFP transgenes at the same genomic insertion site but without any *eya* related regulatory sequence (Figure 6 B,K). however during eye development mw expression started behind the morphogenetic furrow (zone 2 in Figure 8) suggesting *white* specific regulation because eya specific regulation is initiated anterior of the morphogenetic furrow (zone 1 in Figure 8) (Figure 8F-J). Because the lines REGFPΔen and REGFPΔenΔPRE displayed lighter eye colors compared to 16A*mw*, it is likely that *eya* regulatory sequence in REGFP is able to inhibit the activity of *white* specific regulation. The PRE / TRE is even able to completely block *white* specific regulation in the posterior 2/3 of

the eye in homozygous REGFPΔen flies, while this is not the case when the PRE / TRE is removed as seen in REGFPΔenΔPRE (compare homozygous flies in Figure 6 N,O and Figure 7 G,H). This result shows that the *eya* PRE / TRE is capable of pairing sensitive silencing and mediates a repressive effect on the *mw* promoter in the absence of the enhancer. This repression was genetically dependent on *PcG* mutations, indicating that this silencing is achieved through PcG mediated silencing over the PRE / TRE (Figure 7 G,H). However in the presence of the enhancer, deletion of the PRE / TRE led to a dramatic upregulation of *mw* (compare Figure 6 L and M), indicating that in the intact REGFP construct, the PRE / TRE acts to strongly limit the activity of the enhancer on the *mw* promoter. Thus for the *mw* promoter, the enhancer and the PRE / TRE act in opposition to each other. Taken together with the GFP results described above, these results indicate that the *eya* enhancer drives expression of both promoters in the REGFP construct, and that the PRE / TRE acts to limit the effect of the enhancer on the *mw* promoter.

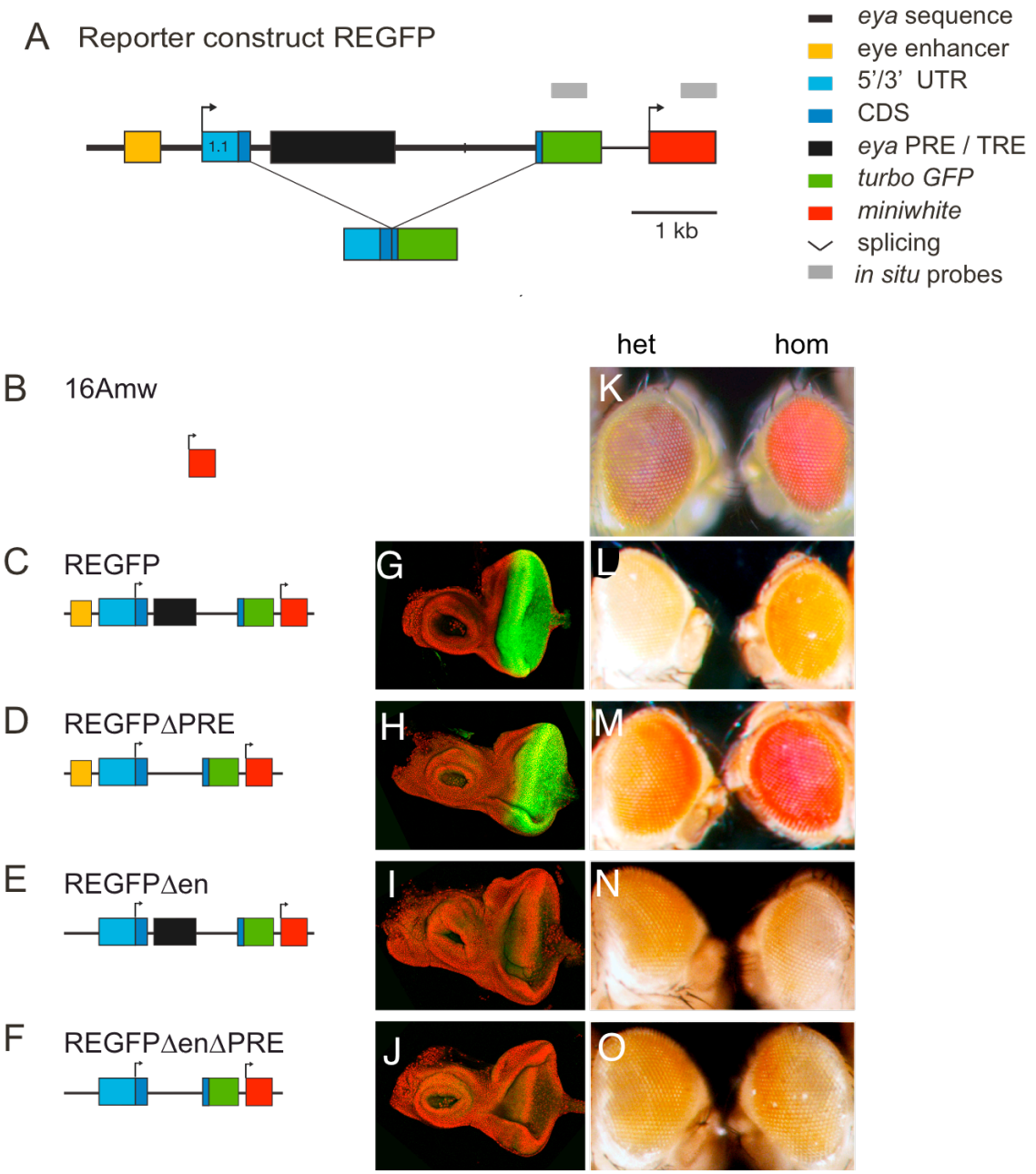


Figure 6: The REGFP reporter distinguishes roles of the enhancer and the PRE / TRE in *eya* regulation.

(**A**) The reporter construct "REGFP" consisting of the regulatory region of *eya* and the reporters *gfp* and *mw* contains genomic sequences from the *eya* locus, fused to reporter gene sequences. The first *eya* sequence (2L:6544449..6548972, genome version FB2010_06) contains the eye specific enhancer, the first alternative exon 1.1 and the *eya* PRE / TRE. The second (2L:6530964..6535677) consists of intronic sequence including a branch site and the first three codons of exon 2 in order to enable splicing. The *turbogfp* sequence (referred to as *gfp*) is fused in frame to the

second exon, so that an mRNA encoding functional GFP protein is produced by splicing. The second reporter gene *miniwhite (mw)* (see Materials and Methods) is located downstream of the PRE / TRE and *gfp* sequence. *In situ* probes used to detect *gfp* and *mw* transcripts in Figure 8 K-M and P-R are shown as grey bars above the reporter gene illustration. The REGFP reporter construct recapitulates the architecture of the endogenous locus by the positioning of the enhancer upstream of the first promoter, and the PRE / TRE in between the two promoters. (**B**) 16Amw transgenes carry a *mw* reporter gene with the same minimal promoter used in REGFP variants. The transgenic insertion site is identical to the insertion site of all REGFP transgenes. (**K**) Adult pigmentation of 16A*mw* transgenes. Male flies were aged for 8 days. Left, heterozygote. Right, homozygote. (**C-F**) schematic representation of REGFP reporter (**C**) and variant constructs lacking the PRE (**D**), the enhancer (**E**) and both elements (**F**). (**G-J**) live GFP imaging of eye- antenna imaginal discs from 3rd instar larvae homozygous for each transgene in (**C-F**). Anterior is to the left, posterior to the right. (**L,M**) Eye colors of adult male transgenic flies, 3 days old, are shown. Left: flies heterozygote for the transgene, right, homozygotes. (**N,O**) eye colors of adult male transgenic flies, 8 days old, are shown.

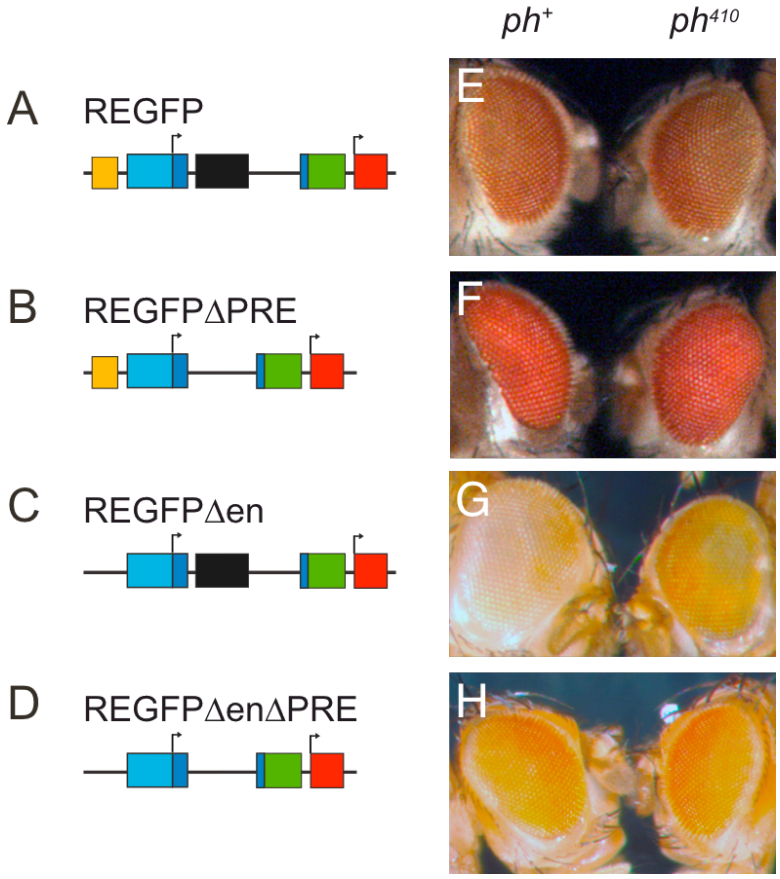


Figure 7: The PRE / TRE in the REGFP construct mediates PcG dependent repression.

(**A-D**) schematic representation of REGFP reporter (**A**) and variant constructs lacking the PRE (**B**), the enhancer (**C**) or both elements (**D**). (**E-H**) Each of the transgenic lines carrying constructs shown in A-D was crossed into a ph^{410} mutant background. The figure shows male, 8 day old flies homozygous for the transgene on chromosome II, and in which the X chromosome is either ph^{+}, w^{-} (left) or ph^{410}, w^{-} (right). In the absence of the enhancer, *mw* levels are derepressed in the ph^{410} mutant background (**G**), this effect was not detectable in the absence of the PRE (F,H) or in the presence of the enhancer (**E**).

2. Functions of the eya PRE / TRE in eye development

2.1. The *eya* PRE / TRE is capable of mediating pairing sensitive silencing, a characteristic feature of PRE / TREs

The adult eye pigmentation of the transgenic lines REGFPΔen and REGFPΔenΔPRE gave the possibility to study the function of the *eya* PRE / TRE without the influence of the eye specific enhancer (Figure 6 E,I,N and F,J,O). Indeed, *eya* specific regulation of *gfp* is absent, indicating that neither of the reporters is regulated in an *eya* specific manner in these transgenes as they lack the eye specific enhancer. Nevertheless both lines display *mw* specific eye pigmentation (Figure 6 E,I,N and F,J,O). Importantly, the *mw* promoter is exposed to *white* specific regulation starting from behind the morphogenetic furrow (zone 2 in Figure 8) onwards as seen with the *mw* control line 16A*mw* (Figure 6 B,K and Figure 8 F-J). Strikingly, both transgenes, REGFPΔen and REGFPΔenΔPRE were able to dramatically reduce the dose of *mw* dependent pigmentation in adult flies compared to 16A*mw* indicating that the *eya* regulatory sequence lacking the eye enhancer reduces the transcriptional output of the *mw* promoter (compare Figure 6 K to N,O). Even in the presence of the eye specific enhancer, the transcriptional output of 16A*mw* could not be reached: REGFP transgenes displayed a lighter eye color both in hetero- and homozygotes compared to 16A*mw* (compare Figure 6 K to L). REGFPΔPRE transgenes displayed a similar eye color both in heterozygotes and homozygotes compared to 16A*mw*. This is surprising because REGFPΔPRE transgenes express *mw* from zone 1 onwards (instead from zone 2 onwards) and should therefore display stronger pigmentation if the *eya* sequence did not have a reductive effect on the *mw* promoter per se. Consequently, the REGFPΔen and REGFPΔenΔPRE lines may be good indicators for *mw* promoter activity as a response to *white* specific regulation in the context of *eya* regulatory sequence. The comparison between REGFPΔen and REGFPΔenΔPRE transgenes revealed a lighter eye color in REGFPΔen

both in heterozygotes and homozygotes indicating that the PRE / TRE sequence has a repressive effect. Strikingly in homozygotes, REGFPΔen (containing the PRE / TRE) displayed a complete lack of pigmentation in the posterior part of the eye. In contrast, REGFPΔenΔPRE showed pigmentation in the posterior part of the eye showing that in the absence of the eye specific enhancer the PRE / TRE element is able to completely silence the activity of the *mw* promoter and prevent activation by *white* specific regulation (compare Figure 6 N to O and Figure 7 G to H). Importantly, the silencing is achieved in homozygous but not heterozygous flies. Therefore, the *eya* PRE / TRE fulfills the characteristic property of pairing sensitive silencing (reviewed in (Ringrose and Paro, 2004)). Strikingly, pairing sensitive silencing was not established in a ph^{410} mutant background showing that pairing sensitive silencing is PcG dependent and requires the PRE / TRE (Figure 7 G,H).

2.2. The expression of the endogenous *eya* isoforms is not uniform during eye developmental progression

I first asked to what extent the transcripts arising from the two endogenous *eya* promoters (1.1 and 1.2, see Figure 5 A) are comparable to those of the two REGFP reporters. Double *in situ* hybridizations on wild type eye imaginal discs using probes specifically directed against exon 1.1 and exon 1.2 revealed a striking difference in the profiles of the two transcripts, both in individual discs (Figure 8 A-C) and in averaged intensity profiles (Figure 8 D,E). Exon 1.1 was detectable most strongly in the cells immediately anterior and posterior to the morphogenetic furrow (zones 1 and 2 on Figure 8), and was reduced in a steep gradient towards the posterior part of the disc at the

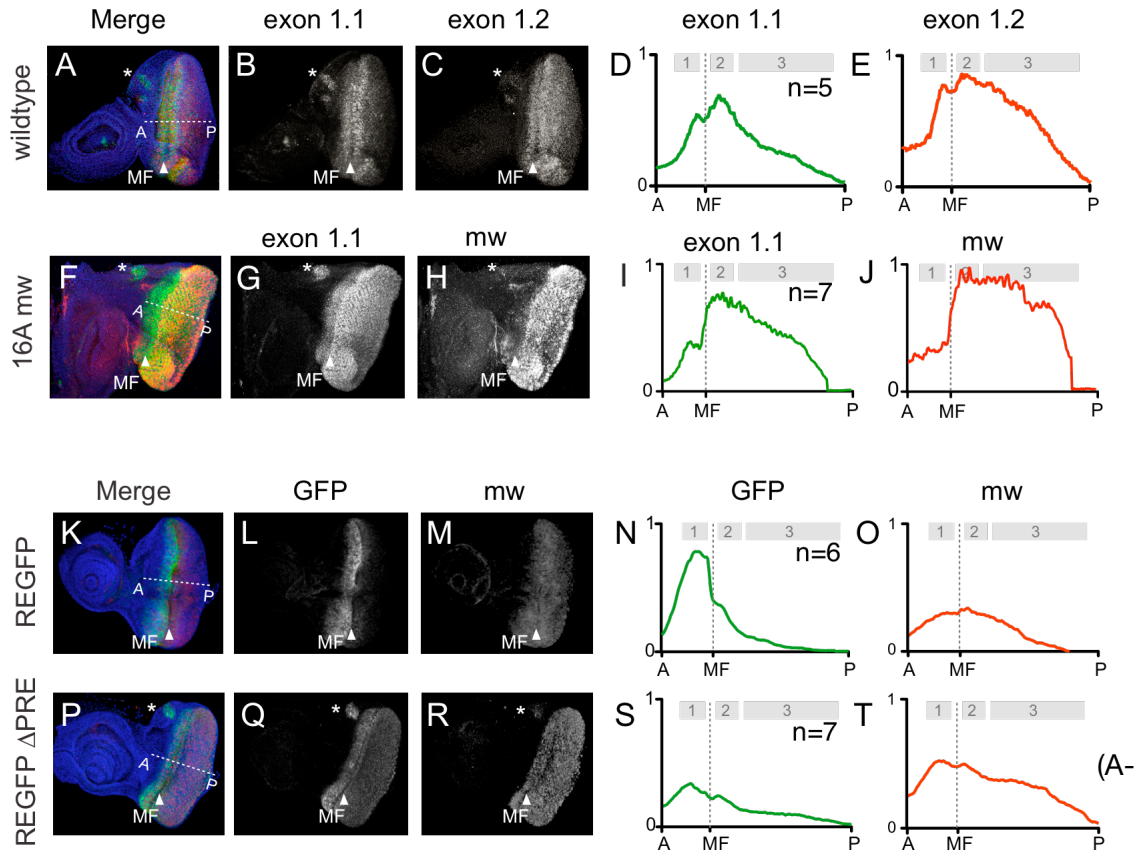


Figure 8: Deletion of the *eya* PRE / TRE disrupts the spatial expression profiles of both REGFP reporters.

(**A-C**) double *in situ* hybridization on wildtype 3rd instar larval eye- antenna imaginal discs, detecting transcripts containing exon 1.1 (**B**) and 1.2 (**C**) of the *eya* gene. Anterior is to the left, posterior to the right. Arrowheads indicate the morphogenetic furrow. Asterisks indicate future ocelli. (**A**) merge: blue, DAPI; green, 1.1; red, 1.2. See Figure 8 for *in situ* probes. Dotted line shows position of line scan in (**D,E**). (**D,E**) Average signal intensity profiles extracted from double *in situ* hybridization images of 5 discs for exon 1.1 (**D**) and 1.2 (**E**) along the anteroposterior disc axis, as shown by dotted line in (**A**). Line scans were performed perpendicular to the morphogenetic furrow and aligned as described in Materials and Methods. Grey boxes above the line scan plots indicate three zones of the disc: (1) mitotic cells anterior to the morphogenetic furrow; (2) zone of photoreceptor differentiation immediately posterior to the furrow; (3) zone of maturing photoreceptors at posterior of disc. Vertical scale represents relative signal intensity, the same scale is used in all plots. (**F-H**) double in situ hybridization on 3rd instar eye-antenna imaginal discs of 16A *mw* transgenes. These transgenes carry a *mw* reporter at the same genomic location as REGFP transgenes and are a control for *mw* expression independent of *eya* regulatory

sequence. (**I,J**) average line scans for exon 1.1 (**I**). and *mw* (**J**, note that *mw* expression starts in zone 2, not 1; signal intensity of the mw profile in zone 1 represents background staining). (**K-M, P-R**) double *in situ* hybridization on 3rd instar eye-antenna imaginal discs of larvae transgenic for the REGFP reporter (**K-M**) or the REGFP ΔPRE reporter (P-R), detecting transcripts of the *gfp* (**L,Q**) or *mw* (**M,R**) reporters. (**K,P**) merge: blue, DAPI; green *gfp*; red, *mw*. See Figure 6A for *in situ* probes. Dotted lines show positions of line scans in (**N,O,S,T**). Asterisks in P,Q,R indicate ocelli, in which expression of both reporters is detectable in REGFPΔPRE. (N,O) average line scans for *gfp* (N) and *mw* (O) from 6 REGFP discs; (S,T) average line scans from 7 REGFPΔPRE discs. Line scans were performed as for (D,E).

stage of crawling larvae (zone 3; Figure 8 B,D). In contrast, exon 1.2 was expressed from the same anterior border but in a broader domain towards the posterior of the disc indicating that the endogenous transcripts are differentially regulated in zones 2 and 3 of eye discs from crawling larvae (Figure 8 C,E). The same combination of in situ probes in prepupal eye discs showed that relations of the profiles to each other change. At both developmental stages both endogenous *eya* transcripts have in common that zone 2 expression is higher compared to zone 1. The difference of the profiles however was characterized by a change in ratio of the isoforms in zone 1 compared to zone 2 taking place between the stage of crawling larvae and prepupae (Figure 9). Furthermore, the difference in zones 2 and 3 between the isoforms was not detectable at the later stage of eye development (Figure 9). These results indicate that *eya* regulation is not uniform during eye development. Interestingly, the REGFPΔen line was not able to block *white* specific regulation of the *mw* promoter at anterior parts of the eye suggesting that the influence of the PRE / TRE ceases with anterior location (see homozygote in Figure 6 N). Given the differential expression of the endogenous isoforms at different stages of development it seems possible that the PRE / TRE is involved.

