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RESEARCH ARTICLE

Polycomb group (PcG) proteins and Pax6 cooperate to inhibit *in vivo* reprogramming of the developing *Drosophila* eye

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ABSTRACT

How different cells and tissues commit to and determine their fates has been a central question in developmental biology since the seminal embryological experiments conducted by Wilhelm Roux and Hans Driesch in sea urchins and frogs. Here, we demonstrate that Polycomb group (PcG) proteins maintain *Drosophila* eye specification by suppressing the activation of alternative fate choices. The loss of PcG in the developing eye results in a cellular reprogramming event in which the eye is redirected to a wing fate. This fate transformation occurs with either the individual loss of Polycomb proteins or the simultaneous reduction of the Pleiohomeotic repressive complex and Pax6. Interestingly, the requirement for retinal selector genes is limited to Pax6, as the removal of more downstream members does not lead to the eye-wing transformation. We also show that distinct PcG complexes are required during different developmental windows throughout eye formation. These findings build on earlier observations that the eye can be reprogrammed to initiate head epidermis, antennal and leg development.

KEY WORDS: *Drosophila*, Eye, Wing, Pax6, Polycomb, Antennapedia, Teashirt, Transdetermination

INTRODUCTION

During metazoan development, pluripotent stem cells that take different development paths differentiate into diverse tissues, organs and cell types, as originally described by Waddington's landscape model (Waddington, 1957). The pluripotency of these cells decreases as development proceeds: once a cell commits to adopting a certain fate, it differentiates autonomously even when it is placed into a different environment, indicating that its fate has already been determined. The imaginal discs of the fruit fly *Drosophila melanogaster* are larval sac-like epithelial structures. Through the process of metamorphosis, they give rise to various adult structures, including the compound eye, antenna, wings, legs, halteres and genitalia (Weismann, 1864). Earlier studies have shown that transplantation of larval imaginal discs or disc primordia from fragmented embryos into different larval hosts will not, under most circumstances, change the fate of the transplanted tissue. This demonstrates that the fate of the imaginal discs is determined during embryogenesis (Schubiger et al., 1969). However, despite the intense interest in tissue fate specification, the molecular mechanisms underlying the developmental commitment of tissues are not well understood.

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Although they most often maintain the initial programmed fate, transplanted disc fragments or embryonic primordial cells can, in some instances, transform into alternative adult appendages. For example, a fragment of the leg disc can give rise to an antenna, whereas regions of the eye disc can be transformed into a wing. This phenomenon, called transdetermination (Hadorn, 1968, 1978), results from abnormal gene expression, such as the ectopic activation of selector genes or the mis-expression of patterning signals like Wingless (Wg) and Decapentaplegic (Dpp) (Beira and Paro, 2016; Worley et al., 2012). It is therefore important to continually repress transcription of unnecessary genes during organogenesis. Indeed, it has been shown that when imaginal discs are transplanted into larvae hosts with reduced Polycomb group (PcG) protein levels, the frequency of transdetermination events rises (Lee et al., 2005). This is because PcG proteins, which are known as epigenetic silencers, maintain transcriptional silencing of developmental genes in cells where these factors are not needed (Schwartz and Pirrotta, 2007; Steffen and Ringrose, 2014). Thus, the loss of PcG proteins during development induces the de-repression of normally silenced developmental genes and this results in transformations of tissue fate (Grimaud et al., 2006).

In *Drosophila*, PcG proteins function within several distinct complexes: Pc repressive complex 1 (PRC1), Pc repressive complex 2 (PRC2), Pc repressive deubiquitinase (PR-DUB) and Pleiohomeotic (Pho) repressive complex (PhoRC) (Czermin et al., 2002; Klymenko et al., 2006; Müller et al., 2002; Scheuermann et al., 2012; Shao et al., 1999). In order to establish and maintain the transcriptional memory of an inactive gene, PRC2 and PRC1 are recruited to Polycomb response elements (PREs) within the genome by PhoRC, which consists of Scm-related gene containing four mbt domains (Sfmbt) and Pho. Sfmbt establishes a bridge, via protein-protein interactions, between the DNA-binding protein Pho and PRC2/PRC1. After the initial recruitment, Enhancer of zeste [E(z)], a member of PRC2, adds tri-methylation marks to the nucleosomes (at the H3K27 position) both at the PREs and along the gene body. This modification is then recognized by Pc, a member of PRC1, which, in turn, ubiquitylates H2A119 via Sex combs extra (Sce), another PRC1 member, and stabilizes PRC2. The accumulation of PRC1 and PRC2 within gene bodies results in the compaction of local nucleosomes and the further silencing of the inactive genes (Kassis et al., 2017; Simon and Kingston, 2009; Wang et al., 2004).

Although PcG proteins have been extensively studied in *Drosophila*, specifically using changes in imaginal disc development as readouts for their roles in transcriptional silencing (Herz et al., 2014; Janody et al., 2004), we still lack a comprehensive understanding of the mechanisms by which PcG proteins actually regulate fate determination *in vivo*. For example, the four PcG complexes are thought to cooperate with each other to silence gene targets but distinct phenotypes are elicited when different PcG proteins are removed. The upregulation of cell proliferation pathways is often associated with loss of PRC1 but not with

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removal of PRC2 (Classen et al., 2009; Martinez et al., 2009). Moreover, each type of imaginal disc responds differently to the loss of PcG activity. When PcG genes such as *Sce* and *pho* are removed, the eye imaginal disc does not show typical Hox gene de-repression, nor does it exhibit any tissue fate transformation as observed in other imaginal disc tissues, such as the wing and antennal discs (Gutierrez et al., 2012; Kim et al., 2008). The resistance to tissue fate changes in the eye disc suggests that maintaining gene silencing is more complicated than has been previously appreciated and that the developing eye employs a different mechanism of silencing gene transcription than do other tissues.

The *Drosophila* compound eyes are derived from a pair of monolayer epithelia called eye-antennal discs (Fig. 1A) (Haynie and Bryant, 1986). The disc primordium forms within the dorsal head region during late embryogenesis (Fig. 1A). By the early second larval instar, the disc is broadly subdivided into two separate fields: the eye field and the antennal field (Fig. 1A). Eventually, the pair of eye-antennal discs is further subdivided and gives rise to most of the adult head, including the compound eyes, the ocelli, the antenna, the maxillary palps and the surrounding head epidermis (Haynie and Bryant, 1986) (Fig. 1A). Previous studies have shown that the fate of the eye is established during the early second larval instar, but it can be redirected towards non-ocular tissue fates by removing retinal determination (RD) network genes such as *Pax6*, overexpressing Hox genes such as *Antennapedia* (*Antp*), as well as by manipulating the EGF receptor and Notch signaling pathways (Kumar and Moses, 2001; Kurata et al., 2000; Weasner and Kumar, 2013). PcG proteins and Trithorax group (TrxG) proteins are also known to play roles in eye fate specification (Janody et al., 2004), but the molecular mechanisms are not well understood. One report has shown that the eye can be transformed into a wing by overexpression of Winged-eye (Wge), a TrxG protein (Katsuyama et al., 2005). However, most other studies on PcG proteins in the eye disc have mainly focused on their roles in tissue growth and tumor suppression (Classen et al., 2009; Martinez et al., 2009). Here, we report our findings on the developmental mechanisms by which PcG proteins control fate determination of the eye. Our results describe the dynamics of PcG activity and the role that Pax6 plays in reducing the developmental capacity of the eye to adopt alternative fate choices over time.