Taken together, these results show that the endogenous isoforms are expressed in a changing ratio both in zone 1 and zones 2 and 3 with ongoing

eye development. Given the behaviour of the *eya* PRE / TRE in the lines REGFPΔen compared to REGFPΔenΔPRE it is possible that the PRE / TRE is required for this type of dynamic differential regulation.

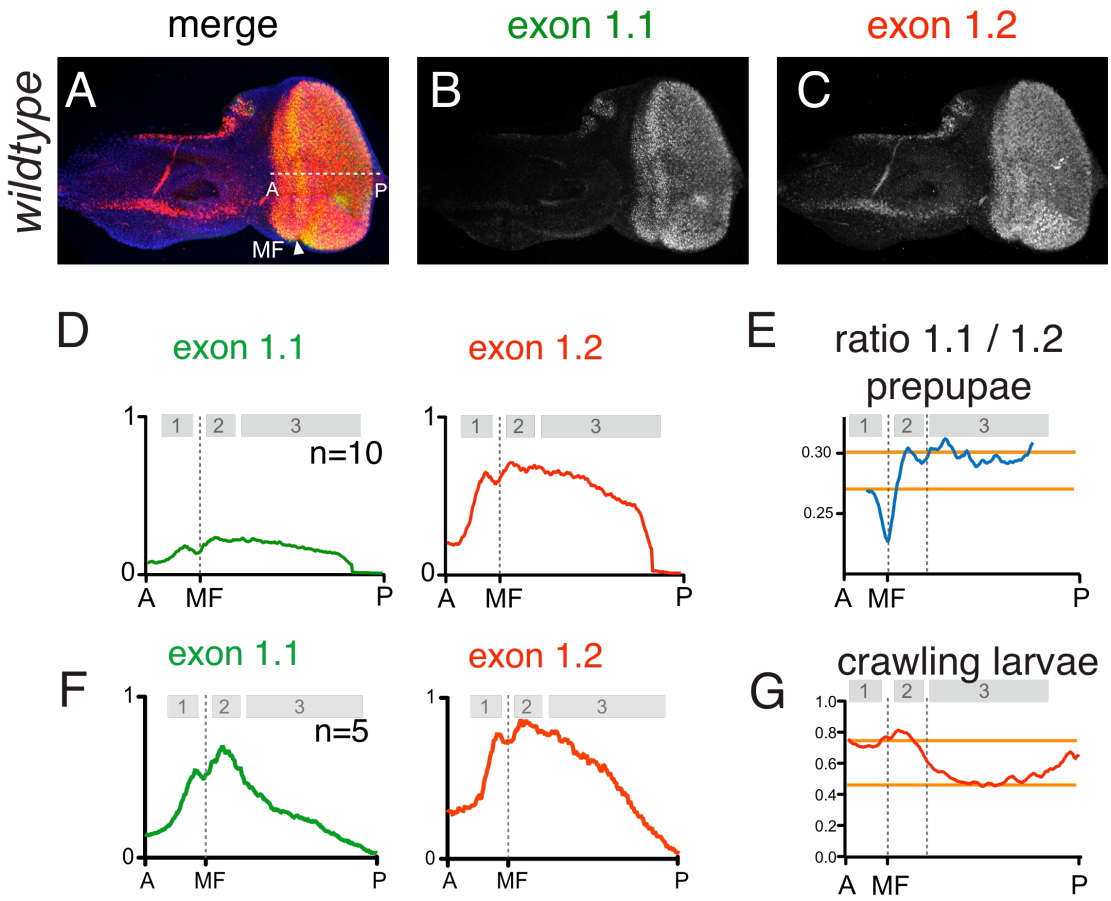


Figure 9: The *eya* isoforms are expressed at different ratios in zone 1 compared to zones 2 and 3.

Double *in situ* hybridizations using probes to detect transcripts of either *eya* isoform (**A**, merge and DAPI; **B**, isoform 1.1; **C**, isoform 1.2) (see also Figure 5 A) were used on prepupal tissue. The colors in (A) are as follows: exon 1.1, green; exon 1.2, red; DAPI; blue. The signal intensity profiles were generated by averaging the profiles of 10 eye antennal discs (**D**, left: *eya* exon 1.1, right: *eya* exon 1.2). (**E**) The ratio of both isoforms is distinct in zone 1 compared to zones 2 and 3 in prepupae. No change in ratio is detectable in zone 2 and 3. (**F**) Expression profiles of exon 1.1 and exon1.2 extracted from 5 eye antennal discs of crawling larvae (see also Figure 8). (**G**) The ratio of the two isoforms is similar in zone 1 and 2 in crawling larvae. This is in contrast to the observations in prepupae. Furthermore, in zones 2 and 3 the differential regulation of both isoforms becomes well visible by the dip in ratio.

2.3. The *eya* PRE / TRE is essential for the distinct spatial expression profiles of the two REGFP reporters

As reported above, live GFP imaging did not detect any effect of deleting the PRE / TRE from the REGFP construct (Figure 6 G,H). However, the same PRE / TRE deletion produced profound effects on *mw* levels in adult eyes (Figure 6 L,M). Thus, I reasoned that deletion of the PRE / TRE may cause quantitative differences in *gfp* transcript levels that were undetectable by live GFP imaging. In order to obtain a more precise readout of both promoters, and to compare their outputs at the same developmental stage, I performed double *in situ* hybridizations to detect both transcripts in 3rd instar larval eye imaginal discs of crawling larvae.

The analysis of images and averaged profiles of the two reporter gene transcripts in REGFP transgenes revealed that the reporter gene expression (1) differed from the endogenous *eya* expression profiles and that (2) the profiles of the two reporter genes differed from each other. Compared to the exon 1.1 profile in crawling larvae, the *gfp* expression profile of REGFP transgenes showed a significantly higher expression in zone 1 compared to zones 2 and 3 that are characterized by very low levels of *gfp* expression. On the other hand, the sharp decrease of transcript in zones 2 and 3 can be seen as a similarity between endogenous exon 1.1 and transgenic *gfp* expression (compare Figure 8 B,D and L,N). The overall different appearance of the *gfp* expression profile may be due to differences in regulation between REGFP and the endogenous locus or due to different transcript stabilities. The *mw* profile in REGFP transgenes looked similar to the endogenous expression of *eya* exon 1.2, with both giving lower expression in zone 1 than zone 2, and a broad domain of expression across zone 3 (compare Figure 8 C,E and M,O). Nevertheless, the *mw* profile may be the result of two independent events acting on the *mw* promoter. For one, *mw* was expressed starting from zone 2 in 16A *mw* transgenes. These transgenes carry only the *mw* gene without any *eya* specific sequence (Figure 8 H, J). This indicates that the *mw* promoter is exposed to *white* specific regulation from zone 2 onwards as this pattern is

essentially identical to that of the endogenous *white* gene in wildtype larvae (Figure 8 H and J and not shown). On the other hand, REGFP transgenes express *mw* from zone 1 (the preproneural zone) onwards in an *eya* specific manner (Figure 8 M,O and Figure 2). Consequently, the *mw* profile could be the result of an additive effect coming from *white* specific regulation (zones 2 and 3) and the *eya* eye specific enhancer (zones 1, 2 and 3).

When compared to each other, the REGFP specific *gfp* and *mw* expression profiles substantially differed from each other. The main detectable difference was the higher relative level of *gfp* expression in the pre-proneural zone (zone 1) anterior to the furrow while *mw* was expressed at low levels (Figure 8 N, O, zone 1). Furthermore in zone 2, *gfp* expression was dramatically downregualted and very low in zone 3 while *mw* was expressed in a broad domain covering zones 2 and 3. Thus, especially in zone 1 the two reporter genes are regulated differently from each other while the higher *mw* expression levels in zone 2 and 3 might be a consequence of *white* specific regulation (compare Figure 8 L,N and M,O). In summary, the profiles of the two REGFP reporters are different to each other. In zones 2 and 3, the *mw* expression profile may be the consequence of both *eya* and *white* specific regulation. Differences of the REGFP profiles compared to the endogenous *eya* profiles may be due to a different regulation compared to the endogenous locus or different transcript stabilities.

To determine to what extent the distinct spatial profiles generated by the two REGFP promoters are affected by the PRE / TRE, I compared the double *in situ* hybridizations of REGFP to eye discs of the PRE / TRE deletion line REGFPΔPRE (Figure 8 P-T). Remarkably, the deletion of the PRE / TRE produced profound effects on the spatial expression of both transcripts. Although the data do not allow for direct quantitative comparisons between experiments, the differences in intensity across each averaged profile (Figure 8 N,O,S,T), allow for comparisons to be made between different regions of the disc (zones 1 to 3 on Figure 8). For example in zone 3, containing maturing photoreceptors, both *gfp* and *mw* transcripts were substantially derepressed in the absence of the PRE / TRE, detectable by an extension of the posterior

border of the profile towards the posterior edge of the disc (compare Figure 8L,N to Q,S and M,O to R,T). In zone 2, in which photoreceptor differentiation is taking place, the *mw* transcript showed higher expression than in the more posterior cells, suggesting upregulation (Figure 8 T), whereas the *gfp* transcript was not detectably affected (Figure 8 S). The most striking effect of PRE / TRE deletion was observed in zone 1, containing pre-proneural cells immediately anterior to the furrow. In these cells a similar level of *mw* expression compared to that in zone 2 was observed whether or not *white* specific regulation may be influencing *mw* expression from zone 2 onwards (Figure 8 T), again suggesting upregulation. Strikingly, for *gfp* expression in zone 1, comparable levels of transcript to that in zone 2 were observed (Figure 8 S), in marked contrast to the strong difference between zones 1 and 2 observed in REGFP discs (Figure 8 N). This result strongly suggests that the loss of the PRE / TRE leads to downregulation of *gfp* in the pre-proneural zone (zone 1). In summary, this analysis indicates that loss of the PRE / TRE leads to an upregulation of *mw* in all zones of the disc, consistent with the adult eye color phenotypes described above (Figure 6). In contrast, the deletion of the PRE / TRE leads to upregulation of *gfp* in zone 3, but to downregulation in zone 1. Thus, the PRE / TRE is essential for the distinct spatial expression profiles of the two REGFP reporters.

2.4. The distinct readouts of the two REGFP reporters are unique to the *D. melanogaster eya* PRE / TRE

In the above experiments I have shown that the *eya* PRE / TRE modulates the spatial expression patterns of the two REGFP reporters differentially in different zones of the eye disc. Furthermore I have shown that the activity of both of these reporters is dependent on the eye specific enhancer. These observations suggest that the PRE / TRE operates by modulating the enhancer activity either by acting as a mediator to communicate with the promoters or by modulating the interaction between the enhancer and each of the two promoters in a cell type specific manner. I next wished to address

whether this enhancer- PRE / TRE interaction could be performed by any PRE / TRE, or whether the *eya* PRE / TRE has evolved specifically to control the activity of the *eya* enhancer. To do so, I replaced the *eya* PRE / TRE sequence in the REGFP construct with one of four other PRE / TREs, and generated transgenic lines carrying this series of replacement constructs at the same genomic location by ΦC31 mediated site-directed integration. The *eya* PRE / TRE and the replacement PRE / TREs are shown in Figure 10. It was previously shown that the DNA motifs required for PRE / TRE function are similar in different *Drosophila* species, but that PRE / TREs show a high degree of evolutionary plasticity in terms of motif turnover and number of PRE / TREs per locus (Hauenschild, et al., 2008). In *D. pseudoobscura,* the orthologous sequence to the *D. melanogaster eya* PRE / TRE contains a high density of typical PRE / TRE motifs (Figure 10 B), and thus obtains a high score in computational predictions (Figure 10 G, peak A). However the number and order of motifs has changed in evolution (Figure 10 A,B), as has the number of predicted PRE / TREs at the locus (Figure 10 F,G). thus I wished to evaluate whether this orthologous *D. pseudoobscura* sequence would interact correctly with the *D. melanogaster eya* enhancer in the REGFP reporter construct. In addition, the *vestigial (vg)* PRE / TRE from *D. melanogaster* was chosen as an example of a relatively well- characterized PRE / TRE from a gene outside the *Hox* complexes (Lee, et al., 2005) (Figure 10 C). Finally, the *bxd* PRE / TREs from both *D. melanogaster* and *D. pseudoobscura* were chosen as two examples of PRE / TREs from the *Hox* gene *Ubx* (Figure 10 D,E) (Simon, et al., 1993, Chan, et al., 1994, Hauenschild, et al., 2008). The *D. melanogaster bxd* PRE / TRE is one of the best-characterized PRE / TREs in the fly genome, and has provided many of the current paradigms for models of PRE / TRE function, thus I wished to evaluate whether the *eya* PRE / TRE and the *bxd* PRE would have equivalent functions in the context of the REGFP reporter assay.

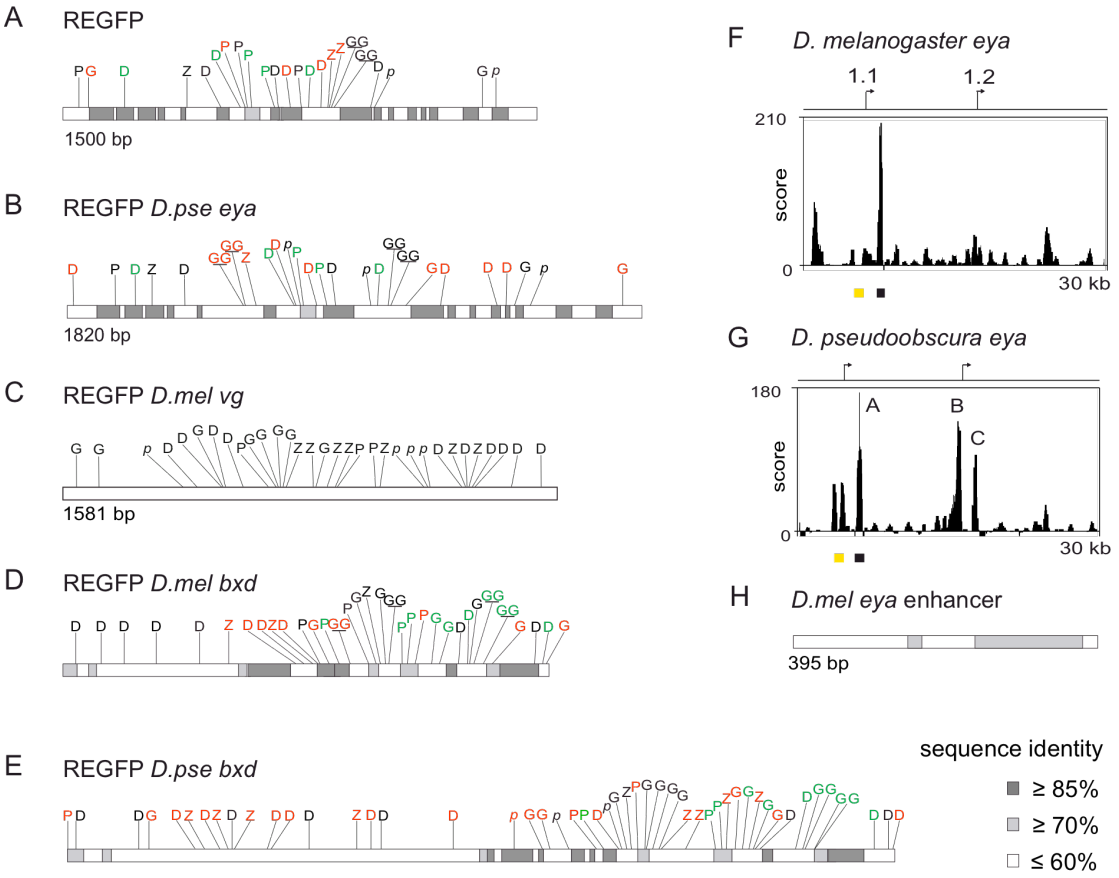


Figure 10: PRE / TREs used in REGFP replacement constructs.

(**A-E**) diagrams showing motif composition and conservation of the PRE / TRE sequence used in the original REGFP construct (**A**) and variants in which 1.5kb of *eya* PRE / TRE was replaced by the orthologous sequence from *D. pseudoobscura* (**B**), the *vestigial* PRE / TRE from *D. melanogaster* (**C**) the bxd PRE / TRE from *D. melanogaster* (**D**) or *D. pseudoobscura* (**E**). Motifs defined as in (Hauenschild et al. 2008). D, Dsp1; G, GAF; P, PHO extended site; *p*, PHO core site (GCCAT); Z, Zeste. Underlined motifs indicate overlapping runs of motifs separated by two bases. (**A**,**B** and **D**, **E**): green, motifs conserved between both species; black, motifs have shifted their position; red motifs are found in one species but not the other. Regions of conservation between *D. melanogaster* and *D. pseudoobscura* are indicated. Note for (**C**), the conservation to *D. pseudoobscura* is not depicted. Genomic coordinates of PRE / TRE sequences are given in Table 7. (**F,G**) PRE / TRE score plots for the *eya* locus in *D. melanogaster* (**F**) and *D. pseudoobscura* (**G**) using the jPREdictor algorithm (Fiedler and Rehmsmeier, 2006). Arrows above each score plot indicate the positions of the two alternative promoters. The *D. melanogaster* enhancer and orthologous *D. pseudoobscura* sequence are shown as yellow boxes below the

score plots. The sequences used in the REGFP (**A**) and REGFP *D. pseudoobscura eya* (**B**) constructs are shown as black bars. (H) 395bp containing the *D. melanogaster eya* enhancer, and deleted in the REGFPΔen construct, is shown with conservation to the orthologous *D. pseudoobscura* sequence. Both conserved regions are at least 90% identical.

To obtain a rapid readout of the effect of each PRE / TRE on the *mw* promoter the eye color in adults flies was examined (Figure 11A-E). In addition, to visualize both the *gfp* and *mw* transcripts I performed double in situ hybridizations on 3rd instar larval eye imaginal discs from crawling larvae (Figure 11 F-W). Comparison of the eye pigment levels of adult flies carrying the D. *pseudoobscura eya* PRE with those of the REGFP line showed a dramatic loss of repression of *mw* in both heterozygote and homozygote flies. The eye pigment levels were nevertheless lower than those of the REGFPΔPRE line (Figure 6 M), indicating that the ability of the D. *pseudoobscura eya* PRE / TRE to repress the interaction of the *D. melanogaster eya* eye enhancer with the *mw* promoter is greatly reduced but not entirely lost (Figure 11 B). Consistent with this observation, a clear difference in the spatial expression profile of the *mw* transcript across the eye disc in REGFP*Dpse-eya* and REGFP larvae was observed, that was most pronounced in zones 1 and 2 (Figure 11 H,K and U,V). Interestingly, the *gfp* expression profile generated by the *D. pseudoobscura* element displayed both similarities and differences to that of the *D. melanogaster* element (Figure 11 G,J and U,V). The most striking differences were observed in zone 2, directly behind the morphogenetic furrow. In these cells, the *D. pseudoobscura* element showed the same level of *gfp* signal intensity compared to zone 1, in contrast to the pattern observed for the *D. melanogaster* PRE / TRE (Figure 11 U,V). However, the *gfp* profiles of the two constructs also displayed a similarity: the *gfp* transcript levels were strongly reduced at the trasnsition from zone 2 to 3. Taken together these results indicate that the ability of the *D. pseudoobscura* PRE / TRE to interact

correctly with the *D. melanogaster* enhancer to regulate the upstream promoter is strongly dependent on cell type. Furthermore, the comparison of *gfp* and *mw* profiles in Figure 11 V indicates that the *D. pseudoobscura* PRE / TRE has to some extent lost the ability to produce differential expression patterns of the upstream and downstream promoters.