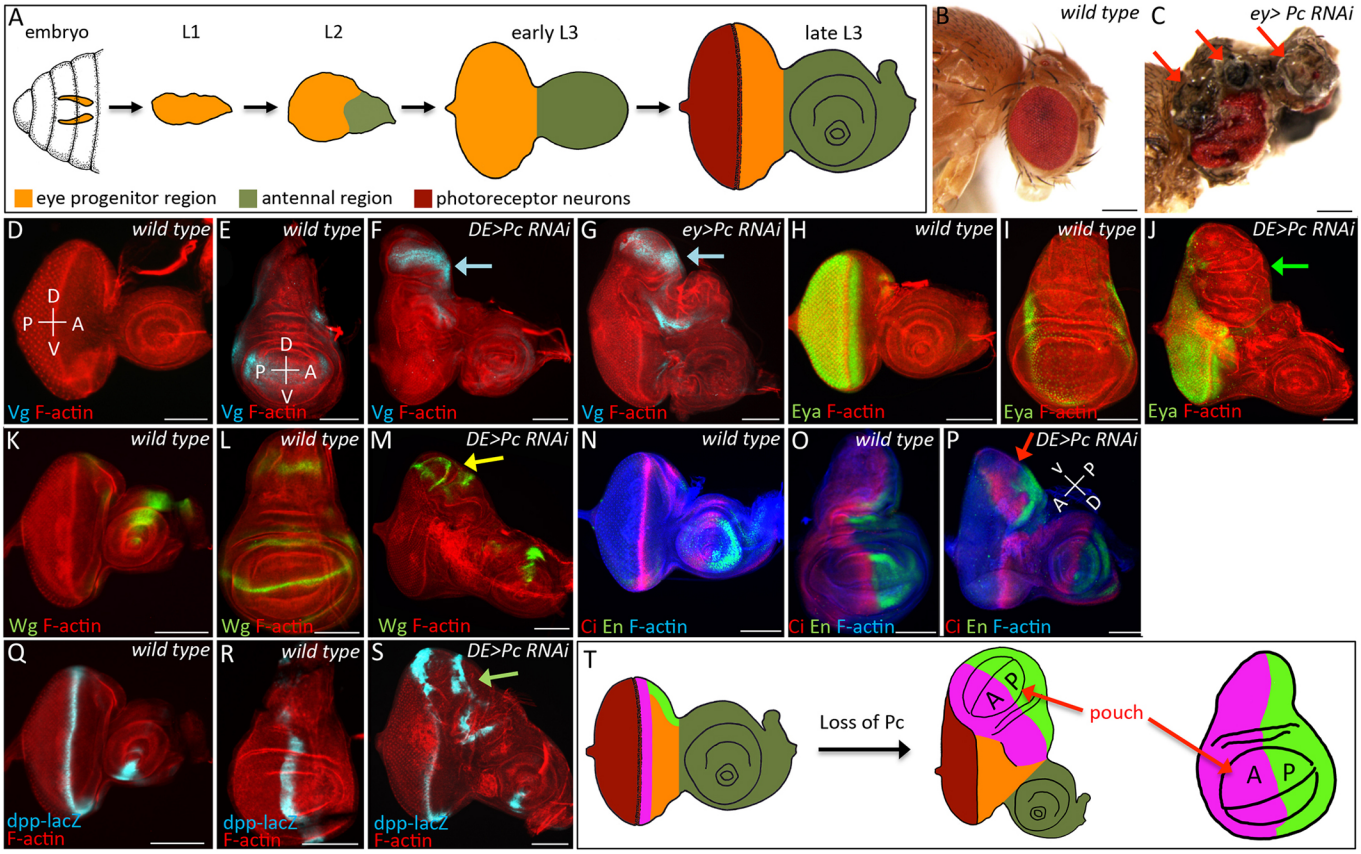

Fig. 1. The loss of Pc induces transformation of the eye into a wing. (A) Diagram of eye-antennal disc development. (B,C) Light microscope images of adult heads from wild-type (B) and *ey-GAL4>Pc RNAi* (C) flies. Ectopic tissue is found on *ey-GAL4>Pc RNAi* fly heads (C, red arrows). (D-S) Light microscope images of third instar eye-antennal and wing discs. Anterior is to the right. (D,E) Third instar wild-type eye-antennal (D) and wing disc (E) stained using an antibody against Vg. (D) Vg is not detected in the eye-antennal disc. (F,G) When Pc is knocked down with either (F) *DE-GAL4* or (G) *ey*-GAL4, *vg* is activated in the dorsal eye field (blue arrows). (H,I) Third instar wild-type eye-antennal (H) and wing (I) discs stained using an antibody against Eya. (H) Eya is activated in the eye progenitor region and photoreceptors in the eye disc. (I) Eya is present in the peripodial cells in the wing disc. (J) When Pc is knocked down with *DE-GAL4*, expression of *eya* is reduced in the dorsal eye field (green arrow). (K,L) Wild-type eye-antennal (K) and wing (L) discs stained using an antibody against Wg. (M) When Pc is knocked down with *DE-GAL4*, expression of *wg* shifts to a wing-like pattern in the dorsal eye field (yellow arrow). (N,O) Wild-type eye-antennal (N) and wing (O) discs stained using antibodies against Ci (red) and En (green). (P) In the *DE-GAL4>Pc RNAi* flies, a new A/P compartment (indicated by expression of *ci* and *en*, red arrow) forms within the ectopic wing. (Q,R) *dpp* expression in the wild-type eye-antennal (Q) and wing (R) discs marked by *dpp-lacZ*. (S) A new A/P axis formed in the eye disc of *DE-GAL4>Pc RNAi* flies indicated by *dpp-lacZ* expression (green arrow). (T) Diagram of compartment boundaries during the eye-to-wing transformation indicated by expression of *ci* (purple) and *en* (bright green). Scale bars: 100 µm.