The *D. pseudoobscura* sequence used in the above experiments was defined by computational means, and has not been tested previously in transgenic assays. Thus I wished to test other PRE / TREs that have been more extensively characterized. The *vestigial* PRE / TRE behaves as a *PcG* dependent pairing sensitive silencer when placed upstream and directly adjacent to a *mw* reporter (Lee, et al., 2005). Remarkably, when placed in the context of the REGFP reporter, the *vestigial* PRE / TRE was unable to silence *mw* (Figure 11 C) giving adult eye pigment levels in both heterozygotes and homozygotes that were higher still than those observed for the *D. pseudoobscura* PRE / TRE. This loss of repression was also reflected in the in situ profiles, in which high levels of *mw* expression were observed across the disc (Figure 11 W). However the *mw* profile was qualitatively different from that given by the *D. pseudoobscura* PRE / TRE construct, giving the highest levels in zone 3, again underlining the dependence of cell type for the enhancer-PRE interaction for the downstream promoter. Strikingly, the *gfp* profile generated by the *vestigial* PRE / TRE was very similar to that of the *D. melanogaster eya* PRE / TRE in zones 1 and 2, but showed a relative increase of *gfp* signal in zone 3, again pointing to cell type dependent interaction of the *vestigial* PRE with the *eya* enhancer (Figure 11 M,W). In summary, these results demonstrate that the *vestigial* PRE / TRE is able to partially recapitulate the function of the *eya* PRE / TRE in zones 1 and 2, can to some extent reproduce the differential expression profiles of the two promoters, but fails to repress both promoters in zone 3.

Finally, I examined the effect of replacing the *eya* PRE / TRE in REGFP with the *D. melanogaster bxd* PRE / TRE from the *Ubx* gene. For comparison I also

included the *D. pseudoobscura* ortholog (Figure 11 D,E). The *bxd* PRE / TREs from both species act as *PcG* and *trxG* dependent pairing sensitive silencers when placed adjacent to a *mw* reporter gene, and both elements show variegation at some genomic sites (Simon, et al., 1993, Chan, et al., 1994, Hauenschild, et al., 2008). In addition the D. mel*anogaster bxd* PRE / TRE has been shown to function as a maintenance element when placed in the correct regulatory context of the *Ubx* promoter and enhancer (Simon, et al., 1993, Chan, et al., 1994). Strikingly, when placed in the context of REGFP, both PRE / TREs induced strong variegation of both the *mw* and the *gfp* reporters, visible both in adult flies (Figure 11 D,E) and in larval discs (Figure 11 O-T). Due to this strong variegation, it was not possible to plot average profiles, however several intriguing features are discernable from the in situ images. Firstly, whereas cells that expressed *mw* did so at a similar level in all zones of the disc (Figure 11 Q,T), the expression level of *gfp* was markedly higher in cells of zones 1 and 2 for both the bxd PRE / TREs and did not reach low expression levels in zone 3 which is characteritic for the eya PRE / TRE in the REGFP construct (compare Figure 11 P,S to G). Secondly, the variegation of the two reporters in a single disc did not occur in a mutually exclusive manner, as would be expected if the PRE / TRE mediates competition between the two promoters for the enhancer. Instead, the variegation of the two reporters occurred in parallel, with patches of cells expressing either both transcripts or neither of them. This behaviour was observed with the *bxd* PRE / TRE from both species (Figure 11 O-T). This result indicates that a single PRE / TRE can block the interaction of an enhancer with two promoters simultaneously in the same cells. Likewise the same PRE / TRE can also simultaneously facilitate the interaction of the enhancer with both promoters. Taken together, the results of the PRE / TRE replacement experiments clearly demonstrate that each of the PRE / TREs tested interacts specifically and differently with the *D. melanogaster eya* enhancer, and thus that the distinct readouts of the two REGFP reporters given by the *D. melanogaster eya* PRE / TRE are unique to this PRE / TRE.

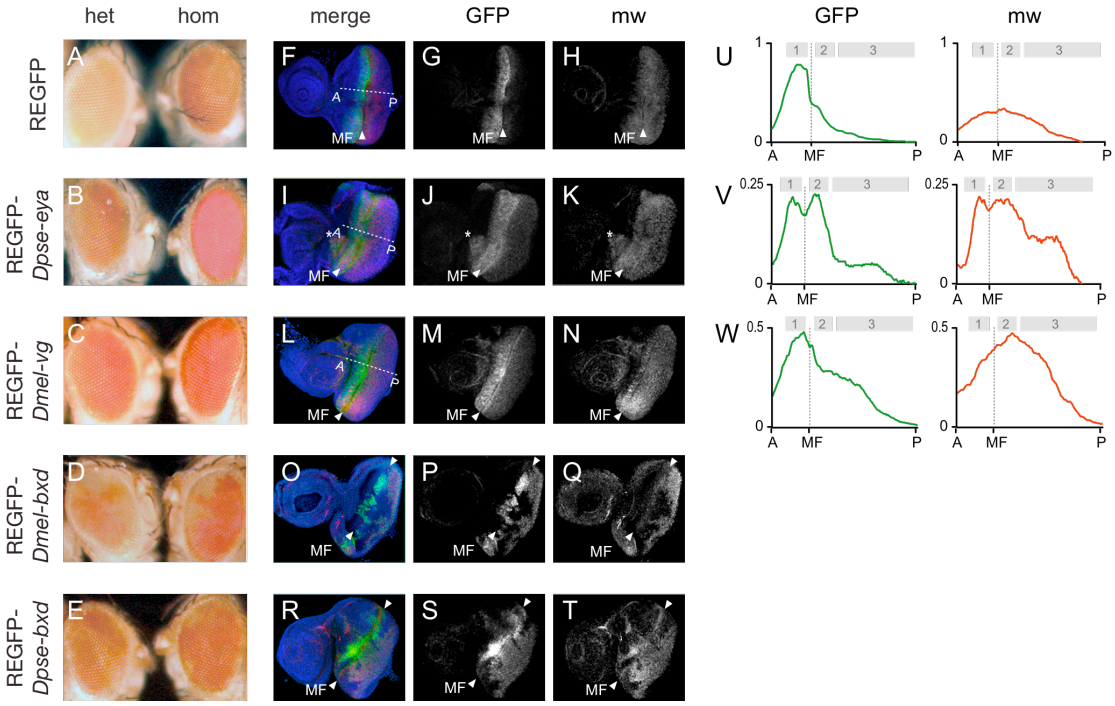


Figure 11: REGFP PRE / TRE replacement constructs show distinct behaviours. (**A-E**) Eye colors of flies heterozygous (left) or homozygous (right) for REGFP (**A**) and each of the replacement constructs (**B-E**) as indicated on the left of the figure. Adult male flies, 5 days old, are shown. *bxd* lines are 10 days old to show variegation. (**F-T**) double *in situ* hybridization on 3rd instar eye-antenna imaginal discs of larvae transgenic for each construct as indicated on the left of the figure. (**F-H,U**) data from Figure 8 for REGFP are shown for comparison. (**I-K**) asterisk indicates folded disc edge, not ectopic expression. (**G,J,M,P,S**) *gfp* reporter. (**H,K,N,Q,T**) *mw* reporter. (**F,I,L,O,R**) merge: blue, DAPI; green *gfp*; red, *mw*. See Figure 11 for *in situ* probes. Dotted line shows positions of line scans in (**U,V,W**). (**U,V,W**) average line scans for *gfp* (left) and *mw* (right) from REGFP discs as in Figure 8 ; (**V**) average line scans from 3 REGFP-*Dpse-eya* discs. (**W**) average line scans from 5 REGFP-*Dmel-vg* discs. Line scans were performed as in Figure 8 . Grey bars above plots indicate 3 zones of the disc, see Legend to Figure 8 for details. The vertical scale on all line scan plots shows relative signal intensity, the same scale is used for all experiments. Line scans were not performed for the *bxd* replacement lines (**O-T**) due to the variegated nature of expression of both reporters.

2.5. The gradient of *mw* pigmentation is an inherent property of *eya* sequence

One interesting observation is that the two lines REGFPΔen and REGFPΔenΔPRE displayed increased pigmentation in the anterior third of the eye (homozygotes in Figure 6 N and O). Even in the REGFP line a gradient of pigmentation increasing from posterior to anterior was detectable in homozygotes (Figure 6 L). To test whether this increasing pigmentation is a property of the *eya* regulatory sequence or alternatively, caused by *white* specific regulation, the signal intensity profiles of the REGFP line were compared at different time points of L3 eye development by analyzing the profiles from crawling larvae (earlier eye development) and prepupae (later eye development) (compare profiles of Figure 8 O and Figure 12 D, right). Strikingly, the *mw* expression in zone 1 displayed different intensities when compared to zone 2 in eye discs of crawling larvae and prepupae. The *mw* expression levels in zone 1 were lower compared to zone 2 in crawling larvae (Figure 8 O). In contrast, prepupae displayed higher levels of *mw* expression in zone 1 compared to zone 2 (Figure 12 D). Consequently, the increasing pigmentation towards the anterior part of the eye must be an inherent function of the *eya* regulatory sequence as this relative change in *mw* expression levels was detected in zone 1 where *eya* specific regulation takes place. Comparision of the *gfp* profile of crawling larvae and prepupae was comparable in all three zones characterized by a strong upregulation in zone 1, lower levels in zone 2 and very low levels of signal intensity in zone 3 (Figure 8 N and Figure 12 D, left). As the *eya* PRE / TRE mediates differential regulation of the two reporters it this finding indicates that the ratio of both reporters in zone 1 changes with developmental progression as observed for the endogenous transcripts (see above Figure 9 E,G) and requires the PRE / TRE.

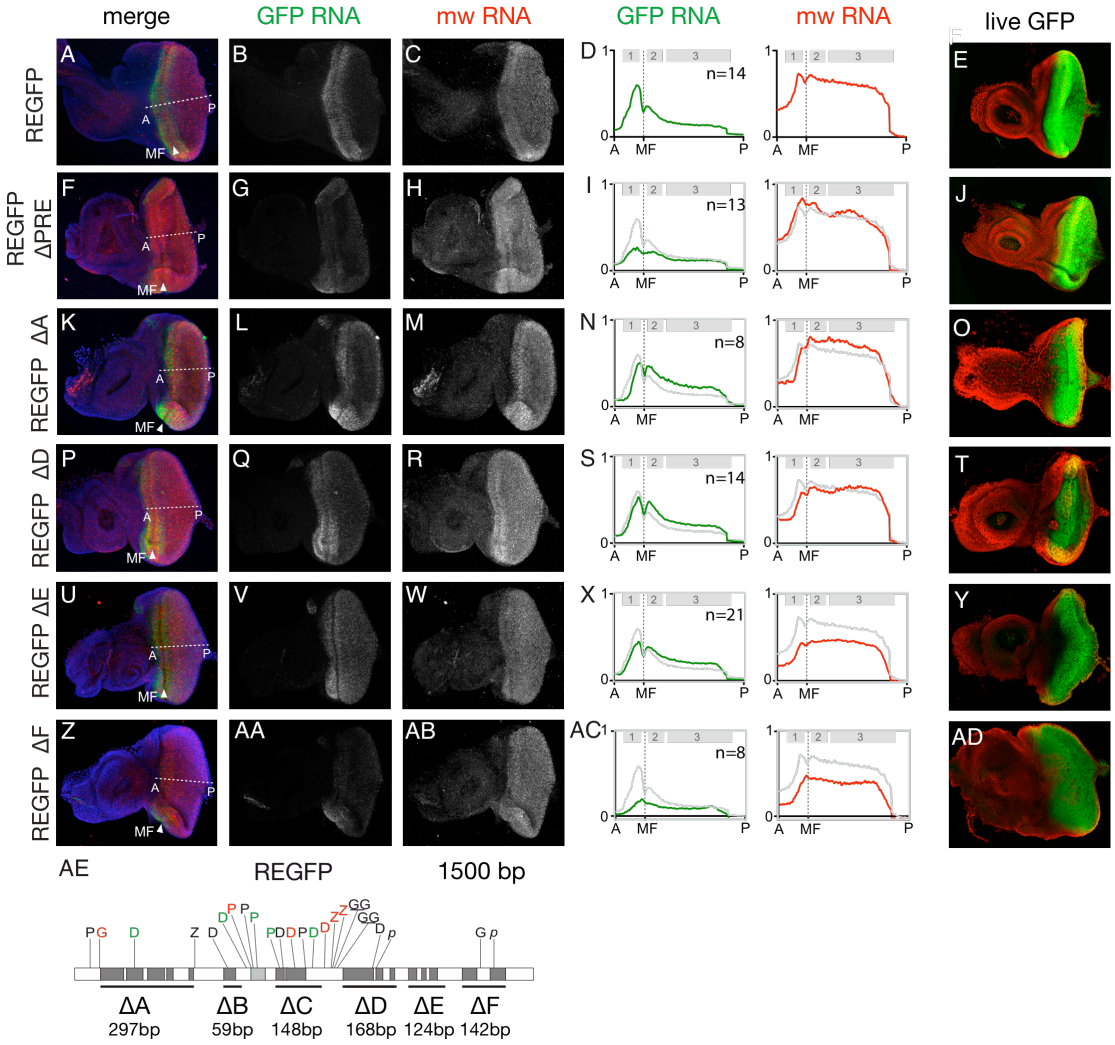


Figure 12: The dissection of the *eya* PRE / TRE reveals two functional categories of the *eya* PRE / TRE in prepupal eye discs.

(**A – C**) Double *in situ* hybridizations on REGFP prepupal larvae using probes to detect *gfp* (**B, G, L, Q, V, AA**) and *mw* (**C, H, M, R, W, AB**) transcripts. (**A, F, K, P, U, Z**) merge: blue, DAPI; green, *gfp*; red, *mw*. Arrowheads indicate the morphogenetic furrow. Dotted line shows position of line scan in (**D, I, N, S, X, AC**). (**D**) Average signal intensity profiles extracted from double in situ hybridization images of 14 eye discs of REGFP transgenes for *gfp* (green) and *mw* (red) along the anteroposterior disc axis. The area of the discs posterior to the morphogenetic furrow is normalized by graphical transformation to standard length. Grey boxes above the line scan plots indicate three zones of the disc: (1) mitotic cells anterior to the morphogenetic furrow; (2) zone of photoreceptor differentiation immediately posterior to the furrow; (3) zone of maturing photoreceptors at posterior of disc. Vertical scale represents

relative signal intensity, the same scale is used in all plots. The REGFP signal intensity profiles shown in **D** are plotted as grey line on the profiles of the REGFP variants ΔPRE (**I**) and ΔA - ΔF (**N**, **S**, **X**, **AC**) for comparison. The number of discs used to generate the average signal intensity profiles is indicated on the upper right corner of each *gfp* profile. (**E, J, O, T, Y, AD**) *gfp* live imaging of 3rd instar eye discs. (**A – E**) REGFP (**F – J**) REGFP ΔPRE, (**K – O**) REGFPΔA, (**P – T**) REGFPΔD, (**U – Y**) REGFP ΔE, (**Z – AD**) REGFP ΔF. (**AE**) PRE / TRE diagram illustrating the deleted sequences in the REGFP dissection constructs ΔA - ΔF. The transgene REGFPΔPRE lacks the complete 1500 bp defined as PRE / TRE in this study. The REGFP lines ΔB and ΔC could not be obtained thus far. For explanation of the binding motifs see Figure 10.

2.6. The *eya* PRE / TRE contains sequence sub-elements required for specifying expression levels

In order to learn more about the regulatory sequence that determines the characteristic attributes of the *eya* PRE / TRE, the most conserved clusters between *D. melanogaster* and *D. pseudoobscura* were deleted resulting in the REGFP dissection variants (Figure 10 A,B and Figure 12 AE). To date all but the REGFP variants REGFPΔB and REGFPΔC were obtained as transgenic lines.

The comparison of the *gfp* profiles in prepupal eye discs of the lines REGFP and REGFPΔPRE showed one striking difference: The strong *gfp* signal in zone 1 of REGFP eye discs was abolished in REGFPΔPRE independent of whether the profiles represented the average signal intensity in eye discs of crawling larvae (Figure 8 N and S) or prepupae (Figure 12 D and I, left) indicating an activating function of the *eya* PRE / TRE in zone 1. Interestingly in REGFP, the high *gfp* signal intensity found in zone 1 was dramatically reduced in zones 2 and 3 raising the question of whether the relative downregulation of *gfp* expression within these cells is dependent on the *eya* PRE / TRE or whether the observed downregulation is caused independent of the PRE / TRE, for example by dilution of the transcripts in the course of proliferation and differentiation (Figure 8 N and Figure 12 D, left).

The comparison between the *gfp* profiles of REGFP and REGFPΔPRE in zones 2 and 3 did not permit any conclusions about whether the *eya* PRE / TRE is involved in downregulation in zones 2 and 3 as the signal intensities behind the morphogenetic furrow could be a consequence from the initial lack of activation in zone 1 (Figure 8 S and Figure 12 I, left). However, the dissection of the *eya* PRE / TRE (see Figure 12 AE) allowed analysis of its role in the zones 2 and 3. The *mw* profiles of the lines REGFPΔA and ΔD were at similar levels compared to REGFP allowing direct comparisons of the *gfp* profiles between the lines in all three zones (Figure 12 K-M and N, right; Figure 12 P-R and S, right). In contrast, the *mw* profile of the line REGFPΔE displayed lower *mw* signal compared to REGFP resulting in a higher level of the *gfp* signal relative to *mw* when directly compared to REGFP making the gfp signal even more significant (Figure 12 U-W and X, right).

In zone 1, the *gfp* profiles of REGFPΔA, ΔD and ΔE resembled the characteristic high *gfp* signal intensity of REGFP (Figure 12 N, S and X, left). This indicates that the sequences deleted in REGFPΔA, ΔD and ΔE are not involved in the upregulation of the *gfp* reporter in zone 1 as levels similar to REGFP were achieved without these sequences. However in zone 2 and 3, the profiles of REGFPΔA, ΔD and ΔE differed from the *gfp* profile of REGFP transgenes. In zone 2, the *gfp* signal intensities of the lines REGFPΔA, ΔD and ΔE were at similar levels compared to zone 1 indicating that the sequences deleted in REGFPΔA, ΔD and ΔE are required for downregulation of *gfp* expression after the transition through the morphogenetic furrow (Figure 12 N, S and X, left). Furthermore, the *gfp* signal intensities in zone 3 were higher in REGFPΔA, ΔD and ΔE compared to REGFP while the *mw* signal intensities in zone 3 were similar (REGFPΔA and ΔD) or even lower (REGFPΔE) compared to the *mw* profile of REGFP transgenes (Figure 12 N, S and X). This indicates that the sequences deleted in REGFPΔA, ΔD and ΔE are required for specifying low levels of *gfp* expression in zone 3.

Taken together, the above results show that the sequences deleted in REGFPΔA, ΔD and ΔE are sub-elements of the 1500 bp *eya* PRE / TRE that are required for downregulation of *gfp* expression in the zones posterior to the

morphogenetic furrow. This supports a model of PRE / TRE dependent downregulation of the *gfp* transcript rather than simple dilution throughout the course of proliferation and differentiation.

The dissection of the *eya* PRE / TRE revealed not only sequence elements involved in downregulation behind the morphogenetic furrow but also one sequence sub-element involved in specifying high levels of reporter gene expression. The transgenic line REGFPΔF displayed similar *mw* signal intensities compared to REGFPΔE in all three zones (Figure 12 Z-AB and AC, right and U-W and X, right). In contrast, the *gfp* signal intensities of REGFPΔF were lower in the zones 1, 2 and 3 compared to REGFPΔE indicating that the sequence deleted in REGFPΔF is required for activation of *gfp* expression at least in zone 1 while the low signal intensities measured in zones 2 and 3 could be a consequence of the initial low level expression in zone 1 (Figure 12 X and AC, left). Interestingly, the *gfp* profile of REGFPΔF resembled the profile of REGFPΔPRE indicating that the lack of activation in zone 1 is due to the absence of the sequence deleted in REGFPΔF in both cases (Figure 12 I and AC).