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RESULTS

Pc is required in the dorsal region of the eye disc to suppress wing formation

To understand how PcG proteins control the determination of eye fate, we expressed RNAi lines targeting individual PcG members in the eye disc using *ey-GAL4* and *Dorsal-eye* (*DE*)-*GAL4* drivers. Both drivers are universally expressed in the embryonic eye-antennal primordium. During larval stages, *ey-GAL4* continues to drive expression throughout the entire eye progenitor region, but the expression from *DE-GAL4* becomes restricted to the dorsal compartment of the eye disc (Hauck et al., 1999; Morrison and Halder, 2010; Zhu et al., 2017). We found that whereas *DE-GAL4>UAS-Pc RNAi* flies die early in pupal development, *ey-GAL4>UAS-Pc RNAi* flies survive until the pharate stage and have outgrowths along the dorsal head epidermis and eye (Fig. 1B, C, red arrows). We initially thought that these growths might be ectopic wing tissue, as we could see wing bristles and, in some instances, wing veins. An analysis of third instar eye-antennal discs from these Pc knockdown mutants showed a transformation of the dorsal half of the eye disc into a wing disc. We observed expression of several wing selector genes, including *vestigial* (*vg*) (Fig. 1D-G, blue arrows), the elimination of the neuronal marker *embryonic lethal abnormal vision* (*elav*) (Fig. S1A-C, green arrows), and a reduction in the expression of the eye selector genes *eyeless* (*ey*), *twin of eyeless* (*toy*) and *eyes absent* (*eya*) (Fig. 1H-J; Fig. S1D-I, green arrows). Genes that are normally required in both the eye-antennal and wing discs, such as *wg*, *distal-less* (*dll*) and *cut* (*ct*) (Fig. 1K-M; Fig. S1J-O, yellow arrows), showed a switch in their expression patterns so that the eye-antennal disc-specific patterns were converted into wing-appropriate patterns. The expression of *cubitus interruptus* (*ci*) and *engrailed* (*en*) (Fig. 1N-P, red arrows) within the newly formed wing disc suggests that it contains both anterior (A) and posterior (P) compartments. A new A/P boundary within the ectopic wing is marked by activation of *decapentaplegic* (*dpp*) (Fig. 1Q-S, green arrow). The new A/P compartment of the transformed eye disc appears to be derived from the endogenous patterns of *ci* and *en* in the eye disc (Fig. 1T). Although the expression patterns within the ectopic wings are similar to those seen in the normal wing, they are far from perfect. We assume that the dorsal eye/head tissue from which the ectopic wings are derived lacks key positional information that would allow for a complete eye-wing transformation.

Interestingly, even when Pc levels are knocked down in both dorsal and ventral eye discs using *ey-GAL4*, the eye-to-wing transformation is observed only in the dorsal compartment (90%, *n*=40). At the molecular level, *Drosophila insulin-like peptide 8* (*dilp8*), which is expressed in cells undergoing either cell proliferation or regeneration (Colombani et al., 2012; Garelli et al., 2012), is similarly activated within only the dorsal region of *ey-GAL4>UAS-Pc RNAi* eye discs (95%, *n*=20) (Fig. S2A,B, green arrow). These data indicate that the dorsal eye disc is more prone to adopting alternative tissue fates when Pc is removed.

Pc is required to suppress the Hox gene *Antp* within the eye-antennal disc

Eye-to-wing and antenna-to-leg transformations have been observed in some mutant alleles of the Hox gene *Antp*. In these instances, *Antp* is ectopically expressed within the eye-antennal disc (Schmid, 1985; Struhl, 1981). These transformations are consistent with the role that Antp plays in controlling the fate of the mesothorax (Struhl, 1982). Because PcG proteins are known to target and silence Hox genes (Beck et al., 2010), we set out to determine whether the absence of PcG activity in the eye-antennal disc results in the ectopic activation of *Antp* or other Hox genes. As expected, when Pc was knocked down with *DE-GAL4*, *Antp* was broadly activated in the dorsal eye disc, starting at the early third larval instar (Fig. 2A, yellow arrows). Interestingly, *Ultrabithorax* (*Ubx*), which remained silenced during the early stages of the eye-to-wing transformation (Fig. 2B, red arrow), was activated once the dorsal region of the eye disc transformed into a wing disc (Fig. 2C, blue arrows). Other Hox genes, such as *proboscipedia* (*pb*), *labial* (*lab*) and *Abdominal B* (*Abd-B*), remained silenced (Fig. S3A-D,I). *Deformed* (*Dfd*) was activated in a few cells in the dorsal transforming region (Fig. S3E,F, green arrows). *Sex combs reduced* (*Scr*) was also detected in the dorsal transforming region, where ectopic tissue resembling a leg disc is formed near the border of the antenna (Fig. S3G,H, yellow arrows).

Our results contrast with those of previous reports that have shown a de-repression of *Ubx* and *Abd-B* in PcG loss-of-function mutant clones (Janody et al., 2004; Loubiere et al., 2016). To investigate this difference between the various studies, we assayed Pc protein levels in *DE-GAL4>UAS-Pc RNAi* eye discs. We found that *DE-GAL4* driving *UAS-Pc RNAi* successfully eliminates Pc proteins in the dorsal eye disc (Fig. 2B, red arrow). However, Pc levels are restored once the dorsal region of the eye disc transforms into a new wing disc (Fig. 2C). The restoration of *Pc* expression is due to the changes in tissue-specific expression of the *DE-GAL4* driver. *DE-GAL4* is expressed not only in the dorsal eye disc (Fig. 2D) but also in the wing disc, mostly in the notum and in a few cells in the pouch (Fig. 2E). Thus, once the dorsal region of the eye disc transforms into a new wing disc, the *Pc RNAi* line is turned off in much of the new pouch, except in a few cells that still express *DE-GAL4* (Fig. 2F, green arrow). As a result, *Ubx* expression is activated in regions that still lack Pc proteins (Fig. 2C,C′, blue arrows). The activation of *Antp* in the eye disc and *Ubx* in the wing disc suggests that when Pc is lost, Hox genes that control the development of more-posterior regions of the body are de-repressed in the order of their position within the genome. In contrast to our study, in which we used genomic enhancers to drive expression of RNAi lines, other studies permanently removed PcG proteins through the use of loss-of-function mutant clones. As a result, more-posterior Hox genes, including *Abd-B*, were de-repressed (Classen et al., 2009; Martinez et al., 2009). The lack of activation of Ubx in the transformed eye disc from *ey-GAL4>UAS-Pc RNAi* flies further confirms our hypothesis (Fig. S3K,L). Because *ey-GAL4* is not expressed in the developing wing, it is likely that *Pc* expression returns after the initial eye-wing transformation; thus, *Ubx* is not de-repressed.

Teashirt (Tsh) is required for activation of *Antp* in the eye disc

Although anterior-to-posterior tissue transformations, such as wing to haltere and antenna to leg, are commonly observed in PcG mutants, posterior-to-anterior tissue transformations have also been described (Lewis, 1978). In fact, many alleles of *Pc* or *extra sexcombs* (*esc*) were first characterized by a transformation of the second and third thoracic legs into first thoracic legs, resulting in ‘extra’ or ‘poly’ sex combs on these transformed legs (Lewis, 1947; Slifer, 1942). These transformations occur because *Scr*, which is normally expressed in the first pair of leg discs, is ectopically activated within the second and third pairs (Glicksman and Brower, 1988). As both A-P and P-A transformations can occur from the loss of Pc, endogenous gene regulatory networks (GRNs) may play important roles in determining which Hox genes are activated.

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Previously, we have shown that when Pax6 proteins are eliminated from the eye-antennal disc, forced expression of *teashirt* (*tsh*), a gene that is required for the growth/specification of both eye-antennal and wing discs (Azpiazu and Morata, 2000; Peng et al., 2009; Singh et al., 2002), can partially restore growth of the eye-antennal disc. In these discs, *Antp* is activated and ectopic wings are found on the heads, thereby indicating the occurrence of a head-to-mesothorax transformation (Zhu et al., 2017). These findings suggest that when the eye program is disrupted, endogenous Tsh in eye progenitors might activate *Antp*, thereby triggering the eye-to-wing transformation. Indeed, when Pc and Tsh are simultaneously knocked down with *ey-GAL4*, Antp is not detected in the eye progenitor region (Fig. 3A,B, white dotted line). Expression of *Antp* is activated in only a few cells within the overlying peripodial epithelium of the double knockdown mutants (Fig. 3A′,B′, yellow arrowheads; disc structure is shown in Fig. 3C). Compared with Pc single knockdown mutants, knocking down Tsh simultaneously with Pc blocked the activation of *Antp* in the dorsal eye disc proper (Fig. 3A″,B″, green arrow). As a result, the frequency of the eye-to-wing transformation and the size of the eye field were both dramatically reduced (Fig. 3D,E). These data suggest that, during normal development, Pc maintains the silencing of the *Antp* locus within the eye field. But in the absence of Pc, endogenous Tsh within retinal progenitor cells can activate *Antp*, thereby triggering the tissue fate change (Fig. 3F).

Loss of different PcG complexes affects the eye-antennal disc in distinct ways

We next asked whether knocking down other PcG proteins induces the eye-to-wing transformation. We found that simultaneously reducing Polyhomeotic proximal (Ph-p) and Polyhomeotic distal (Ph-d) induces de-repression of *Antp*. However, instead of forming a new wing disc, the mutant eye disc underwent neoplastic growth and a loss of cell polarity (Fig. 4A-C), which is consistent with previous studies showing that Ph is required for tumor suppression (Classen et al., 2009; Loubiere et al., 2016). Knocking down Sce, another PRC1 member, in the developing eye disc showed no obvious phenotype (Fig. 4A) (Gutierrez et al., 2012), providing further evidence that PcG proteins in the same complex have distinct functions in maintaining cellular memory. In addition, loss of E(z) failed to transform the eye into a wing despite the de-repression of several Hox genes (Fig. 4A,D,E) (Loubiere et al., 2016). This is likely due to the reduction in cell proliferation within the E(z) knockdown disc (Classen et al., 2009; Martinez et al., 2009).

We also knocked down the PhoRC member Sfmbt. The responses to the loss of Sfmbt showed tissue specificity – when *Sfmbt*[1] loss-of-function clones were generated, *Antp* was de-repressed only within the antennal field, but not in the eye field (Fig. 5A, purple arrows). Consistent with this result, knocking down Sfmbt in the eye disc using *DE-GAL4* failed to induce the eye-to-wing transformation (Fig. 5B), as *vg* expression was not activated (Fig. 5C), and Antp was detected in only a few peripodial cells of both the eye disc and antenna fields (Fig. 5D,K, green arrows). Similarly, knocking down another PhoRC member, Pho, also failed to induce the eye-to-wing transformation (Fig. 5K).

Reducing Pax6 levels returns the eye to a more-plastic developmental state

Next, we asked what is the cause of this tissue specific response to loss of PhoRC members? We propose that the difference in how the eye and antennal fields respond to the loss of Sfmbt is due to differences in the GRNs that are present in the two tissues. To test this idea, we knocked down members of the RD network to determine whether it increased the ability of the eye disc to adopt

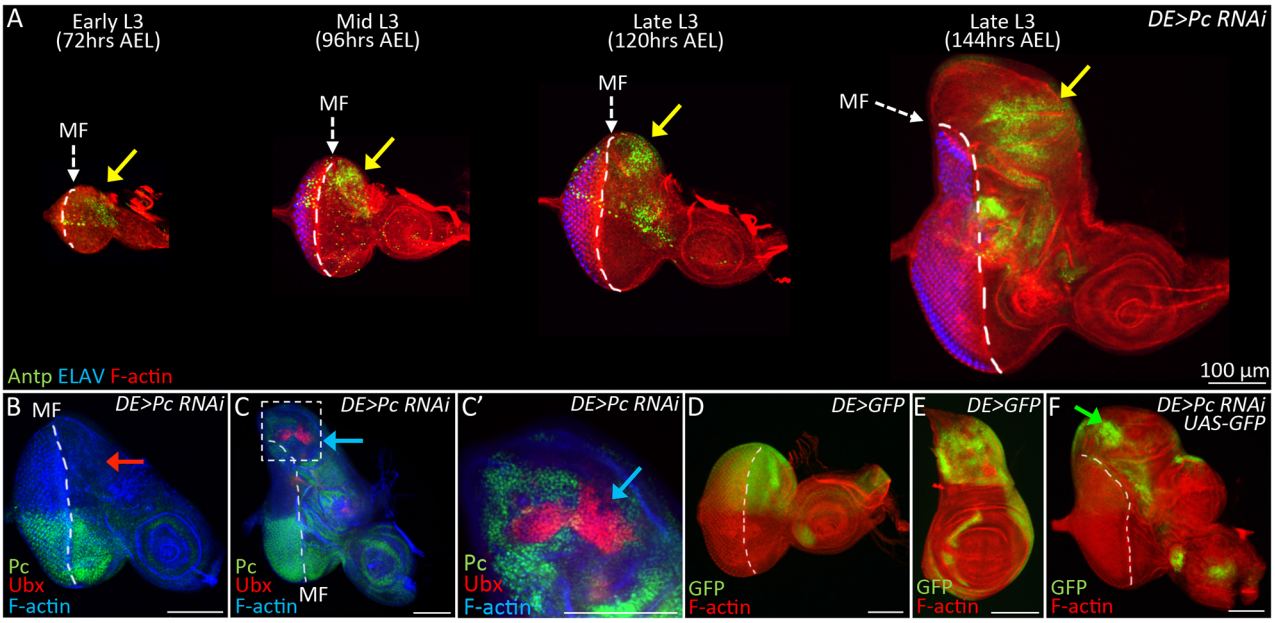

Fig. 2. Hox genes are sequentially de-repressed during the eye-to-wing transformation. (A) Third instar larval eye-antennal disc of *DE-GAL4>Pc RNAi* flies stained using antibodies against Antp (green) and Elav (purple). *Antp* is activated in the dorsal eye disc throughout the third instar larval stage (yellow arrows). (B-C′) Third instar larval eye-antennal disc of *DE-GAL4>Pc RNAi* flies stained using antibodies against Pc (green) and Ubx (red). Pc is removed from the dorsal eye disc of *DE-GAL4>Pc RNAi* flies but Ubx is not detected in the eye disc at this stage (B, red arrow). Expression of *Pc* is restored in the dorsal eye-to-wing transforming region and Ubx is detected in the newly formed wing pouch (C,C′; blue arrows). (D-F) Third instar larval eye-antennal disc and wing disc of *DE-GAL4>UAS-GFP* flies and *DE-GAL4>Pc RNAi; UAS-GFP* flies. GFP marks the expression pattern of *DE-GAL4*. (F) When Pc is knocked down, *DE-GAL4* is only activated in a few cells of the dorsal transforming region (green arrow). MF, morphogenetic furrow. Anterior is to the right. Scale bars: 100 µm.