2.7. The establishment of the adult eye color observed in REGFP transgenes requires the integrity of the *eya* PRE / TRE sequence

As mentioned above adult REGFP flies and REGFPΔPRE transgenes differed in the adult eye color whereby REGFP flies displayed a orange eye color when homozygous (Figure 13 A, C, E, G, upper left) and a yellow eye color when heterozygous (Figure 13 B, D, F, H, I, upper left). In contrast, REGFPΔPRE flies displayed a red eye color when homozygous (Figure 13 A, C, E, G, upper right) and a brown eye color when heterozygous (Figure 13 B, D, F, H, I upper right) showing that the *eya* PRE / TRE is involved in the generation of a lighter eye color both in the heterozygous and homozygous situation. In order to identify the critical sequence components responsible for the generation of the

lighter eye color the *eya* PRE / TRE dissection lines REGFPΔA, ΔD, ΔE and ΔF were compared to REGFP and REGFPΔPRE flies both as homozygotes (Figure 13 A, C, E, G) and heterozygotes (Figure 13 B and I, D, F, H). In homozygotes and heterozygotes, the *eya* PRE / TRE dissection lines REGFPΔD - ΔF displayed an adult eye color comparable to the REGFPΔPRE line indicating that the functional integrity of the *eya* PRE / TRE is perturbed by any of the above alterations on the *eya* PRE / TRE sequence because a lighter eye color was never obtained within the fly populations (Figure 13 A-H). In contrast, the line REGFPΔA resulted in two variants of eye colors that were stably inherited. The first variant referred to as “REGFPΔA-red” (REGFPΔAr) reproduced the eye color of REGFPΔPRE both in homozygotes and heterozygotes (Figure 13 A and B). The second variant - referred to as “REGFPΔA yellow” (REGFPΔAy) - reproduced the yellow eye color of REGFP flies in heterozygotes (Figure 13 I). Individual REGFPΔAy flies frequently arose in the progeny of the heterozygous founding population of the first variant (REGFPΔAr). Interestingly, the darker eye color behaved dominant allowing maintaining the lighter eye color only in a pure REGFPΔAy stock.

In summary, the presented results show that the establishment of the lighter eye color seen in REGFP flies categorically requires the sub-elements D, E and F while the A element seems to be important for the stability of silenced states as these transgenes are able to mimic the lighter eye color of REGFP transgenes.

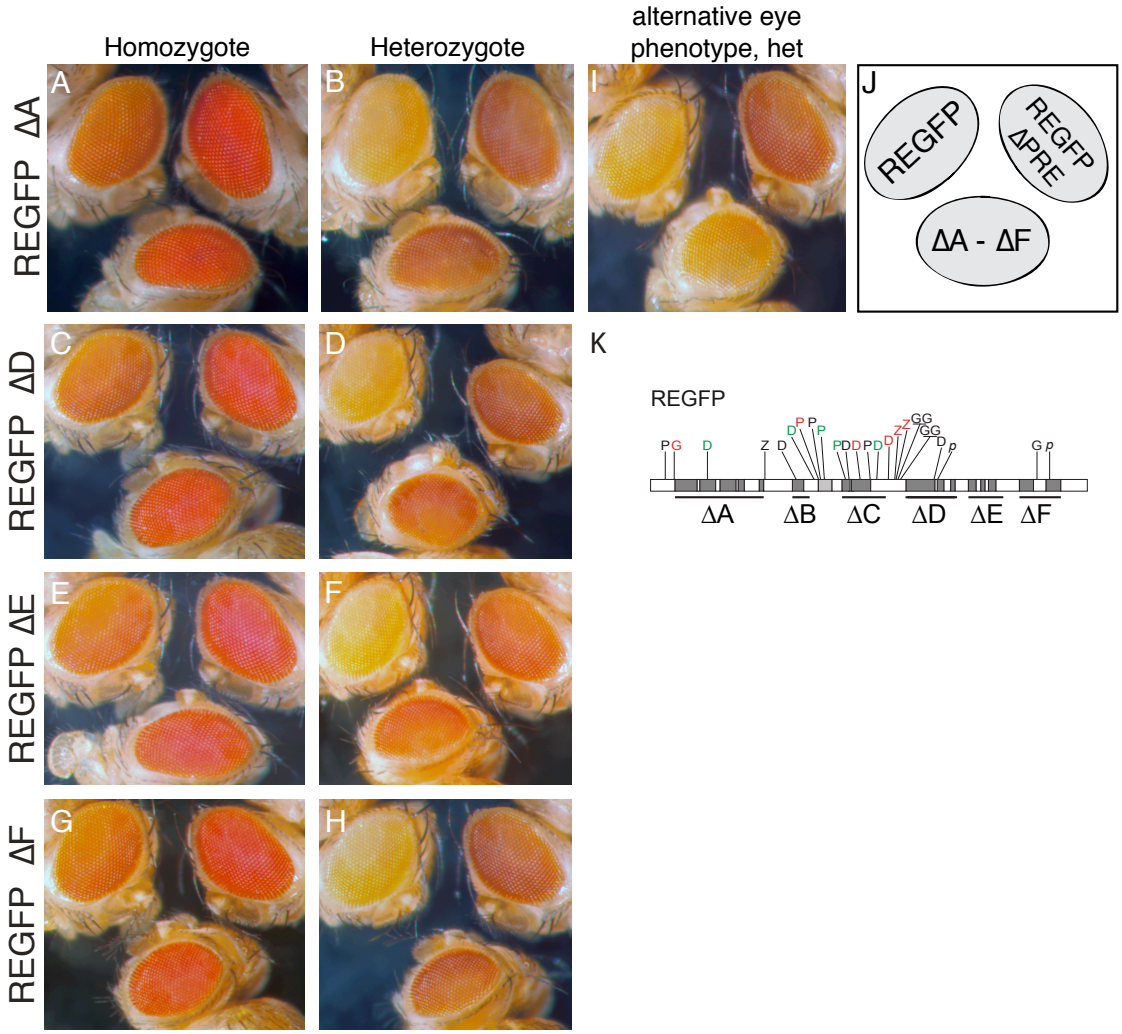


Figure 13: Most alterations of the *eya* PRE / TRE used in REGFP lead to a loss of silencing capacity in the adult eye.

All images shown in this figure follow the format illustrated in **J** where a REGFPΔA - ΔF transgene (bottom) is compared to a REGFP (upper left) and a REGFPΔPRE transgene (upper right). All flies shown are males aged for 6 days. *eya* PRE / TRE dissection constructs REGFPΔA (**A,B,I**), REGFPΔD (**C, D**), REGFPΔE (**E,F**), REGFPΔF (**G, H**) are compared as homozygotes (**A, C, E, G,**) and heterozygotes (**B, D, F, H, I**). REGFP ΔA – REGFP ΔF flies (bottom of each image) display lack of silencing compared to REGFP and have a phenotype comparable to REGFP ΔPRE both in homozygotes and heterozygotes. One exception is REGFPΔA that displays two stable phenotypes in heterozygotes: One comparable to REGFPΔPRE (**B**) and another comparable to REGFP (**I**) with yellow eye color. (**K**) PRE / TRE diagram illustrating the deleted sequences within the *eya* PRE / TRE *u*sed in the REGFP dissection constructs ΔA - ΔF. Black lines under the diagram represent the deletions to scale. The respective naming and exact length of the deletion is indicated

underneath each of these lines. The REGFP lines ΔB and ΔC could not be obtained thus far. For explanation of the binding motifs see Figure 10.

3. The *eya* PRE / TRE contains an optic lobe enhancer

3.1. The REGFP reporter gene expression reflects the expression of the endogenous *eya* expression in the larval optic lobes.

In addition to the expression of both isoforms in the 3rd instar eye-antennal imaginal disc *eyes absent* is expressed in two domains of the larval brain: the lamina of the optic lobe and a subset of cells within the central brain (Bonini, et al., 1998). In order to compare the expression pattern of *eyes absent* with the expression of the two REGFP reporters *gfp* and *mw*, two independent double *in situ* experiments were performed using probes to detect exon 1.1 and exon 1.2 transcripts in wildtype (Supplementary material, Figure 19, A-D) and probes to detect *gfp* and *mw* transcripts on REGFP prepupal brains (Figure 14, A-D). Expression specific to the lamina of the optic lobe was detected for both, endogenous *eya* (arrowheads in Figure 19, A-D) and transgenic REGFP transcripts (arrowheads in Figure 14 , A-D). These results demonstrate that the *eya* sequence used in the REGFP reporter construct contains the full sequence information to drive optic lobe specific expression for both REGFP reporters.

3.2. The eyes absent PRE / TRE contains a functionally conserved optic lobe enhancer

The two *eya* isoforms and the two REGFP reporters *gfp* and *mw* were expressed in lamina cells of the optic lobe forming a pattern that resembles a horseshoe when viewed from a lateral perspective (Figure 14 Z and E, J, O, T, Y). The innervation scheme of retinal axons is shown in Figure 14 AA. Thereby, the physiological location lamina cells that are synaptically connected

to photoreceptor neurons at the midline of the eye field lie between the two ends of the horseshoe (M' in Figure 14 AA), while the two ends of the horseshoe represent lamina cells that are synaptically connected to the most dorsal and ventral retinal neurons (D' and V' in Figure 14 AA) (Yoshida, et al., 2005, Fischbach and Hiesinger, 2008). To test whether the *eya* PRE / TRE or the eye specific enhancer in the REGFP construct are involved in the regulation of brain specific expression, double *in situ* hybridizations were performed using probes to detect *gfp* and *mw* transcripts in REGFP (Figure 14 , A-E) were compared to the REGFPΔPRE, REGFPΔen and REGFPΔenΔPRE lines (Figure 14 , F-J, K-O, P-T). In addition, to test for functional conservation of the *eya* PRE / TRE in optic lobe specific expression, the REGFP *D. pse.* PRE / TRE line was included to the experimental set (Figure 14 , U-Y, see also Figure 10 B). Interestingly, the removal of the *eya* PRE / TRE in REGFPΔPRE led to a complete loss of expression in the optic lobe domain for both reporters (Arrowheads in Figure 14 F-I) indicating that the 1500 bp sequence defined as *eya* PRE / TRE contains critical sequence information to drive expression in the optic lobes. In contrast, the removal of the eye specific enhancer in REGFPΔen (Figure 14 K-O) did not have any effect on the expression pattern in the brain compared to REGFP showing that the eye specific enhancer is not involved in regulation of gene expression in the brain. Consistent with the results encountered with REGFPΔPRE and REGFPΔen, the removal of both sequences in the REGFPΔenΔPRE line (Figure 14 P-T) led to lack of expression of both reporters in the optic lobe domain mimicking the phenotype observed for the REGFPΔPRE line. Finally, the replacement of the *D. melanogaster eya* PRE / TRE with the othologous sequence of *D. pseudoobscura* (see Figure 10 A and B) recovered the optic lobe specific expression of the *gfp* and *mw* reporters showing that the optic lobe specific enhancer is conserved between both species (Figure 14 , U-Y). In summary, the *eya* PRE / TRE contains sequence information to drive expression specifically in the lamina of the optic lobe. This function is conserved within the orthologous sequences of *D. melanogaster* and *D. pseudoobscura* while the PRE / TRE function is not.

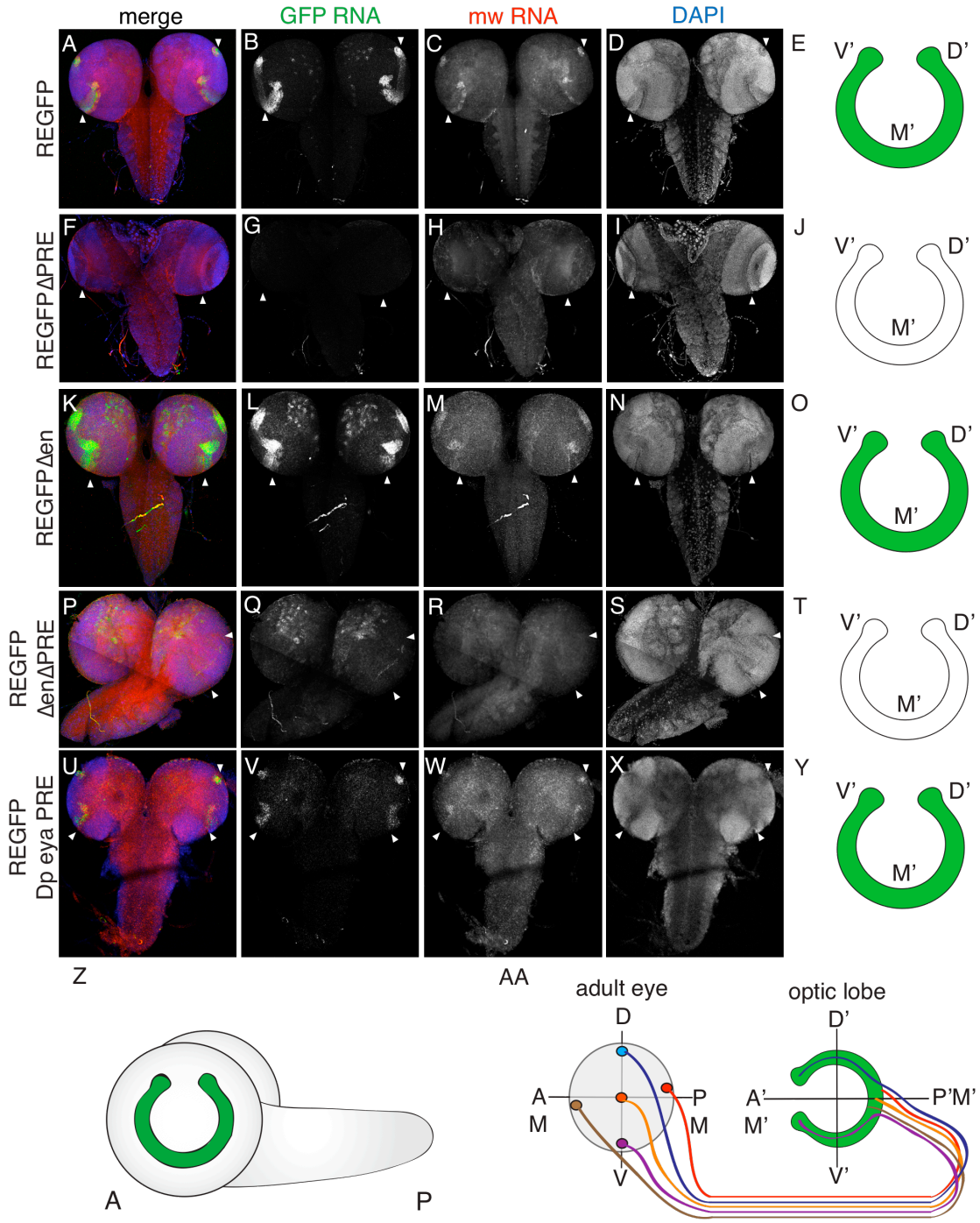


Figure 14: The *eya* PRE / TRE contains an optic lobe enhancer.

(**A–E**) REGFP, (**F–J**) REGFPΔPRE, (**K–O**) REGFPΔen, (**P–T**) REGFPΔenΔPRE, (**U-Y**) REGFP *D. pse. eya* PRE*. Double *in situ* hybridizations on L3 prepupal larval brains using probes to detect *gfp* (**B,G,L,Q,V**) and *mw* transcripts (**C,H,M,R,W**). The central nervous system (CNS) morphology is visualized by DAPI (**D,I,N,S,X**). Merge; blue, DAPI; green, *gfp*; red, *mw*. Arrowheads indicate the border between lamina and medulla of the optic lobe. (**E,J,O,T,Y**) „Horseshoe“ diagrams illustrating the

expression of *gfp* RNA in the lamina. Green, expression; white, no expression; V', innervation of ventral photoreceptor neurons. D', innervation of dorsal photoreceptor neurons. M' innervation of photoreceptor neurons located at the midline of the eye. (**Z**) The lamina forms a pattern resembling a „horse shoe". This pattern is illustrated within the context of the entire third instar *Drosophila* central nervous system and equals the expression domain of *eya* in wildtype flies or the *gfp* and *mw* reporters in REGFP flies. Anterior to the left, lateral view. (**AA**) Innervation scheme of retinal axons into the lamina of the optic lobe (based on (Yoshida, et al., 2005)). The adult eye is illustrated with the dorso-ventral axis defined by the extremities D and V and the antero-posterior axis defined by its extremities A and P. the AP axis is located at the midline M. fort his reason the extremities are referred to as AM and PM. Retinal axons innervate into the lamina following a stringent pattern whereby D and V axons innervate to the D' and V' regions of the lamina, respectively. The D' and V' positions in the lamina are located at the ends of the horseshoe pattern. Retinal axons from photoreceptor neurons located along the midline of the eye innervate into the center region M' of the lamina. Thereby, posterior cells innervate to the P' position of the lamina while anterior cells innervate to the A' position. As the formation of the lamina is an inductive process – induced by innervation of retinal axons - this tissue grows from P' to A' reflecting the differentiation of the eye from posterior to anterior.
Note that the dorso-ventral extremities of the lamina could not be assigned in Figures **E**,**J**,**O**,**T**,**Y** due to difficulties in determining which of the sides D / V came to lie on top on microscopic slides. Consequently, the D' and V' positions are for illustration only.

3.3. The optic lobe enhancer within the *eya* PRE / TRE contains two different categories of subfragment

To address optic lobe enhancer functions within the 1500 bp of the eya PRE / TRE, the dissection variants were analyzed for lamina specific expression (Figure 15 U). The REGFPΔA and ΔD - ΔF transgenes were analyzed by double *in situ* hybridization using probes to detect the *gfp* and *mw* reporters. Interestingly, two different phenotypes could be observed with respect to the

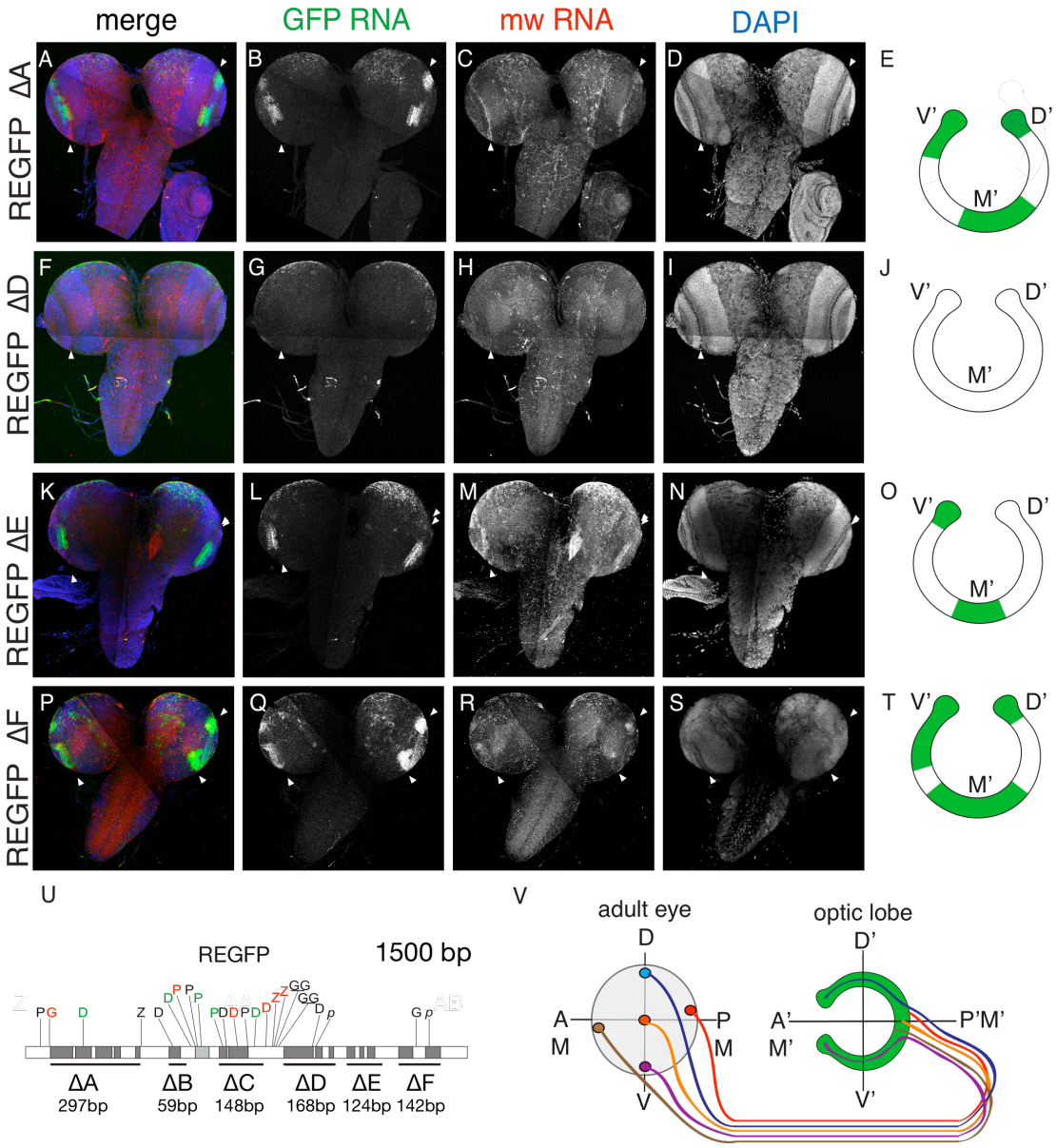


Figure 15: The optic lobe enhancer within the *eya* PRE / TRE can be functionally dissected into different fields of function.