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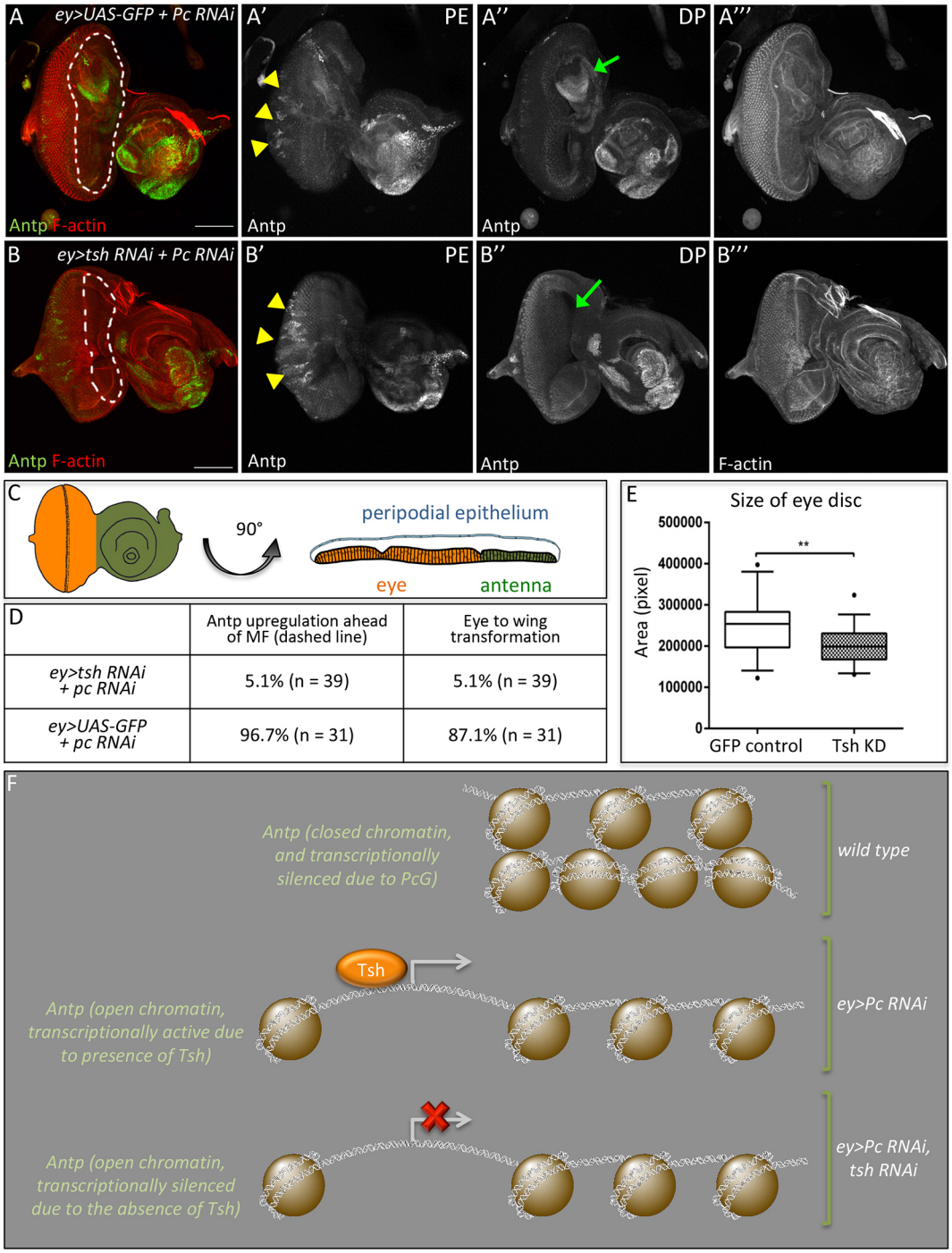

Fig. 3. Teashirt is required for the activation of Antp during the eye-to-wing transformation. (A,B) Light microscope images of third instar eye-antennal discs of *ey-GAL4>Pc RNAi; UAS-GFP* flies (A) and *ey-GAL4>Pc RNAi; tsh RNAi* flies (B) stained using an antibody against Antp (green). Anterior is to the right. Antp is detected in the eye progenitor region (white dotted line) of Pc single knockdown flies (A) but not in the Pc and Tsh double knockdown flies (B). Antp is detected in the peripodial epithelium cells in both mutants (A′ and B′, yellow arrowheads). *Antp* is activated in the eye disc proper of Pc single knockdown mutant (A″, green arrow), but not in the Pc and Tsh double knockdown mutant (B″, green arrow). A‴ and B‴ both show the outlines of the eye-antennal discs that are shown in panels A and B. Scale bars: 100 µm. (C) Schematic diagram of the structure of the eye-antennal disc. (D) Quantification of Antp upregulation and eye-to-wing transformation frequency. (E) Quantification of eye disc size, *n*=31 and 39, respectively. Error bars represent the 5-95% percentile. $^{**}P \leq 0.01$. (F) Our model for the regulation of *Antp* by PcG and the retinal determination factor Tsh. Compared with the wild-type eye disc, when Pc is knocked down, nucleosomes on the *Antp* locus are not compacted, which leads to a more accessible *Antp* locus. Endogenous Tsh serves as the activator of *Antp*, so knocking down Tsh simultaneously with Pc blocks the de-repression of *Antp*.

alternative tissue fates. We started by simultaneously reducing the expression levels of Sfmbt with each of the Pax6 genes *ey* and *toy*, because these are the most upstream factors within the entire RD network and are expressed in the eye progenitor region since embryogenesis (Fig. 1A, orange region) (Czerny et al., 1999; Quiring et al., 1994). Surprisingly, the knockdown of Sfmbt with either Pax6 gene led to a robust eye-to-wing transformation that was equivalent to the loss of Pc. In these double knockdown mutants,

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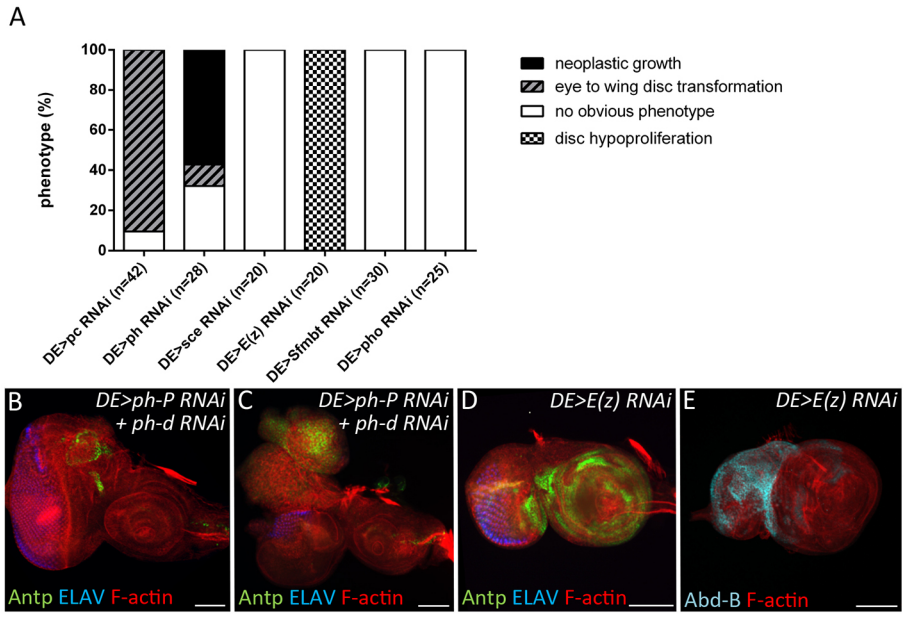

Fig. 4. Loss of different PcG members affects the eye disc in distinct ways. (A) Quantification of the different types of effects on eye development when individual PRC1 and PRC2 are removed. (B-E) Light microscope images of third larval instar eye-antennal discs from *DE-GAL4>ph-d RNAi; ph-p RNAi* flies stained using antibodies against Antp (green) and Elav (blue). When both Ph-d and Ph-p are knocked down with *DE-GAL4* in the eye disc, Antp is activated in the dorsal eye disc (B,C). Neoplastic growth is also induced in the Ph knockdown discs (C). (D,E) Light microscope images of third larval instar eye-antennal discs from *DE-GAL4>E(z) RNAi* flies stained using antibodies against Antp, Elav (D) and Abd-B (E). Knocking down E(z) causes ectopic activation of Antp (D) and Abd-B (E) with reduced eye disc size. Anterior is to the right. Scale bars: 100 µm.

ectopic wings formed on adult fly heads (Fig. 5E,H, red arrows), while *vg* (Fig. 5F,I, blue arrows) and *Antp* (Fig. 5G,J, green arrows) were activated in the mutant disc. Similar results were obtained when we expressed the *Sfmbt RNAi* line in the ey^2 loss-of-function mutant (Fig. S4A,B) or knocked down Pho (the other PhoRC member) in concert with either Pax6 gene (Fig. 5K). Finally, the expression pattern of *Antp* in the *Sfmbt* and *Pax6* double knockdown at various developmental stages mimicked that in the Pc single knockdown mutants (Fig. S4C,D).

To test whether removing other RD network genes could also increase the frequency of this fate change, Sfmbt was knocked down in concert with the Pax6 downstream targets *sine oculis* (*so*) and *eya*. These two selector genes participate in the initiation of eye development, and loss of either gene prevents eye development (Kumar, 2010). However, unexpectedly, when Sfmbt was removed in the eya^2 mutant discs, in which expression of *eya* and *so* is not activated, neither the eye-to-wing transformation (Fig. 6A,D,M) nor the activation of *vg* or *Antp* in the mutant eye disc (Fig. 6E,F) was observed. In fact, these discs did not look significantly different from the eya^2 mutant discs (Fig. 6B,C). If, however, either Ey or Toy was knocked down in concert with Sfmbt in the eya^2 mutant background, then we observed robust eye-to-wing transformations in these triple mutants (Fig. 6G,J,M, red arrows) as well as activation of *vg* (Fig. 6H,K, blue arrows) and *Antp* (Fig. 6I,L, green arrows). These data suggest that simply blocking eye development at any level is not sufficient to reprogram the eye into a wing (even if Sfmbt/Pho is removed). Because Pax6 expression starts during stage 15 of embryogenesis whereas the expression of both *so* and *eya* is initiated during the second larval instar (Czerny et al., 1999; Quiring et al., 1994; Weasner et al., 2016), it is possible that the developmental window during which the eye can be reprogrammed into a wing precedes the expression of *so* and *eya*.

Dynamic temporal requirements of PRCs in the maintenance of eye fate

We next determined the developmental stages at which Pc, Pax6 and Sfmbt are required. To address this issue, we employed a temperature-sensitive GAL80 protein to control RNAi expression (Fig. 7A). At 18°C (the permissive temperature), GAL80 is functional, inhibits the activity of GAL4 and prevents the RNAi construct from being expressed. At 30°C (the non-permissive temperature), GAL80 is non-functional and therefore the RNAi is activated. By toggling between 18°C and 30°C, we could control the timing of RNAi expression and determine when Pc, Pax6 and Sfmbt were required to suppress wing formation (Fig. 7B). When determining the critical windows, we took into account that development of *Drosophila* proceeds twice as fast at 30°C as it does at 18°C (Bliss, 1926), and that it takes 10-12 h for endogenous Ey and Toy proteins to be eliminated after RNAi induction (Zhu et al., 2017). After egg laying (AEL), embryos and larvae were raised at 18°C for different lengths of time before being shifted to 30°C. Mutant eye-antennal discs were then dissected from wandering later third instar larvae and screened for eye-to-wing transformations (Fig. 7B).

When Pc was removed before the mid-first larval instar stage, *Antp* was activated and the dorsal eye disc transformed into a wing with 100% penetrance (Fig. 7C,D; Fig. S5A). However, if Pc was removed during the late first larval instar (18°C for 60 h AEL), then only 20% of the mutant discs were transformed into wings despite the activation of *Antp* (Fig. 7C; Fig. S5A). If Pc was removed at the start of the second larval instar, then neither activation of *Antp* nor the eye-to-wing transformation was observed (Fig. 7C,D; Fig. S5A). These data suggest that the critical window for Pc activity is from late embryogenesis to the mid-first larval instar. In contrast, Ey or Toy must be removed in concert with Sfmbt during late embryogenesis in order to induce the eye-to-wing transformation (Fig. 7C; Fig. S5B-E). Knockdown of these proteins after late embryogenesis had little to no effect on the fate of the eye (Fig. 7C; Fig. S5B,C). These data demonstrate that the PhoRC complex is required earlier than Pc in the eye to recruit other PRCs to initiate silencing.

DISCUSSION

Although imaginal discs have long been used as a model system for studying the function of PcG proteins, the molecular mechanisms and developmental dynamics of PcG activity in these tissues are still poorly understood. In this report, we describe how different Pc complexes cooperate to maintain the fate of the eye.