3rd instar prepupal CNS of (**A–E**) REGFPΔA, (**F–J**) REGFPΔD, (**K–O**) REGFPΔE, (**P–T**) REGFPΔF. Double *in situ* hybridizations on L3 prepupal larval brains using probes to detect *gfp* (**B,G,L,Q,**) and *mw* transcripts (**C,H,M,R**). The central nervous system (CNS) morphology is visualized by DAPI (**D,I,N,S**). (**A,F,K,P**) merge: blue, DAPI; green, *gfp* transcripts; red, *mw* transcripts. Arrowheads indicate the border between lamina and medulla of the optic lobe. „Horseshoe“ diagrams illustrate the *gfp* expression in REGFP ΔA - ΔF transgenes (**E,J,O,T**). The functional categories of the deletions are subdivided into the categories „incomplete patterning“ as seen in

REGFPΔA, ΔE and ΔF (**A-E**, **K-O and T-Y**) and "complete lack of expression" as seen for REGFPΔD (**F-I**). **Z**, PRE / TRE diagram illustrating the deleted sequences in the REGFP dissection constructs ΔA - ΔF. Black lines under the diagram represent the deletions to scale. The respective naming and exact length of the deletion is indicated underneath each of these lines. The lines REGFPΔB and ΔC were not obtained thus far. (**AA**) innervation scheme of retinal axons, see Figure 14 AA. Note that the dorso-ventral extremities of the lamina expression domain could not be assigned in Figures E,J,O,T due to difficulties in determining which of the sides D / V came to lie on top on microscopic slides. Consequently, the D' and V' positions are for illustration only and do not qualify for functional assignments of the sub-elements in respect to specific dorsal and ventral functions.

optic lobe specific expression of both reporters: First, the REGFP deletion lines REGFPΔA, ΔD and ΔF displayed a phenotype of interrupted expression along the horseshoe pattern. In REGFPΔA and ΔF transgenes, expression of both reporters was detected at both the dorsal (D') and ventral (V') ends of the horseshoe as well as in the domain at the center of the horseshoe (M') (Figure 15 E, T). A stronger phenotype was detected in REGFPΔE transgenes that was characterized by reporter gene expression in the center region and only one of the two dorso-ventral ends of the horseshoe (Figure 15 O). These findings indicate that the sequences deleted in REGFPΔA, ΔE and ΔF are involved in driving optic lobe specific expression into regions of the lamina with moderate dorsal and ventral positions (ΔA and ΔF) and moderate to extreme dorsal / ventral positions (ΔE) (Figure 15 A-E, K-O and P-T). The white regions of the horseshoe reflect the spatial specificity of the deleted element. These domains are synaptically connected to the corresponding dorsal and ventral retinula cells as illustrated in Figure 15V (see also (Fischbach and Hiesinger, 2008)). Second, the line REGFPΔD displayed complete lack of expression of both reporters within the horseshoe expression domain (Figure 15 F-J). The lack of expression within the entire optic lobe domain is remarkable as it indicates a hierarchical organization of

the optic lobe enhancer whereby the D sub-element is indispensable for enhancer activity while the A-, E, and F fragments contribute to specifying the expression domain. Taken together, the results presented above show that the optic lobe enhancer within the *eya* PRE / TRE is composed of regulatory sequence indispensable for enhancer function (sub-element D) and sequence specifying moderate to extreme dorsal / ventral positions (A- ,E- and F- sub-elements). It remains to be seen whether sequences required for the other dorso-ventral extremity are located within the B and C sub-elements or alternatively, outside the 1500 bp region of the *eya* PRE / TRE.

IV.DISCUSSION

Genome wide studies have identified several hundred potential PRE / TRE elements in *Drosophila* and mammals. The models of PRE / TRE function are largely based on *Hox* gene regulation which displays a static behaviour. However, gene regulatory networks such as the RD gene network follow a different sequential order of gene regulation, cross-regulate each other and are dynamically expressed. The presence of PRE / TREs at most RD genes suggests that these elements operate in a fundamentally different manner compared to *Hox* gene PRE / TREs.

The data presented in this thesis led to the development of a model for the *eya* PRE / TRE where it functions in adjusting specific expression levels for the two promoters at different time points of eye developmental differentiation. These properties are unique to the *eya* PRE / TRE among those tested and require specific interactions between the PRE / TRE and enhancer. Furthermore, analysis of the PRE / TRE sub-elements identified regulatory sequence required for specifying high and low levels of gene expression. Finally, the *eya* PRE / TRE contains a conserved optic lobe specific enhancer while the PRE / TRE function is not conserved. This study defines novel properties of PRE / TREs and has multiple implications for the functions of these elements in dynamic differentiating tissues.

1. The *eya* PRE / TRE: a novel model for PRE / TRE function in dynamically developing tissue

This work documents a differential regulation of the two *eya* promoters in the eye disc. The endogenous transcripts arising from the two alternative *eya* promoters have been detected by Northern blot in eye discs (Leiserson, et al., 1998) but have not previously been subjected to in situ analysis. Using the REGFP reporter construct to dissect the role of the PRE / TRE and the eye specific enhancer in this differential regulation, the data presented here shows that the enhancer sets up a similar pattern for both promoters across

the eye disc, and that the PRE / TRE refines this into a differential pattern for each promoter.

2. Potential limitations of the REGFP construct

During eye development, the expression of both reporters, *gfp* and *mw*, coincide with the expression domain of the endogenous *eya* gene. This makes the regulatory region of *eya* that was used in this assay a suitable tool to study regulatory properties of the *eya* gene. However, there are several potential limitations that could complicate the interpretation of the presented results. While the expression domain of the reporters and the endogenous gene are identical, the expression profile of the *gfp* reporter differs from the endogenous *eya* 1.1 profile (compare Figure 8 N to Figure 8D and Figure 12D to Figure 9D). The difference of the *gfp* profile compared to the endogenous *eya* 1.1 profiles could be caused by two reasons: the use of only partial *eya* sequence in the REGFP construct or alternatively, a lower RNA stability of the *gfp* mRNA. In addition, the *mw* profile may be the result of two different sources of gene regulation. (1) *eya* specific regulation via the REGFP construct, and (2) *white* specific transcriptional regulation from zone 2 onwards (Figure 8 F-J). Therefore, it is possible that the *mw* expression profile only resembles the *eya* 1.2 profile superficially. For this reason, the question must be asked to what degree REGFP regulation reflects *eya* regulation and whether this influences the conclusions drawn about the function of the *eya* PRE / TRE.

2.1. The *eya* regulatory sequence used in the REGFP construct lacks specific regulatory information that is present at the endogenous locus

Indeed, at least one confirmed EY binding motif is missing from the reporter gene construct. The eya3 binding motif has been shown to bind the EY protein in vitro (Ostrin, et al., 2006). This binding motif is located several kb downstream of the PRE / TRE and has not been included in the REGFP

reporter construct. Similarly, additional unknown regulatory sequences may be missing in the REGFP reporter that consists of 5 kb while the entire *eya* region spans approximately 20 kb (FlyBase ID FBgn0000320; genomic location: 2L:6,527,447..6,546,972 [-]). Therefore, it seems plausible that the REGFP reporter construct recapitulates only a subset of the endogenous *eya* regulation. Nevertheless, this potential limitation does not limit the conclusions drawn from the comparison of the REGFP and REGFP-variant lines. Indeed, the PRE / TRE element plays a role in reporter gene regulation as alteration, replacement or removal of this element changes the profiles of reporter gene expression (Figure 8, Figure 11 and Figure 12). Although the *gfp* profile is different from the profiles of the endogenous *eya* isoforms (especially in zone 1 but not zone 2 and 3, see Figure 8 D,E and N,O), the findings presented in this thesis support the idea that the *eya* PRE / TRE plays a role in the regulation of *eya* during eye development where it is involved in differential regulation of the two promoters. Nevertheless, it is required to consider that endogenous *eya* regulation may be more complex than REGFP regulation.

2.2. The RNA stability of the *gfp* mRNA may be lower than the endogenous *eya* transcripts

An alternative explanation for the difference of the eya 1.1 and the *gfp* expression profiles could be a difference in mRNA stability. Generally, mRNA stability is enhanced with the length of its poly-A tail (Beilharz and Preiss, 2007). Additionally, the nucleotide sequence of the 3'UTR is known to play a major role for RNA stability by providing binding specificity for RNA binding proteins or short non-coding RNAs, involved in mRNA decay (Mukherjee, et al., 2011, Wu and Brewer, 2012). The stability of the *gfp* mRNA may be lower compared to endogenous *eya* transcripts because the 3'UTR contains only basic attributes such as two SV40 polyadenylation signals. Additionally, this minimal 3' UTR does not have any sequence attributes related to eye development. If the *gfp* mRNA stability is lower compared to endogenous *eya*

mRNA, the *gfp* profile may approximately reflect the transcriptional activity in zones 1, 2 and 3 while the same transcriptional activity from the endogenous *eya* gene would be masked by the higher transcript stability resulting in an even distributed profile. Therefore it is potentially possible that the main source of *eya* transcripts is zone 1, and the real transcriptional activities of the *eya* gene are approximately reflected by the *gfp* profile.

In summary, the *gfp* profile may differ from the endogenous *eya* 1.1 profile either due to the lack of regulatory sequence or due to different transcript stabilites. However, the conclusions drawn about the function of the *eya* PRE / TRE are nevertheless valid because *gfp* expression is strictly dependent on the *eya* eye enhancer and is modulated by alteration, exchange or absence of the PRE / TRE (Figure 6, Figure 8 and Figure 11).

2.3. The *eya* eye specific enhancer interacts with the *mw* promoter in an *eya* specific manner

The transgenic line 16A*mw* contains only the *mw* reporter without any *eya* related sequence. *mw* expression in 16A*mw* is induced behind the morphogenetic furrow in zone 2 (Figure 8 J). As the transgenic insertion site of 16A *mw* and all REGFP based constructs is identical it must be assumed that the same factors that act on the *mw* promoter in 16A*mw* are present in the REGFP variants as well and are acting on the *mw* promoter. These factors are likely to be *white* specific regulatory factors. Therefore, it is likely, that the *mw* profiles and the adult eye colors obtained from distinct REGFP variant lines are the result of an additive effect consisting of *eya* and *white* specific regulation (Figure 8 O,T and Figure 6 L,M,N,O). However, the transgenic line REGFPΔen (lacking the enhancer but containing the PRE / TRE) does not express *gfp* in eye imaginal discs suggesting that *eya* specific expression may not only be absent for *gfp* but also for *mw* as is to be expected by the absence of the eye specific enhancer (Figure 6 E,I,N and F,J,O). Nevertheless, adult, homozygous flies display *mw* specific pigmentation at the anterior part of the eye while the posterior 2/3 of the eye lack pigmentation completely.

Although the source of this gradient remains elusive it shows that *white* specific factors are indeed acting on the *mw* promoter because REGFP lines lacking the enhancer do show pigmentation to some extent.

2.4. The *eya* enhancer is the unique effector of *mw* expression in zone 1

The eye enhancer is able to transfer regulatory information to the *mw* promoter because in absence of the enhancer *mw* expression is severely reduced (REGFPΔenΔPRE) or even absent (REGFPΔen) (Figure 6 E,I,N and F,J,O). This is further supported by the induction of *mw* expression in zone 1 as shown for the REGFP transgenes containing the enhancer (Figure 8 O,T). Zone 1 specific induction is a characteristic for *eya* specific regulation (Figure 8 D,E). Consequently, these results show that the *mw* promoter qualifies as an informative substitute of the second *eya* promoter in order to study *eya* specific regulation in the context of REGFP although the *mw* promoter is not specific to the *eya* gene

2.5. The presence of *eya* regulatory sequence has a restrictive effect on *white* specific regulation

Hetero- and homozygous flies of the line REGFPΔenΔPRE have a darker eye color compared to REGFPΔen (containing the PRE / TRE), respectively (Figure 6N,O and Figure 7G,H). Nevertheless, the pigmentation does not reach the level of 16A*mw* transgenes (Figure 6 B,K). It may be, that the *eya* regulatory sequence used in REGFP has – independent of the PRE / TRE - the property to constantly limit interaction of *white* specific regulatory factors with the *mw* promoter in all REGFP lines. Indeed, the different eye colors of homozygous REGFPΔen, REGFPΔenΔPRE, REGFP, and REGFPΔPRE follow a sequential order allowing this conclusion. REGFPΔen flies have one source for the regulation of the *mw* promoter (*white* specific regulation) which are blocked by the PRE / TRE. REGFPΔenΔPRE flies have the same source of factors. These are not blocked by the PRE / TRE but limited by *eya* regulatory

sequence. REGFP flies have two sources for *mw* regulation, the eye enhancer that is under control of the PRE / TRE and *white* specific regulation that may be blocked by the PRE / TRE (Figure 8 O, T). Finally, REGFPΔPRE flies have two sources for *mw* regulation: *white* specific regulation with limited access to the promoter and the *eya* eye specific enhancer without restrictions by the PRE / TRE. REGFPΔPRE flies obtain the same eye colors as 16A*mw* flies both in heterozygotes and homozygotes although these 16A*mw* flies have only *white* specific regulation as a source for *mw* regulation and express *mw* only from zone 2 onwards instead from zone 1. Consequently, the *eya* regulatory sequence may limit the activity of *white* specific factors to the same degree in all REGFP lines independent of the PRE / TRE to the same degree.

In future it will be required to generate REGFP lines containing the second *eya* promoter instead of the *mw* promoter in order to exclude *white* specific effects. However, the use of the *mw* promoter revealed that the PRE / TRE is able to block *white* specific factors from interaction with the *mw* promoter at least in the posterior part of the eye. Additionally, it helped to identify that the influence of the PRE / TRE ceases with eye developmental progression.

3. The *eya* PRE / TRE contains sub-elements that reduce and enhance promoter activity

The dissection of the well studied *bxd* PRE / TRE at its endogenous locus identified only sequence components required for silencing (Sipos, et al., 2007). The authors monitored *Ubx* regulation and deleted small sequences from a 3 kb region encompassing the *bxd* core PRE / TRE. Here, I show that the *eya* PRE / TRE consists of sub-elements with distinct functions including roles not only in silencing but also activation. Three sub-elements are involved in down regulation of the *gfp* reporter in zones 2 and 3 (elements A, D and E, see Figure 12 K-O, P-T U-Y). Additionally, one sub-element is required for strong activation of *gfp* and *mw* in zone 1 (elements F, see Figure 12 U-Y). Interestingly, several other PRE / TREs tested have the ability to

mediate strong activation of *gfp* in zone 1 (the *vg* and *bxd* PRE / TREs. See Figure 11 M,W and P,S). However, the regulation in zones 2 and 3 cannot be substituted by these elements. This indicates that the activation of the upstream promoter in zone 1 is a task that can generally be performed by PRE / TREs (Figure 11). As this finding applies to the bxd PRE / TRE as well, it indicates that this property must be coded within the bxd PRE / TRE but could not be identified by monitoring the *Ubx* regulation (Sipos, et al., 2007). Accordingly, the potential of the bxd PRE / TRE to mediate activation has been reported previously (Rank, et al., 2002). The strong activation of *gfp* in zone 1 is likely to be specific to TrxG proteins as all tested PRE / TREs were able to mediate this activation. Furthermore, *trxG* mutant clones displayed a reduction in *eya* levels within zone 1 (Figure 2) (Janody, et al., 2004), indicating that the strong activation in this zone may indeed depend on TrxG proteins.

4. General model of PRE / TRE function during eye development of crawling larvae

The results presented in this thesis suggest a model of *eya* PRE / TRE function where the PRE / TRE interacts with the enhancer to individually regulate the expression of the first and second promoter. Thereby, the PRE / TRE interactions with the enhancer are distinct at different stages of differentiation (zones 1, 2 and 3 in Figure 16 A,B,C,D,E). The adult eye color of REGFPΔen flies reflects that the repressive function of the PRE / TRE ceases with an increasingly anterior position as otherwise *white* specific factors would not be able to act on the *mw* promoter (Figure 6 N and Figure 7G). For this reason I present a model of *eya* PRE / TRE function based on the findings encountered with crawling larvae (Figure 16 A-E). In absence of the PRE / TRE, the eye specific enhancer interacts with both promoters resulting in profiles of both reporters that are similar to each other in all three zones. Thereby, the expression in zone 1 is higher compared to zones 2 and 3 (Figure 16 B,C and Figure 8 S). In presence of the PRE / TRE both reporters

are regulated differently in zone 1 where the PRE / TRE acts as a strong activator of the first promoter while the second promoter displays low level activity. In zones 2 and 3, the PRE / TRE is responsible for low levels of expression from the first promoter but also from the second promoter as reflected by the lighter eye color of REGFP transgenes and the corresponding *mw* profile in zone 3 compared to REGFPΔPRE (Figure 16 D,E and Figure 8 N,O). Strikingly in zone 2 and 3, the profiles of the endogenous isoforms and the REGFP reporters reflect that the repression of the first promoter is stronger than the repression of the second promoter in crawling larvae (Figure 8 D,E,N,O). In parallel to *eya* specific regulation, *white* specific regulatory factors may interact with the second reporter gene promoter in zones 2 and 3. However both *eya* regulatory sequence and the PRE / TRE itself have shown to have restrictive functions on these factors as discussed above (Figure 16 C,E).

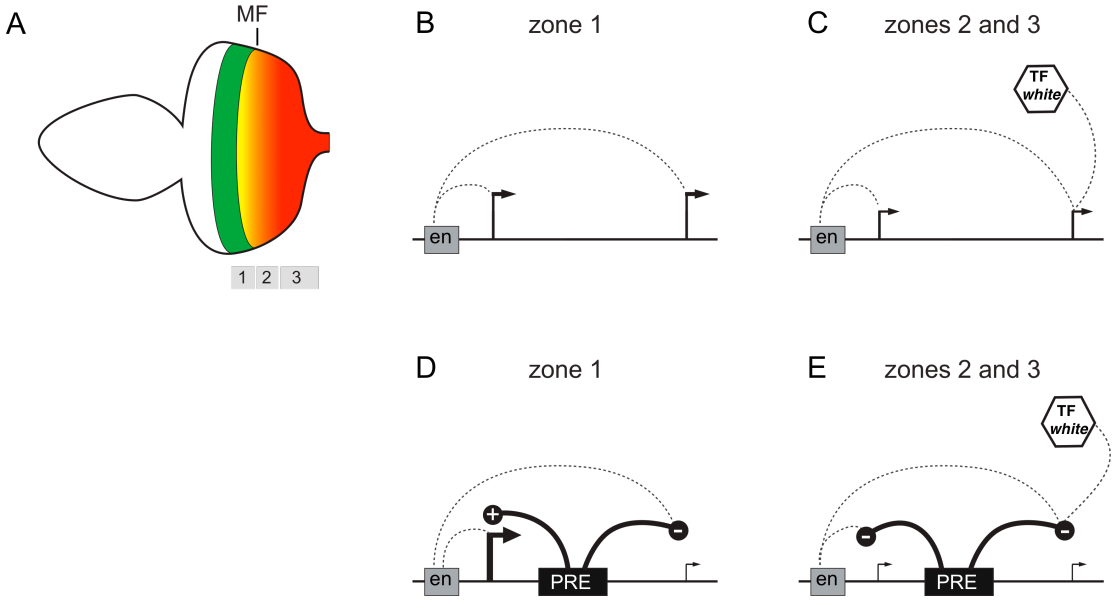


Figure 16: Model of *eya* PRE / TRE function at the stage of crawling larvae.
(**A**) Schematic representation of an eye antennal imaginal disc. Green, zone 1 – the preproneural zone. Yellow, zone 2 behind the morphogenetic furrow. Red, zone 3, retinal tissue with terminally specified retinula cells R1 – R8. (**B**) in absence of the PRE / TRE, the eye specific enhancer is able to communicate with both promoters in zone 1. The promoter activity is elevated compared to zones 2 and 3. (**C**) zones 2 and 3 in the absence of the enhancer. The enhancer is able to directly interact with the two reporter gene promoters. The expression levels are moderately lower compared to zone 1. Within zones 2 and 3 the *mw* promoter may be targeted by *white* specific regulatory factors. This effect is rather small as REGFP lines lacking the enhancer have only weak pigmentation. (**D**) In presence of the *eya* PRE /TRE the first and second promoter are regulated differently in zone 1. The first promoter is expressed at high levels while the second promoter is expressed at low levels. This is encountered in crawling larvae. In contrast, prepupae do not show the disproportional regulation of the two promoters suggesting that the repressive effect on the second promoter ceases with developmental progression. (**E**) zones 2 and 3. In presence of the PRE / TRE the expression from both promoters is very low (*gfp*) and low (*mw*) which is also reflected by the relatively light eye color of REGFP flies compared to REGFPΔPRE (See Figure 6).

5. The *eya* PRE / TRE: A combined PRE / TRE and optic lobe enhancer

eya expression in lamina cells of the optic lobe has been reported previously (Bonini, et al., 1998) and the location of the optic lobe specific enhancer has been identified in this study. Interestingly, the *eya* optic lobe enhancer is located within the *eya* PRE / TRE containing optic lobe specific regulatory information distributed over the 1500 bp defined as PRE / TRE (Figure 14). The dissection of the PRE / TRE (optic lobe enhancer) in the REGFPΔA-F lines also revealed that the optic lobe enhancer is separated into units that regulate the dorsal and ventral expression in the lamina (Figure 15 E,O,T). Interestingly, it was not possible to generate gaps in all regions of the horseshoe pattern. This indicates that the sequences defining the midline specific expression domain (middle of the horseshoe) and the counterpart of the E-element that defines one of the two extreme dorsal / ventral expression domains (Figure 12Figure 15 O, green domain at V') must be located either within the D sub-element that is indispensable for OL expression (Figure 12 J) or alternatively within the B and C sub-elements (no transgenes obtained) or outside the 1500 bp of the *eya* PRE / TRE. The replacement with the orthologous sequence from *D. pseudoobscura* showed that the optic lobe enhancer is conserved but it cannot distinguish between the two alternatives. As the *eya* PRE / TRE indeed contains sequence information sensitive to dorsal and ventral factors it will be interesting to determine whether these properties are being used in eye development by generating dorso-ventral profiles in eye discs of REGFP variants. However, the *mw* promoter should be composed of the second *eya* promoter to avoid any undesired regulatory effects.

6. Implications for endogenous *eya* regulation

6.1. What is the role of the two *eya* isoforms?

The comparison of the profiles obtained from crawling larvae and prepupae revealed a change in ratio of the endogenous isoforms in zone 1 compared to zone 2 (Figure 9). In addition, when considering the profiles of the reporter genes in REGFP crawling larvae and prepupae, the *mw* expression in zone 1 changed from levels lower compared to zone 2 in crawling larvae to levels higher compared to zone 2 in prepupae (compare Figure 8 O to Figure 12D). This indicates that both endogenous and REGFP regulation are subject to a successively modified regulation of both promoters in zone 1 where exclusively *eya* specific expression takes place without influence of *white* specific factors. These results may indicate different requirements for either *eya* isoform. The two *eya* isoforms differ from each other at their alternative N-terminal ends that are 19 bp (EYA 1.1) and 23 bp (EYA 1.2) long (Figure 3 and (Bonini, et al., 1993)). The residual 743 amino acids are identical. As both isoforms are able to induce ectopic eyes and rescue *eya* mutant phenotypes it has been assumed that the two isoforms are completely redundant (Bonini, et al., 1997, Bonini, et al., 1998, Leiserson, et al., 1998). However, the two *eya* isoforms are differentially expressed in a highly controlled manner where the ratio of both transcripts changes upon developmental progression in zone 1 (Figure 9). This suggests that the two isoforms are distinguishable from each other by subtle differences. As *eya* is involved in axon pathfinding (Xiong, et al., 2009), it could be that the differential regulation of the *eya* isoforms is involved in axon targeting into the optic lobe. It will be interesting to determine whether EYA 1.1 and EYA 1.2 proteins display a different preference for cytoplasmic or nuclear localization (Xiong, et al., 2009), whether they display distinct preferences for interacting with a preference for distinct sets of Gαi proteins (Embry, et al., 2004)and whether they play different roles in the cytoplasm, the nucleus or in both compartments. Different scenarios spring to mind:

Nuclear difference: The two EYA isoforms may target slightly different target genes for example by preferably binding distinct cofactors. One application of this scenario could be a changing ratio of the two isoforms during eye developmental progression. Here, the regulation of *eya* would require individual regulation of either isoform. Alternatively, the distribution of cofactors may be asymmetric throughout developmental progression making individual regulation of *eya* isoforms unnecessary.

Cytoplasmic difference: As *eya* is involved in axon targeting to the optic lobe one could imagine that the alternative N-termini may influence the interaction with different Gαi proteins (cell-cell communication) or may establish distinct preferences for cytoplasmic substrates (Embry, et al., 2004, Xiong, et al., 2009). A different preference to for G proteins could lead to an asymmetric retention of *eya* isoforms at the plasma membrane and a subsequent asymmetric reimport of *eya* isoforms to the nucleus or alternatively but not mutually exclusive, to an asymmetric dephosphorylation of cytoplasmic substrates. Here, *eya* regulation would not require individual isoform regulation as the nuclear function is redundant and could not trigger regulatory consequences. Nevertheless, gene regulatory effects could be achieved in an indirect way where the asymmetrically phosphorylated substrates signal into the nucleus over other pathways to manifest regulatory changes. Alternatively, axon pathfinding / targeting could be achieved without feedback to the nucleus by making use of asymmetrically dephosphorylated *eya* substrates as effectors of axon targeting.

Nuclear and cytoplasmic difference: This scenario is similar to the scenario “cytoplasmic difference” with the distinction that the asymmetric reimport of EYA itself has regulatory consequences. Accordingly, *eya* regulation would not require isoform specific regulation as an asymmetric distribution is achieved over retention at the plasma membrane. Nevertheless, it may be useful to individually regulate either isoform in order to amplify a signal or re-balance the *eya* isoforms after corrections in axon growth have been executed.

Another scenario is that the two isoforms exhibit different preferences for nuclear and cytosolic localization. Interestingly, the EYA isoform 1.2 has two predictions for nuclear localization while the N-terminus of EYA 1.1 possibly does not contain a NLS at its N-terminus ((Jemc and Rebay, 2007b) and not shown). Isoform specific regulation may thus have a influence on the relative concentrations obtained in the nucleus and cytoplasm. Nevertheless, this could also be achieved by regulating *abl* activity that induces export of EYA into the cytoplasm by its phosphorylation.

6.2. Higher total levels of *eya* expression in zone 2 compared to zone 1 may regulate complex formation

The transition from zone 1 to zone 2 comes along with the initiation of neurogenesis (reviewed in (Treisman and Heberlein, 1998). This transition is also accompanied with higher levels of either isoform in zone 2 compared to zone 1. These higher levels may be required for an adaption to changes in complex formation that happen along the transition from zone 1 to zone 2. For example, the DAC interactors DAN and DANR become downregulated in zone 2 (Curtiss, et al., 2007). Therefore it may be that formation of EYA-DAC complexes increases while the SO-EYA complexes require to be maintained at constant levels. Upregulation of EYA protein levels could satisfy the requirement for increased DAC interactions while keeping the SO-EYA interactions balanced.

7. Distinct PRE / TREs display unique characteristics

Based on their behaviour in transgenic reporter assays PRE / TREs have been proposed to be exchangeable elements because they display similar behaviour in transgenic assays where they act as maintenance elements or mediate pairing sensitive silencing (reviewed in (Ringrose and Paro, 2007). Indeed, PRE / TREs have repeatedly been demonstrated to be interchangeable elements (Kozma, et al., 2008, Pérez, et al., 2011). Indeed,

the exchanged PRE / TRE sequences all displayed the ability of *gfp* activation in zone 1 (Figure 11). However, the different PRE / TREs also display profoundly different properties in the REGFP context.

Remarkably, replacement of the *D. melanogaster eya* PRE sequence with the orthologous sequence from *D. pseudoobscura* resulted in a different readout of both promoters compared to REGFP: In zone 1 both promoters were not differentially regulated. Furthermore, activation of the first promoter was rather low. A similarity compared to the *D. melanogaster eya* PRE / TRE was the strong down regulation from zone 2 to 3 and the low levels in zone 3. (Figure 11). The *D. pseudoobscura* PRE / TRE failed to repress the second promoter in zone 1, giving high expression of *mw* in all 3 zones and a darker adult eye color than the *D. melanogaster* PRE / TRE (compare Figure 11 A,U to B,V). The explanation for this behaviour may be based on the sequence composition of the *D. pseudoobscura eya* PRE / TRE. PRE / TRE motifs have been shown to turn over rapidly in evolution (Hauenschild, et al., 2008). The *D. psudoobscura eya* PRE / TRE contains fewer PHO (6) sites and more GAGA sites (11) than the orthologous *D. melanogaster* sequence (8 PHO sites, 6 GAGA sites). Additionally, the *D. pseudoobscura* locus contains two predicted PRE / TREs, close to each endogenous promoter raising the possibility that enhancer and PRE / TREs have evolved to specifically interact with the corresponding promoter (B and C on Figure 10 G). Finally, the eye specific enhancer of *D. pseudoobscura* is flanked by two potential PRE / TREs with lower score (Figure 10 G, yellow bar). It will be interesting to learn more about the differences in regulation of *eya* in both species by generating construct variants including both the orthologous PRE / TREs and eye specific enhancer and dissect the functions.

The *vestigial* (*vg*) PRE / TRE was able to regulate the first and second promoter in a similar way compared to the *eya* PRE / TRE in zone 1. However, it was not able to downregulate *gfp* and *mw* expression to the same degree as the *eya* PRE / TRE in zones 2 and 3 which is also supported by the darker eye color of the REGFP-vg transgenes compared to REGFP (compare Figure 11 A,F,G,H,U to G,L,M,N,W). However, the *vg* PRE / TRE maintains some

silencing capacity because homozygous flies did not develop a fully red eye but instead a less pigmented brown eye. The *vg* PRE / TRE has been analyzed in different studies and was found to maintain transcription in the absence of initial transcription factors during L3 wing development. Interestingly, this function was not unique to the *vg* PRE / TRE as the same maintenance was achieved with the *Fab-7* PRE / TRE from the Bithorax complex (Pérez, et al., 2011). However, the expression pattern of the endogenous *vg* gene was only approximately obtained leaving space for individual adaptations of PRE / TRE elements to their corresponding genes. Indeed, fundamentally different properties of the *Fab-7* and *vg* PRE / TRE have been reported using reporter gene constructs with these elements at an identical genomic location (Okulski, et al., 2011). The results presented here showed that the *vg* PRE / TRE mediated only a moderate silencing capacity of *gfp* compared to the *eya* PRE / TRE in zones 2 and 3 but similar to it, it was able to mediate differential regulation of both reporters in zone 1 accompanied with high levels of *gfp* expression. This shows that some functions of the *eya* PRE / TRE are interchangeable but others are not thereby reflecting the alien nature of the vg PRE / TRE in the *eya* context.

The *bxd* PRE / TRE is well characterized and has provided many paradigms defining current models of PRE / TRE function (Simon, et al., 1993, Chan, et al., 1994). It is able to maintain embryonic expression patterns that are maintained over multiple cell generations (Chan, et al., 1994, Rank, et al., 2002). Given this characteristic, one might expect that the *bxd* PRE / TRE would maintain the expression patterns of both reporter genes. Surprisingly, the *bxd* PRE / TRE of both species, *D. melanogaster* and *D. pseudoobsura* resulted in a variegated expression pattern. Variegation was biased in such a way that the pigmentation was more absent at the anterior part of the eye possibly indicating that the *bxd* PRE / TRE has an opposed sensitivity to the anteroposterior polarization of the eye imaginal disc compared to the *eya* PRE / TRE making the activation of the transgenic locus become increasingly difficult with eye developmental progression. However, the cells that are able to activate the transgenic locus are able to express high *gfp* levels in zone 1

compared to zone 2 and 3 thereby displaying a property shared with the *vg* and *eya* PRE / TRE (Figure 11 D,O,P,Q and E,R,S,T). This result reflects the interchangeable character of PRE / TREs. Nevertheless, the individual properties of the bxd PRE / TRE are strikingly represented by its ability to mediate variegation thereby showing properties of this element that are likely adaptations to its endogenous gene. Furthermore, the very low signal intensity of gfp in zone 3 - characteristic for REGFP - could not be reached using the bxd PRE / TRE from both species (compare Figure 11 G to P,S). The significance of the gene specific properties of the *bxd* PRE / TRE remain to be investigated. However, regulation of HOX genes by PcG and trxG proteins has been shown to be established early in development and to not be switched indicating a possible reason for the different nature of this element compared to the *eya* and *vg* PRE / TREs in the REGFP context.

8. Conclusions

The results of this study presented new findings about endogenous *eya* regulation. During eye development the endogenous isoforms are differentially regulated indicating that the alternative protein isoforms are not entirely redundant as also indicated by the use of only isoform 1.2 during embryogenesis (Leiserson, et al., 1998).

eya has been reported to be expressed in lamina cells of the optic lobe. In this study, I have identified the optic lobe specific enhancer that is conserved between D. melanogaster and D. pseudoobscura. The optic lobe enhancer is composed of sub-enhancers defining dorso-ventral specificity (A, E and F sub-elements) in the lamina expression domain and of a sub-element (D) indispensable to drive optic lobe specific expression. The optic lobe enhancer is located within the *eya* PRE / TRE.

The eya PRE / TRE is able to mediate pairing sensitive silencing and to prevent the activation of the *mw* promoter even in presence of *white* specific factors. Additionally, the pairing sensitive silencing is sensitive to the *PcG* mutant ph^{410} reinforcing the finding that the *eya* PRE / TRE is responsive to

PcG regulation during eye development and not only an optic lobe specific enhancer. Interestingly, both PRE / TRE and optic lobe enhancer are functionally distributed along the entire 1500 bp region defined as PRE / TRE in this study. To my knowledge, this is the first characterization of such a compound regulatory element containing a PRE / TRE and an enhancer.

The results presented in this thesis make it likely that the differential expression of the endogenous *eya* isoforms is established by the PRE / TRE as the presented data showed that the PRE / TRE is required for this purpose in the REGFP background. Additionally, I have identified sub-elements of the *eya* PRE / TRE that are required for high level expression in the pre-proneural zone anterior to the furrow and sub-elements required to for low level expression behind the morphogenetic furrow.

Previous findings suggested that core PRE / TREs are interchangeable elements. While my data partly agrees with this conclusion I present evidence that the *eya* PRE / TRE is profoundly adapted to its endogenous environment and can functionally not be entirely interchanged with PRE / TREs from other genes. This indicates that core PRE / TREs can serve manifold functions.

9. Future perspectives

To learn more about the functional roles of differential *eya* regulation it will be required to study eye and optic lobe development and axon pathfinding in a system where the differential regulation of *eya* can be manipulated. One important question to address would be whether lamina development requires *eya* expression or whether lamina specific expression of *eya* is involved in targeting of retinal axons. To date, these questions could not be addressed as the characterized *eya* mutants compromise eye development and consequently lamina induction (Selleck and Steller, 1991, Selleck, et al., 1992, Winberg, et al., 1992, Leiserson, et al., 1994). Furthermore, it will be interesting to investigate the role of differential *eya* regulation during eye development. Both *eya* isoforms have been shown to rescue mutant phenotypes when expressed under control of a heatshock promoter – a

rather imprecise method of *eya* regulation both in time and space but also for the regulation of expression levels (Leiserson, et al., 1994). The adult rescued eyes displayed a rough eye phenotype suggesting that precise developmental timing of *eya* expression plays a crucial role for proper eye development. Alternatively, the expression of both isoforms may be required to prevent the rough eye phenotype.

Studying differential *eya* regulation in a system that expresses *eya* in the native spatiotemporal context may help identifying the role of differential *eya* expression in the formation of the highly symmetric compound eye or alternatively, axon targeting into the optic lobe. In order to manipulate the differential regulation of the *eya* isoforms two different approaches appear suitable:

Genetic rescue in mutant background

This approach is complementary to the REGFP reporter constructs as it would involve the generation of construct variants modifying or abolishing the PRE / TRE and the first alternative exons including the promoters. As the *eya* PRE / TRE is likely to affect the differential expression of the isoforms the experiment is designed to study both the role of the PRE / TRE and the function of differential *eya* isoform regulation both in eye development, axon pathfinding and lamina expression. One construct variant could for example consist of the same promoter including the corresponding first exon at both promoter positions. This would address whether both isoforms are redundant or required for proper eye development or axon pathfinding. In order to avoid sources of undesired effects, the entire 20 kb of the *eya* sequence should be included in these rescue variants. Complementation with the endogenous mutant locus due to transvection may influence gene regulation at the transgenic locus thereby diminishing the effect of PRE / TRE removal / modification in rescue construct variants. As reported by Leiserson and colleagues the *eya* locus performs transvection (Leiserson, et al., 1994).

Gene targeting to the endogenous *eya* locus

Alternatively, it could be considered to design a targeting construct for the endogenous locus. For example, the entire *eya* region could be cloned and modified in such a way that the PRE / TRE is flanked by FRT sites that can be removed upon GAL4 mediated FLP activation. More complex construct variants enabling site directed integration to insert PRE / TRE variants are desireable. Using such an approach would enable to generate *eya* variants lacking or containing modified versions of the PRE / TRE at the endogenous locus and allow to study the functional role of differential *eya* expression in the eye and to investigate the role of lamina specific expression without impairing the induction of the lamina by absent or reduced eye tissue.

V. MATERIALS AND METHODS

1. Experimental strategy and cloning

1.1. Cloning strategy for the REGFP reporter construct

The REGFP reporter construct was generated as follows: The *eya* sequences A (primers #12 and #13, 5227 bp) and B (primers #14 and #15, 761 bp, Figure 17 and Table 3) were amplified from genomic DNA using Phusion® polymerase (NEB Cat. No. F-530) and subsequently cloned into the pCR®-BLUNT II-TOPO vector (Invitrogen, Cat. No. K2800) and sequenced. The fusion of sequence B and turboGFP (Evrogen Cat. No. FP512) was achieved by the use of the bridging primers #18 and #20 that are complementary to each other and consist of 15 bp *eya* sequence and 15 bp TurboGFP sequence (Horton, et al., 1989). The *eya* sequence B was amplified from a verified clone using the forward primer #14 and the reverse bridge Primer #20. Additionally, the TurboGFP sequence was amplified using the forward bridge Primer #18 and the reverse primer #19. These two PCR products with an overlapping sequence of 30 bp were joined as template for a PCR reaction and amplified with the primers #14 and #19 to generate the *eya*-TurboGFP fusion sequence BC which was cloned into the pCR®-BLUNT II-TOPO vector and sequenced. To generate the complete REGFP construct consisting of the sequences A and BC the 5208bp HindIII – NotI fragment containing sequence A was combined with the 1737 bp NotI – SpeI fragment containing sequence BC and ligated into the pCR® II vector subjected to restriction digest with HindIII and SpeI. After sequencing the 6880 bp EcoRI fragment was ligated into the EcoRI digested pKC27_*mw*_nohs vector resulting in the final REGFP construct pKC27_REGFP_*mw*_nohs. Complete sequencing was performed with the primers listed in Table 4).