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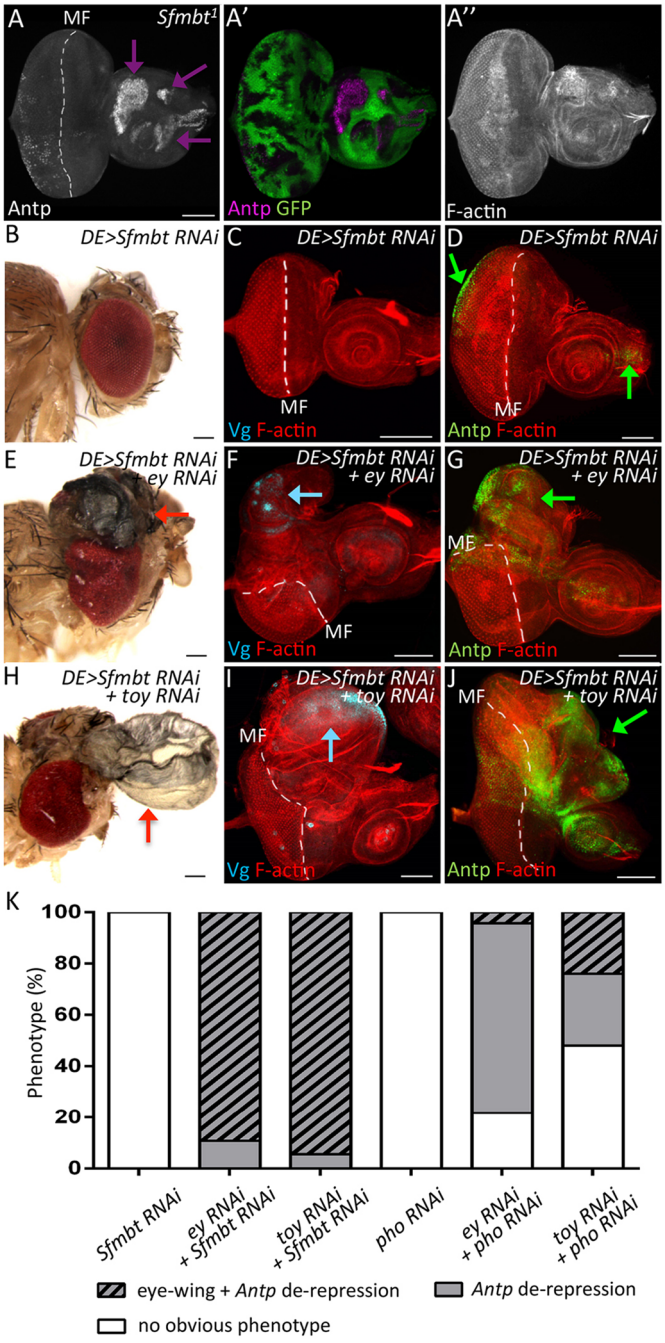

Fig. 5. Simultaneous reductions of Pax6 and Sfmbt induces the eye-to-wing transformation. (A-A″) Third instar larval eye-antennal discs with *Sfmbt* loss-of-function clones (indicated by absence of GFP). Removal of Sfmbt causes de-repression of *Antp* in the antennal disc (purple arrows) but not in the eye disc. (B-D) Eye-to-wing transformation is not observed in the *DE-GAL4>Sfmbt RNAi* flies. (B) Knocking down Sfmbt causes no obvious head phenotype. (C) Vg is not detected in the third instar larval mutant eye-antennal disc. (D) Antp is activated in only a few cells in the mutant antennal disc and in the very posterior region of the eye disc (green arrows). (E-J) Eye-to-wing transformation is found in the *DE-GAL4>Sfmbt RNAi; ey RNAi* (E-G) and *DE-GAL4>Sfmbt RNAi; toy RNAi* (H-J) flies. (E,H) Ectopic wings form on the Pax6 and Sfmbt double knockdown fly heads (red arrows). (F,I) Third instar larval mutant eye-antennal discs show *vg* activation in the dorsal eye-to-wing transforming region (blue arrows). (G,J) Antp is de-repressed in the dorsal transformation region (green arrows). Anterior is to the right. Scale bars: 100 µm. (K) Quantification of *Antp* de-repression and eye-to-wing transformation phenotypes; *n*=30, 37, 36, 20, 23 and 25, respectively.

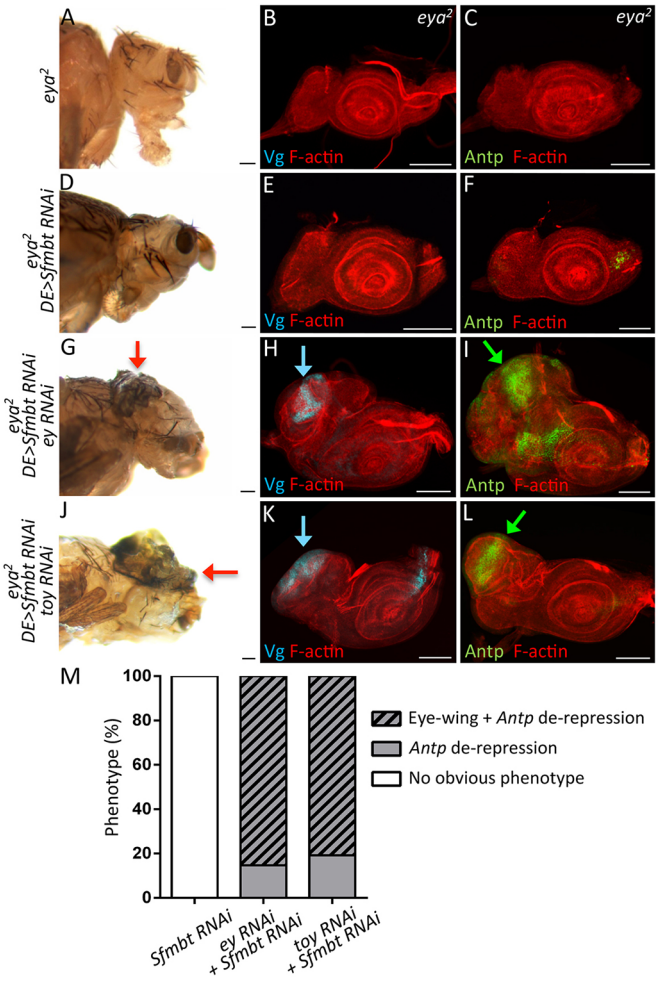

Fig. 6. The loss of Pax6, but not of Eya, is required for Sfmbt dependent eye-to-wing transformation. (A-C) Light microscope images of an *eya*^{2} adult head (A) and third instar larval eye-antennal discs (B,C) stained using antibodies against Vg (B) and Antp (C). Vg and Antp are not detected in the mutant discs. (D-F) Ectopic wing formation is not induced when Sfmbt is knocked down in an *eya*^{2} mutant background. The adult head phenotype of *eya*^{2}; *DE-GAL4>Sfmbt RNAi* flies is not different from that of *eya*^{2} flies (D). *vg* (E) and *Antp* (F) are not activated in the mutant eye disc. (G-L) Knocking down either Ey (G-I) or Toy (J-L) simultaneously with Sfmbt in the *eya*^{2} mutant background leads to eye-to-wing transformation. Ectopic wings are found on adult heads of *eya*^{2}; *DE-GAL4>ey RNAi; Sfmbt RNAi* (G) and *eya*^{2}; *DE-GAL4>toy RNAi; Sfmbt RNAi* (J) mutants. Expression of *vg* (H and K, blue arrows) and *Antp* (I and L, green arrows) is activated in the dorsal eye disc of mutant flies. Anterior is to the right. Scale bars: 100 µm. (M) Quantification of *Antp* de-repression and eye-to-wing transformations in the eye disc from mutants mentioned above; *n*=20, 34 and 26, respectively.