	Primer sequence	Purpose
#12	GCGCACTTAAGTAGCTTAAACAGAC	A fw
#13	ATTTAGACCAGGAGACAACAATGAG	A rev
#14	TGTTTTGAGGGACTTCTTTAGGG	B fw
#15	ATGTGTATCCGTGTGGTCTGTCT	B rev
#18	TTTCAGGTTAAACGTGAGAGCGACGAGAGC	bridging; TurboGFP fw
#19	ATGAGTTTGGACAAACCACAAC	TurboGFP rev
#20	GCTCTCGTCGCTCTCACGTTTAACCTGAAA	bridging; B rev

Table 3: Primers used for the generation of the REGFP construct.

The abbreviations A and B refer to the *eya* sequences amplified by the Primers and illustrated in Figure 17. Forward (fw) and reverse (rev) orientation of the primers refer to the sense strand of the *eya* or TurboGFP gene. The bridging primers #18 and #20 are complementary to each other and consist of *eya* sequence (underlined) and TurboGFP sequence.

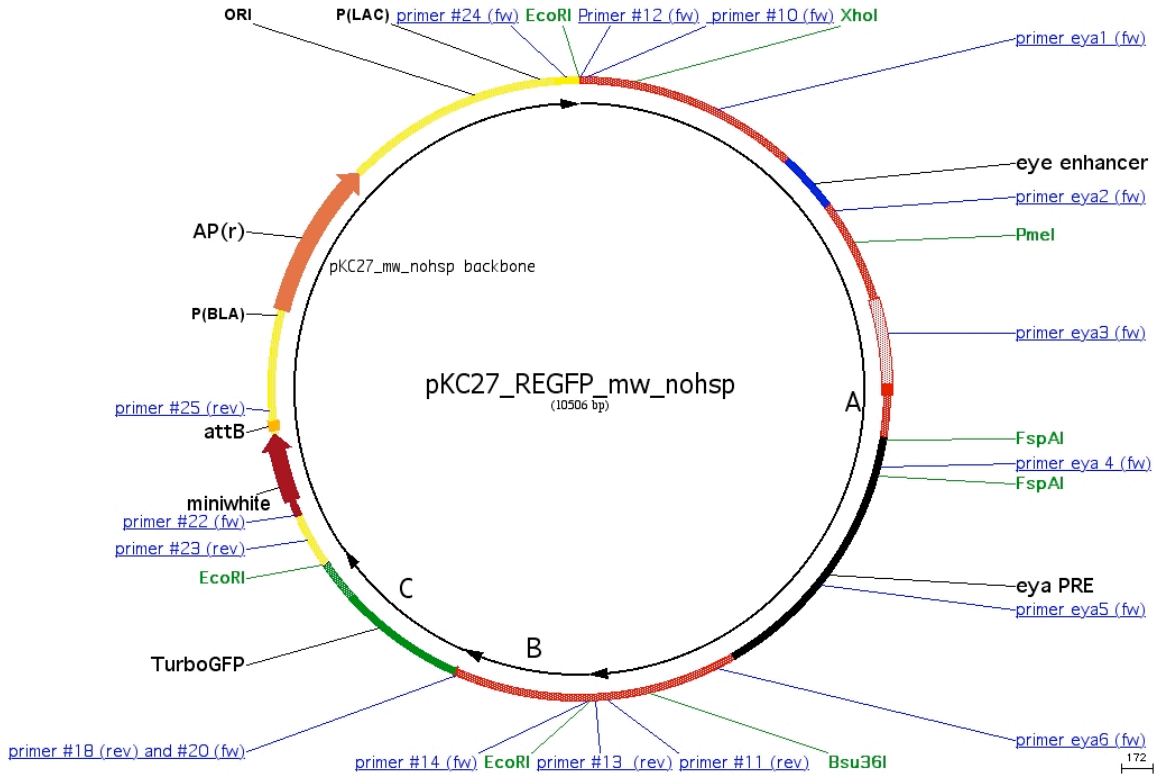


Figure 17: Schematic representation of the finalized REGFP reporter construct.

Primers are depicted in blue with an indication of the amplification direction (forward (fw) clockwise and reverse (rev) counter clockwise, See Tables 3.1.1A and 3.1.1B).

The EcoRI sites used in the final ligation step are indicated in green font. Black arrows indicate the PCR products A, B, and C. Characteristic sequence attributes such as protein coding sequences (Ap(r), *miniwhite* and TurboGFP) or characteristic regulatory DNA sequences (eye enhancer and *eya* PRE) are indicated in black font, restriction sites used during the cloning of REGFP or REGFP variants are indicated in green.

Primer Name	Primer Sequence
Eya1	AATTAGGAAAACGCCCCCTTCT
Eya2	AGTTTATTAATCAATTTTGATA
Eya3	TTGTGTCGCGCGCATCGTGATA
Eya4	AATGCAGGACACAATGCAGACA
Eya5	AAATGCCGTGAGGATAATTTCA
Eya6	GCTCGAATTGGAAACTCTTCTT
#11	GGCCACAGCAAGGTACAAAT
#14	TGTTTTGAGGGACTTCTTTAGGG
#18	TTTCAGGTTAAACGTGAGAGCGACGAGAGC
#22	GTCAATGTCCGCCTTCAGTT
#23	TGAGAGGTAATCGAAAGAACCTG
#24	GCAACTACTGAAATCTGCCAAG
#25	CAAGGCGATTAAGTTGGGTAAC

Table 4: Sequencing Primers for the REGFP construct

1.2. REGFP reporter variants

Three groups of REGFP reporter gene variants were generated using different approaches. The first group of variants REGFPΔen, REGFPΔPRE and REGFPΔenΔPRE were constructed using a strategy involving bridge primers according to Horton et al., 1989 (Horton, et al., 1989) as follows (Figure 6 D-F): sequences flanking the enhancer were fused to design the two complementary bridge primers reg1LEFT_rev and reg1_RIGHT_fw. These primers were used in two independent PCR reactions with the basic REGFP plasmid as template. The PCR reaction amplifying the left fragment was performed using the primer reg1LEFT_fw and the bridge primer reg1_LEFT_rev; the PCR reaction amplifying the right fragment was

performed using the bridge primer reg1_RIGHT_fw and the primer reg1_RIGHT_rev. The two PCR products with overlapping sequence were joined as template in a subsequent PCR reaction using the primers reg1_LEFT_fw and reg1_right_rev generating an *eya* sequence excluding 395 bp of the sequence containing the eye specific enhancer. After cloning into pCR® II the 1118 bp XhoI / PmeI restriction fragment was used to replace the 1513 bp XhoI / PmeI fragment of the basic REGFP Reporter resulting in the REGFPΔen construct. The same 1118 bp XhoI / PmeI restriction fragment was used to replace the XhoI / PmeI fragment of the REGFPΔPRE reporter resulting in the REGFPΔenΔPRE fragment.

Primer Name	Primer sequence	Purpose
reg1LEFT fw	GAGCACGTGTGTGTGCTTCT	product I
Reg1LEFT rev	AACACTTTAAGGATAAAATCGCATACGGCCAGTTTCGTCTCC	bridge, I
Reg1RIGHT fw	GGAGACGAAACTGGCCGTATGCGATTTTATCCTTAAAGTGTT	bridge, II
reg1RIGHT rev	GGGGAAACACAGGCACATAA	Product II
PRE_left_fw	ACCATTCACACCACCAAAAA	Product I
PRE_left_rev	GAGTAAACAAACAAACAAGTTCATTTGAGCACCAGGAGTCAGGTTTG	bridge, I
PRE_right_fw	CAAACCTGACTCCTGGTGCTCAAATGAACTTGTTTGTTTGTTTACTC	bridge, II
PRE_right_rev	CAAAAAGCAGGTCCTTCGAG	Product II

Table 5: Primers used for the generation of REGFP variants lacking the enhancer, the PRE / TRE or both elements.

Underlined sequences of bridge primers belong to the 3' end, sequences in normal font belong to the 5' end of the deletions.

The same strategy was applied to the generation of the REGFPΔPRE construct using the primers and bridge primers listed in Table 5. Here, the 1559 bp PmeI / Bsu36I restriction fragment was used to replace the 3064 bp PmeI / Bsu36I of the basic REGFP construct.

The second group of REGFP variants represent the replacement constructs REGFP_Dpse_eya_PRE, REGFP_Dmel_vg_PRE, REGFP_Dmel_bxd_PRE and REGFP_Dpse_bxd_PRE where the *eya* PRE / TRE sequence was replaced by other PRE / TRE sequences (Figure 10 B-E). This was achieved by placing restriction sites at the 5' and 3' flanks of the *eya* PRE / TRE using a synthesized sequence (Mr. Gene, http://mrgene.com) referred to as "PREvariants". The PREvariants sequence is based on the 1982 bp FspAI / Bsu36I fragment of the *eya* gene with following modifications: The sequence 5'-GCTAGC-3' was inserted directly adjacent to the 3' end of the FspAI site resulting in the recognition sites of NheI and AvrII and defining the 5' edge of the PRE / TRE insertion site. The 40 adjacent bp were retained while the additional 1434 bases towards the 3' end of the PRE / TRE were replaced by the short 5'-TACCGAGCTC-3' sequence importing the KpnI and SacI sites and defining the 3' edge of the PRE / TRE insertion site. The 495 bp sequence until the Bsu36I site mentioned above remained unchanged resulting in a truncated FspAI / Bsu36I fragment of 569 bp of length that contains two restriction sites on each edge of the *eya* PRE / TRE. This fragment was used to replace the 1982 bp FspAI / Bsu36I fragment in the basic REGFP construct. The four different PRE / TRE sequences to be inserted were amplified using forward primers with the overhang sequence 5'-ATGCTAGCCTAGG containing the NheI and AvrII sites and reverse primers with the overhang sequence 5'-TTGAGCTCGGTACCAT containing the KpnI and SacI sites (see Table 6 and 7).

Primer Name	Primer Sequence
Dpse_eya_PRE_fw	atgctagcctaggCCAGATCATATTTTCCGCATC
Dpse_eya_PRE_rev	ttgagctcggtaccatCTGGAGCATATTGGACACCA
vestigial_1.6_kb_fw	atgctagcctaggAAGTCTCCGCCCAATAATG
vestigial_1.6_kb_rev	ttgagctcggtaccatGAGCATATAGAAGTGGTCGAATATT
Dmel_bxd_PRE_fw	atgctagcctaggGCTTGTCGAATTCAAAAAGAATTA
Dmel_bxd_PRE_rev	ttgagctcggtaccatCTCTCTTTCGTTTTCCGCTTCT
D. pse_bxd_PRE_fw	atgctagcctaggGCCATTCGTATTCCACAAGAATTA
D. pse_bxd_PRE_rev	ttgagctcggtaccatGGAAAACTGGTTCGATTTTCTG

Table 6: Primers to amplify PRE / TREs for the REGFP replacement constructs. The sequence 5'-atgctagcctagg written in small letters contains the NheI and AvrII sites. The sequence 5'-ttgagctcggtaccat contains the motifs KpnI and SacI. Sequence in capital letters is specific for the PRE / TRE to be amplified.

The PRE / TRE sequences were amplified using Phusion® polymerase and cloned into pCR® II. NheI / KpnI fragments for each of the four different replacement PRE / TREs were used to replace the NheI / KpnI fragment of the REGFP_PREvariants plasmid leading to the final REGFP variants REGFP_Dpse_eya_PRE, REGFP_Dmel_vg_PRE, REGFP_Dmel_bxd_PRE and REGFP_Dpse_bxd_PRE. All final plasmids were checked for the correct sequence prior to injection for the generation of transgenic flies.

PRE sequence	Genomic coordinates	Genome release
D. pse. *eya* PRE	4_group1: 1613650..1611853	*D. pseudoobscura* (R2.14)
D. mel. *vg* PRE	2R: 8792050..8793632	*D. melanogaster* (R5.31)
D. mel. *bxd* PRE	3R: 12590916..12589364	*D. melanogaster* (R5.31)
D. pse. *bxd* PRE	2:17571442..17568814	*D. pseudoobscura* (R2.14)

Table 7: Genomic locations of the sequences used to replace the *eya* PRE / TRE in the REGFP construct.

The third group of six REGFP variants displaying deletions within the *eya* PRE / TRE sequence were designed *in silico* and synthesized (Mr. Gene™,

http://mrgene.com) containing the above mentioned flanking sequences carrying NheI / AvrII and KpnI / SacI sites. NheI / KpnI fragments of each of the six variants were used to replace the NheI / KpnI fragment of the REGFP_PREvariants plasmid leading to the final REGFP_eyaPREdissection A, B, C, D, E, and F constructs (see Figure 12 AE).

2. Generation of transgenic flies using the ΦC31 system for site directed integration

2.1. The ΦC31 system

The generation of transgenic flies was performed using the ΦC31 system for site directed integration into the *Drosophila* genome. It is based on the presence of a 'landing site' in the genome containing a single recognition site (attP) for the phage ΦC31 integrase enzyme. The integrase is provided on a helper plasmid, which is co-injected with the donor plasmid that contains a second recognition site (attB) (Groth, et al., 2004, Ringrose, 2009).

2.2. Generation of transgenic flies using site-specific integration

All REGFP and REGFP reporter variants were generated by microinjection into embryos carrying the landing site line 16A (Sheetal Bhalerao, IMP / IMBA unpublished) located at the genomic position 46E1 (2R) at genomic location 5,965,083 according to flybase version FB2010_05, with the genotype

43.16a: *yw*; *P*{43.16a},y^+/ *P*{43.16a},y^+; +/+

Microinjection was performed as described in (Ringrose, 2009) using a mixture of 600 ng / µl donor plasmid (REGFP, Figure 17 or REGFP variants) and 250 ng / µl helper plasmid pKC40 expressing the ΦC31 integrase in nuclease free water. Injections were performed by the IMBA Fly House service.

2.3. Verification and maintenance of transgenic flies

The survivors of injection (Parental generation P) were crossed to *yw* ; *Pin* / *CyO* ; + / + and the F1 progeny were screened for red eye pigmentation given by the transformation marker *miniwhite* (see Figure 17) as verification for a successful integration of the reporter construct. To obtain a homozygous stock transgenic flies with the genotype *yw* ; REGFP / *CyO* ; +/+ were crossed to *yw* ; *Pin* / *CyO* ; +/+. F2 siblings with the genotype *yw* ; REGFP / *CyO* ; +/+ were mated producing the F3 progeny with *yw* ; REGFP / REGFP; +/+ . These flies were used as foundation for a homozygous stock.

2.4. Fly strains, genetics and handling

Balancer lines:

yw / *yw* ; *Pin* / *CyO* ;

yw / *yw* ; *sp* / *CyO* ;

Landing site line:

yw / *yw*; *P*{43.16a} , y^{+} / *P*{43.16a} , y^{+} ; +/+

REGFP Basic Construct

yw / *yw*; *P*{43.16a-REGFP, w^{+mw}} , y^{+} / *P*{43.16a-43.16a-REGFP, w^{+mw} } , y^{+} ; +/+

Polycomb Group mutations

In(1)ph^{410} ,$ph\text{-}p^{410}$ w^{1}
Pc^{XL5}/TM3, Sb^{1}, Ser^{1}

Trithorax Group mutations

w*; ash1^{10} P{FRT(w^{hs})}2A/TM6C, Sb^{1} Tb^{1})

w*; ash1^{22} P{FRT(w^{hs})}2A/TM6C, Sb^{1} Tb^{1})

2.5. Fly Food and Handling

Fly food was supplied by the IMP / IMBA services with following composition: 7.5g Agar, 80g Corn meal, 18g dried yeast, 80g Malzym, 0.5ml O-phosphoric acid, 8.4ml Propionic acid, 10g Soya meal, 22g Sugar beets syrup, added to 1L ddH_2O. The flies were kept at 25°C or 18°C with a daily cycle of 12 h light and 12 h darkness.

3. Characterization of the adult eye color

3.1. Imaging

The adult eye color was imaged using flies that were aged for different periods of time depending on the purpose of demonstration: For comparison of adult eye colors between REGFP and REGFP variants both hetero- and homozygous flies were aged for five days before imaging. For the demonstration of pigmentation patterning in the REGFP variants REGFPΔen, REGFPΔenΔPRE, REGFP-Dmel-bxd and REGFP-Dpse-bxd homozygous flies were aged for 10 – 15 days before imaging. For the comparison of the REGFP variants in a *ph^{410}* mutant background and the *ph* wildtype situation flies were separated in two groups: the first group with pigmentation all over the eye consisting of REGFP, REGFPΔPRE, REGFP-Dpse-*eya* and REGFP-Dmel-vg were aged for 5 days prior to imaging. The second group of flies displaying a pigmentation gradient or variegation consisting of REGFPΔen, REGFPΔenΔPRE, REGFP-Dmel-bxd and REGFP-Dpse-bxd were aged for 8 - 10 days prior to imaging. Flies were imaged on a Lumar.V12 stereomicroscope (Zeiss) with the NeoLumar S 0.8 x Forward 80 mm objective using 75-fold magnification. The images were captured with a Spot insight™ 18.2 digital camera using the corresponding imaging software (Spot).

3.2. *ph*^{410} mutant crosses

In order to analyze the adult eye pigmentation in a *ph*^{410} mutant background homozygous transgenic males were crossed to *ph*^{410} / *ph*^{410} mutant females (cross A, Figure 18). At the same time males from the 2nd chromosome balancer line *Sp* / *CyO* were crossed to *ph*^{410} mutant females (cross B, Figure 18). The entire male progeny of both crosses A or B carries the X-linked *ph*^{410} mutation. Males from cross A carrying the transgene on the 2nd chromosome (red eye pigmentation) were back-crossed to *ph*^{410} mutant females (cross C) generating progeny of a pure population in respect to the *ph*^{410} mutation and a mixed population in respect to the transgene on the 2nd chromosome. Female progeny of cross C with red eye pigmentation (*ph*^{410} / *ph*^{410} ; transgene / wt) was crossed with male progeny from cross B carrying the *CyO* 2nd chromosome balancer (cross D). Male and female progeny from cross D carrying the transgene and the *CyO* balancer (*ph*^{410} / *ph*^{410} ; transgene / *CyO*) were crossed (cross E). Progeny of cross E hetero- and homozygous for the transgene were compared to the corresponding transgenes from a *ph* wildtype background as described above by imaging.

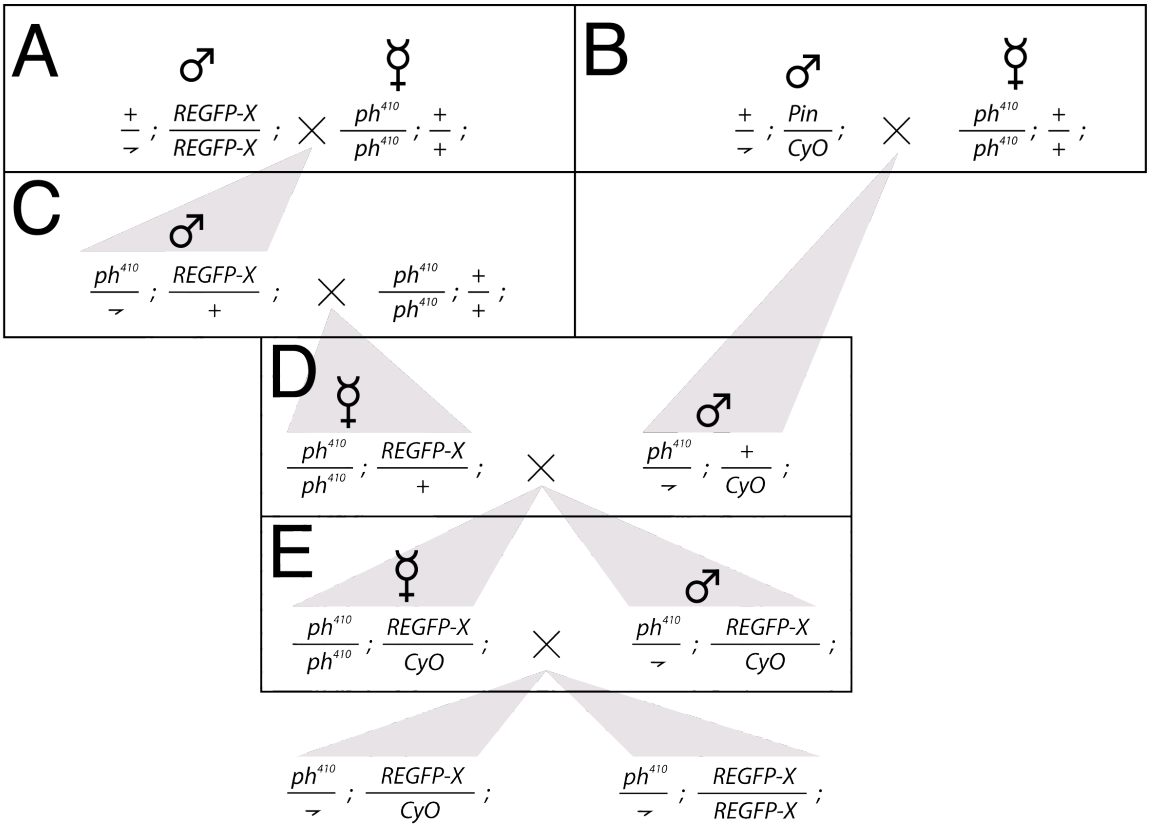


Figure 18: Crossing scheme for the analysis of pigmentation in the adult fly in a *ph*^{410} mutant background.

(A-E) refers to sequential fly generations. REGFP-X stands for any REGFP variant crossed to the *ph*^{410} background. Grey areas with sex symbols indicate the genotype of the progeny that was used for furter crosses.

4. RNA *in situ* hybridization

4.1. Generation of labeled RNA probes

PCR products of the respective probes (see table 3.5.1 below) were cloned into the pCR®II vector (Invitrogen, TA Cloning® Kit dual Promoter, Cat. No. K2027), transformed into competent DH5 alpha cells and plated on Ampicillin Plates (IMP / IMBA Service Department, 1% Agar, LB medium, Ampicillin 50 µg/ml). Four clones were further analyzed and amplified in Ampicillin selective LB medium for 12-16 hours. After plasmid purification (QIAGEN Miniprep Kit) the orientation and correct sequence of the inserts was determined by

sequencing (IMP / IMBA Service Department, in house) using M13 forward and reverse primers that flank the insertion site and are part of the TA cloning vector. For the generation of individual probes one clone was chosen as template for PCR and amplified using the M13 forward and reverse primers. Each PCR product originating from these primers contains the T7 promoter sequence that is included in the TA-cloning vector. The purified PCR product (QUIAGEN PCR Purification kit) was used as template for in vitro transcription (T7 Polymerase, Roche) using the DIG RNA labeling kit or Fluorescein RNA labeling Kit (both from Roche) for the generation of labeled RNA probes. The successful transcription by T7 polymerase was monitored by a test gel where 25 ng of template DNA were compared to an *in vitro* transcription mix made from 25 ng.. Successful transcription by T7 Polymerase appears approximately 10 times stronger on the gel. The in vitro Transcription mix was ethanol precipitated resuspended in Resuspension Buffer (50% Formamid, 0.1% Tween 20, 20 µg/ml heparin, 5xSSC pH 4.5) and stored at -20°C.

oligo system ID	SEQUENCE	Detection
eya_exon_1_fw_probe	GAAAGCGGACCACCATAAAC	exon 1.1
eya_exon_1_rev_probe	AGTTTTGATAGCACGGCACA	
eya_alt_exon_1_fw	CCCGAAGTCGCAGATAAAAA	exon 1.2
eya_alt_exon_1_rev	TTGGCTCGATCATTTTGTCA	
GFP_PROBE_F	GCCATGGAGATCGAGTGC	gfp
GFP_PROBE_R	GGTGTTGCTGTGATCCTCCT	
mw_exon2_for	CTGCATTAACCAGGGCTTC	miniwhite

Table 8: Primers used for the generation of RNA probes.

The PCR products originating from these primers were cloned into pCR®II.

4.2. Fixation of Larval Tissue

Larvae were dissected in PEMS (0.1 M Pipes, 2mM $MgSO_4$, 1mM EDTA, pH 6.9). Using a scalpel the larvae were cut in two parts. The anterior part was inverted using forcipes thereby making the imaginal discs and the brain fully accessible to the outer environment. The tissue was transferred into a 2 ml

Eppendorf tube and fixated in 1.7 ml PEMS and 300 µl Formaldehyde (37 %) for 30 minutes on a turning wheel. After washing twice with Methanol the tissue was stored until further use at -20°C.

4.3. Double *in situ* hybridization and detection

Double in situ hybridization on larval tissue was carried out based on the "Fluorescent in situ hybridization using TSA" protocol from Invitrogen (http://probes.invitrogen.com/media/pis/mp20912.pdf). The detection of Fluorescein labeled probes was performed using the primary Anti-Fluorescein monoclonal antibody from mouse (Roche Cat. No.11426320) with a working concentration 1:500 and the secondary antibody HRP-goat-anti-mouse IgG antibody (Invitrogen Cat. No. T-20912; TSA™ Kit #2) with a working concentration of 1:100. According to the suppliers instructions the visualization was performed using Alexa Fluor 488 Tyramide according to the manufacturers instructions (Invitrogen Cat. No. T-20912; TSA™ Kit #2). The detection of Digoxigenin labeled probes was performed using Anti-Digoxigenin-AP Fab fragments from sheep (Roche Cat. No. 11093274910) with a working concentration of 1:1500. The staining reaction for Digoxigenin labeled probes deviates from the Invitrogen protocol as follows: the larval tissue was transferred into FR-Buffer (0,1 M Tris-HCl, ph 8,2) for the staining reaction with the Alkaline Phosphatase substrate Sigmafast™ Fast Red TR / Naphtol AS-MX Tablets (Sigma Prod. No. F4523) at 4°C. The staining progress was determined by periodical inspection of larval tissue using a Leica MS5 stereomicroscope and stopped by the addition of PBT at the point at which optimal staining was obtained. The staining reaction for the FITC labeled probes was performed according to the invitrogen protocol in amplification buffer. The tissues were mounted with Vectashield® with DAPI (Vector Laboratories Cat. No. H-1200) on microscope slides.

4.4. Fluorescent Confocal Microscopy

Imaging was performed with the confocal microscope LSM 700/Axioimager (Zeiss) using the LD LC I Plan Apochromat 25x / 0.8 LD LCI and Plan-Apochromat 63x/1.4 oil DIC objectives with oil immersion. TurboGFP was

excited with a 488 nm solid-state laser and signal was collected using the 555 nm shortpass filter. DAPI and Fastred were excited simultaneously using the 405 nm and 555 nm solid-state lasers. DAPI signal was collected using the 555 nm shortpass filter and Fastred signal was collected using the 560 nm long pass filter. The beam splitter was set to 555 nm. Laser power and detector gain were set to appropriate levels. Z-stacks were performed with a pinhole aperture of one airy unit and optimal Z-interval settings using the Z-Stack function of the Zen software (Zeiss).

4.5. Generation of Signal Intensity Profiles

Signal intensity profiles were generated by evaluation of maximum intensity projections using the line scan function of the imaging software MetaMorph (Version 7.1.1.0). The line scans were placed in anteroposterior orientation at 90° to the morphogenetic furrow and covered a the full range of the *eya* / TurboGFP / *mw* eye specific expression domain. Average Y-values of each channel (Red/Green/Blue) were obtained by applying a scan width of 50 pixels. Several line scans from individual eye discs of one experiment were aligned with respect to the position of the morphogenetic furrow on the X-axis. The Y-values of several discs were averaged and plotted on a line chart resulting in the average profiles presented in Figure 8, Figure 9 Figure 11, Figure 12).

4.6. Generation of Horseshoe Diagrams

The horseshoe diagrams seen in Figure 15 E, J, O, T were generated by examining several Z-Stacks of the corresponding transgenic lines REGFPΔA-ΔF. The visible expression domains were documented by marking a blank horseshoe. Subsequently, multiple diagrams of each transgenic line were combined into a common horseshoe that represents the maximal expansion of the expression domains. The expression patterns were highly reproducible.

5. Real Time PCR

5.1. Templates for qPCR: Fly strains and tissue preparation

For qPCR analyses following fly strains were used:

Wildtype:

ash1^{10}: *w**; *ash1*^{10} P{FRT(*w*^{hs})}2A / *TM6C*, *Sb*^{1} *Tb*^{1})

ash1^{22}: *w**; *ash1*^{22} P{FRT(*w*^{hs})}2A / TM6C, *Sb*^{1} *Tb*^{1})

ph^{410}: In(1)*ph*^{410} ,*ph-p*^{410} *w*^{1}

Pc^{XL5}: *Pc*^{XL5}*/TM3, Sb*^{1}*, Ser*^{1}

Dissection of eye-antennal imaginal discs from third instar larvae

Eye antennal imaginal discs from third instar larvae were dissected in PBS and transferred into a 1.5 ml eppendorf tube filled with 1 ml PBS + 5 µl RNAse inhibitor (Fermentas RiboLock™ RNase inhibitor Cat. No. EO0382) chilled on ice. After a collection time of 45 minutes the eye disc tissue was spun down, frozen in liquid nitrogen and stored until further use.

RNA isolation

For RNA preparation approximately 200 eyediscs were pooled and transferred into Lysis / Binding Buffer from High Pure RNA isolation kit (Roche, Cat. No. 11828665). The tissue was homogenized with a pestle and the RNA was subsequently extracted according to the High Pure RNA Isolation Kit protocol. In order to remove traces of DNA the RNA preparation was treated with Turbo DNase (Ambion Cat. No. AM2238) according to the suppliers instructions. RNA concentration was determined by absorption at 260/280 using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific).

cDNA Synthesis

cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen, Cat. No. 18064-022). For the reaction mix 1µg of template RNA, Random Hexamer- and Oligo (dT) primers (both at a final concentration of 2,5 µM) were combined and used for reverse transcription following the suppliers' guidelines.

5.2. qPCR Components, Equipment and Settings

qPCR analyses were performed using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma Cat No. S4438) and Realplex Mastercycler (Eppendorf) with the following program:
3 min at 95°C, 40 cycles of (95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec), 15 sec 95°C and a final temperature gradient over 20 min from 60°C to 95°C for the generation of a melting curve to analyze non-specific primer products. Primers for the qPCR analyses are listed in table 9 below.

Primer Name	Primer sequence	Product and orientation
qPCR27	CTTGTGTGTGTGGTGCATTTC	eya 1.1 forward
qPCR28	CATGTGTATCCGTGTGGTCTG	eya 1.1 reverse
qPCR5	AAACGGTCGAGTTCACTACCC	eya 1.2 forward
qPCR6	CATGTGTATCCGTGTGGTCTG	eya 1.2 reverse
qPCR25	CGGTCACTACTCGAACGGTAA	eya exon 3 fw
qPCR26	GTGCGACATCACAGTTGCTC	eya exon 3 rev
TBP_forw2	CATCGTGTCCACGGTTAATCT	TBP forward
TBP_rev2	GAAACCGAGCTTTTGGATGAT	TBP reverse

Table 9: qPCR primers for expression analysis in wt and mutant tissue.
Primers to specifically amplify the two eya isoforms 1.1 and 1.2 and the third exon which is common to both isoforms. These primers were used on cDNA extracted from wildtype, $Ash1^{10}$, $Ash1^{22}$, ph^{410} and Pc^{XL5} mutant tissue. TBP primers were used as reference.

5.3. Quantitation of Results

In order to calculate transcript abundance, template cDNA was subjected to qPCR in a three step 5-fold dilution series using the primers in Table 9. The *eya* 1.1 and *eya* 1.2 transcripts were quantified relative to the housekeeping gene TBP.

The primer efficiency [Ep] was calculated as

Ep = 5^(1/n)

Where **5** is the constant for the dilution factor and **n** is the slope function in an X / Y diagram where the individual Ct values of a dilution series (Y-value) were plotted against the sample number (X-axis).
Primer efficiencies were consulted as quality control for PCR reactions. Efficiencies lower than 1.7 were excluded from the evaluation of the data set.

The abundance of the sample as % TBP [A_{TBP}] was calculated as follows:

A_{TBP}= 2^-(Ct_{TBP} – Ct_{SAMPLE}) x 100
where the constant **2** is the theoretical primer efficiency, **Ct_{TBP}** and **Ct_{SAMPLE}** are the cycle threshold (Ct) values of the reference TBP primers and the sample primers in a PCR reaction with an identical concentration of template. The factor 100 converts the data to % of TBP. Enrichments calculated with this equation were averaged using the mean value of four individual dilution series coming from two biological and two technical replicates (12 values in total). In a few cases, a lower number of enrichment values for the calculation of the mean were used (minimum of 9 values), due to low primer efficiencies and exclusion from the dataset.

6. Live turboGFP imaging

Tissues of interest were dissected from third instar larvae in PBS. After 10 min of incubation in PBS with a 5 µM concentration of SYTO® 59 Red

Fluorescent Nucleic Acid Stain (invitrocen Cat. No. S11363) the tissues were mounted for microscopy and directly imaged on a confocal microscope LSM 700/Axioimager (Zeiss). Imaging was performed at room temperature with a EC plan Neofluor 10x / 0,3 NA objective using a solid-state laser at 488 nm for turboGFP excitation using a detection window from 500 – 540 nm. The SYTO® 59 molecules were excited with the 639 nm solid-state laser and signal was detected using a 560 nm longpass filter. The tissues were scanned using a pinhole aperture of one airy unit and applying the Z-Stack function of the Zen software (Zeiss) with optimal settings for the Z-interval size. The live turboGFP images shown in this thesis represent maximum intensity projections of Z-stacks.

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VIII. SUPPLEMENTARY MATERIAL

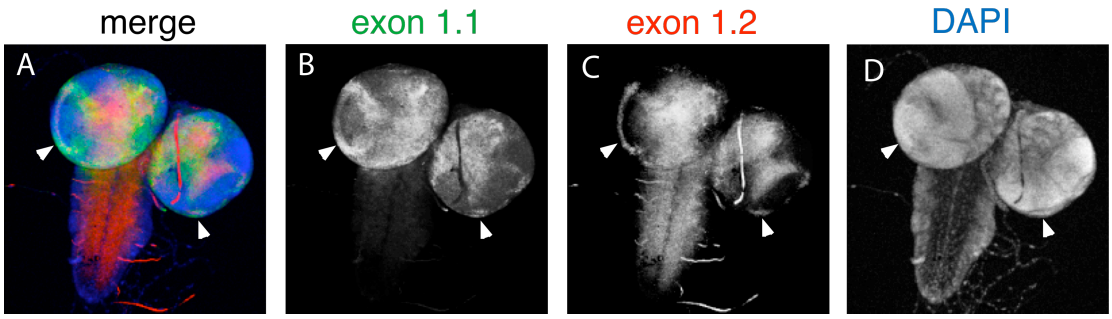

Figure 19: Optic lobe specific expression of the *eya* isoforms 1.1 and 1.2.

Both *eya* isoforms are expressed in the optic lobe and central brain as reported by Bonini and colleagues (Bonini, et al., 1998). Arrowheads indicate lamina specific expression. The enhancer responsible for lamina expression pattern is located within the *eya* PRE / TRE. (**A**) merge, (**B**) *gfp* RNA detection, (**C**) *mw* RNA detection, (**D**) DAPI. For probe sequences see materials and methods and grey bars in Figure 5 A.

Table S1 RNAi phenotypes

			VDRC stock #	off	off target			ey GAL4		GMR GAL4	
	RNAi line	Chr	or other source	targets	gene	CG	FBgn	larval phenotype	adult phenotype	larval phenotype	adult phenotype
trxG	ash1	III	28982	0		CG8887	FBgn0005386	normal	normal	normal	normal
	brm	III	37721	2		CG5942	FBgn0000212	disrupted morphology; small eye disc	small or absent eye	normal	normal
	brm	III	37720	2		CG5942	FBgn0000212	disrupted morphology; small eye disc	small or absent eye	normal	normal
	mor	III	6969	0		CG18740	FBgn0002783	disrupted morphology; small or absent eye disc	lethal; escapers: small or absent eye	normal	normal
	osa	III	7810	0		CG7467	FBgn0261885	disrupted morphology; small or absent eye antenna disc	lethal	normal	normal
	Trl	II	17198	0		CG33261	FBgn0013263	disrupted morphology; overgrown eye disc	lethal	normal	normal
	Trl	III	41095	1	star1	CG33261	FBgn0013263	normal	small rough eye	normal	normal
	trx	II	37715	1	Mgat2	CG8651	FBgn0003862	normal	small protruding rough eye or absent eye	normal	normal
PRC1	ph-p	III	10679	1	ph-d	CG18412	FBgn0004861	disrupted morphology; massively overgrown eye antenna disc	lethal	normal or slightly disrupted morphology	rough eye
	Psc	II	30586	0		CG3886	FBgn0005624	normal	weak rough eye	normal	normal
	Psc	II	30587	0		CG3886	FBgn0005624	normal	weak rough eye	normal	normal
	Sce	III	27465	32		CG5595	FBgn0003330	disrupted morphology; small eye disc	lethal	normal	normal
	Pc	I	pWIZ, Pc2-1	n.d		CG32443	FBgn0003042	normal or slightly disrupted morphology	protruding, rough eye	normal	normal
	Pc	II	pWIZ, Pc 12-1	n.d		CG32443	FBgn0003042	normal or slightly disrupted morphology	normal or protruding, rough eye	normal	normal
	Pc	III	pWIZ, Pc 3-1	n.d		CG32443	FBgn0003042	normal or slightly disrupted morphology	protruding, rough eye	normal	normal
PRC2	E(z)	I	27645	0		CG6502	FBgn0000629	disrupted morphology; small or absent eye disc	lethal		
	E(z)	III	27646	0		CG6502	FBgn0000629	normal	normal	normal	normal
	esc	III	5692	0		CG14941	FBgn0000588	normal	normal	normal	normal
	esc	III	5690	0		CG14941	FBgn0000588	normal	normal	normal	normal
	Su(z)12	III	42422	1	uif	CG8013	FBgn0020887	disrupted morphology; overgrown eye disc	small rough eye or absent eye	normal	normal
	Su(z)12	III	42423	1	uif	CG8013	FBgn0020887	disrupted morphology; overgrown eye disc	lethal	normal	normal
DNA binding	Dsp1	II	41029	548		CG12223	FBgn0011764	disrupted morphology; small or absent eye antenna disc	lethal	normal	lethal
	pho	III	39529	0		CG17743	FBgn0002521	normal	protruding, rough eye	normal	normal
	psq	II	30591	685		CG2368	FBgn0004399	disrupted morphology; small or absent eye antenna disc	lethal	disrupted morphology; small eye disc	small or absent eye
	z	II	37718	394		CG7803	FBgn0004050	normal	normal	normal	normal
	z	III	37717	394		CG7803	FBgn0004050	disrupted morphology; small or absent eye antenna disc	lethal	normal	lethal

Table 10: Full list of phenotypes of RNAi knockdown of *PcG* and *trxG* genes in the developing eye.

Flies carrying a UAS-RNAi construct directed against the gene of interest were crossed to flies carrying both an ey-GAl4 driver and UAS dicer 2, and to flies carrying both a GMR-GAl4 driver and UAS dicer 2. For lines obtained from the Vienna Drosophila RNAi Centre, (VDRC, http://stockcenter.vdrc.at/control/main (Dietzl, et al., 2007), stock numbers are given. RNAi lines against Pc were generated using the pWIZ vector (Lee, 2003). Off target effects predicted by VDRC are shown. For those lines with a single predicted off target, the identity of this gene is given (note ph-p 10679 has a single predicted off target, namely ph-d). For definitions of phenotype descriptions see Legend to Table 2.

IX. ACKNOWLEDGEMENTS

I thank my supervisor Leonie Ringrose for being a great boss and the possibility to work on this exciting project, for her support and patience during experimental and writing phases.

I thank my PhD committee consisting of Jürgen Knoblich and Werner Aufsatz for the constructive suggestions related to my project. In addition, I wish to thank Jürgen Knoblich and Marco Milan for acting as examiners on my thesis.

I enjoyed my time in the Ringrose lab a lot and would like to thank my fabulous colleagues that contributed to the professional and fun working atmosphere.

I thank my family for love, patience and support.