The eye-to-wing fate change

Earlier studies on the transdetermination of imaginal discs have suggested that the eye and wing are serially homologous structures as they both are dorsally derived appendages (Schmid, 1985). Our study provides genetic support for this proposal by showing that the loss of Pc early in development transforms the eye disc into a wing disc (Fig. 1). This eye-to-wing transformation mimics the phenotypes observed when regions of the eye disc are transplanted into host larvae or when both *Antp* and Notch signaling are activated in the eye field (Hadorn, 1968, 1978; Kurata et al., 2000; Schmid, 1985). It also resembles the phenotype observed with the overexpression of *wge*, a putative TrxG factor (Katsuyama et al., 2005). The eye-to-wing transformations that result either from

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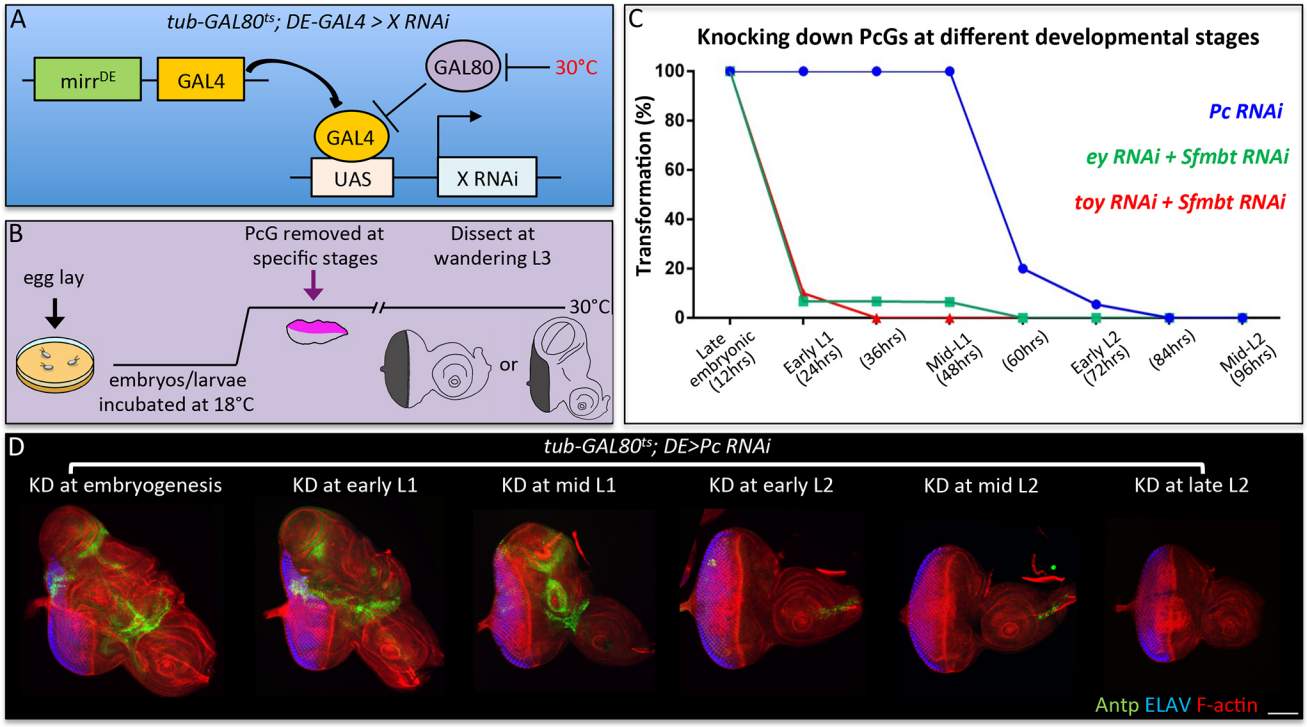

Fig. 7. Pc and Sfmbt are required at different stages to establish the fate of the eye. (A) Schematic diagram of the GAL4/GAL80 system. (B) Schematic diagram of the experimental assay to knock down different proteins at various developmental stages. Embryos of mutant lines were kept at 18°C after egg laying for various hours and then shifted to 30°C before dissection at the wandering third instar larval stage. (C) Summary of phenocritical period of Pc and Sfmbt activity. Pc, Ey with Sfmbt or Toy with Sfmbt are knocked down at different developmental stages (various hours AEL). Knocking down Pc (blue line) before mid-first larval instar (48 h) causes eye-to-wing transformation with 100% penetrance. The ability to induce tissue transformation is significantly reduced when Pc is removed later than 60 h AEL. Knocking down Sfmbt either with Ey (green line) or Toy (red line) before the late embryonic stage (12 h AEL) induces the eye-to-wing transformation with 100% penetrance. Tissue transformation is significantly reduced when these proteins are knocked down later than the early first larval instar (24 h). (D) Light microscope images of third larval instar eye-antennal discs of *tub-Gal80^{ts}; DE-GAL4>Pc RNAi* flies stained using antibodies against Antp and Elav. Pc is knocked down at different developmental stages. Anterior is to the right. Scale bar: 100 µm.

PcG loss or TrxG gain suggest that there is a delicate balance between these antagonistically acting complexes in the maintenance of the epigenetic state of the developing eye (Steffen and Ringrose, 2014).

Interestingly, the dorsal region of the eye disc is more prone to adopting the wing fate than the ventral compartment when Pc is knocked down (Fig. 1; Fig. S2). One possible explanation is that the expression of *wg*, a selector gene for the wing (Maves and Schubiger, 1995), is initiated earlier and is expressed at higher levels in the dorsal region of the eye (Ma and Moses, 1995; Treisman and Rubin, 1995). Another mechanism might involve the expression patterns of *ci* and *en*, which meet along the A/P axis of the normal wing disc. In the eye field, *ci* is present within the morphogenetic furrow, with no obvious difference in the dorsal and ventral compartments; *en* is expressed in a small population of cells within a region of the dorsal compartment (Fig. 1N,T). These cells give rise to the ocellar region of the visual system (Haynie and Bryant, 1986). The *ci* and *en* expression domains in the newly formed wing disc (and corresponding A/P compartments) align physically with the endogenous expression patterns of these two genes (Fig. 1N,T), indicating that the junction between these two A/P patterning genes may be more sensitive to transformation. Finally, the dorsal eye may be more likely to transform into a wing because it contains a weak point: regions of each disc that are more susceptible to transdetermination (Maves and Schubiger, 1999). A screen for regions that support ectopic eye formation identified a putative weak spot within the dorsal anterior margin (Salzer and Kumar, 2010), and the eye-to-wing transformation occurs within this region.

The regulation of Hox genes by PcG proteins

Although it is known that Hox genes are downstream targets of PcGs, a complete understanding of how they are regulated during development remains elusive. In our Pc knockdown experiments, we observed that only a single Hox gene, *Antp*, became broadly activated (Fig. 2). This contrasts with other studies in which additional Hox genes such as *Ubx*, *Abd-A* and *Abd-B* have been detected in *Pc* loss-of-function mutant clones (Classen et al., 2009; Janody et al., 2004; Martinez et al., 2009). These differences may be attributed to the varying methods of Pc removal. In the case of *Pc* mutant clones, *Pc* expression is lost permanently, whereas in our study Pc is removed only temporarily, and is restored in the newly transformed tissue. The prior method prevents further Hox gene activation and additional tissue transformations. The temporary knockdown of Pc allowed us to identify PcG suppression of the eye-to-wing transformation, a phenomenon that has eluded all prior studies to date. In only a few cells that still express Pc RNAi do we observe *Ubx* de-repression. This result is consistent with previous studies showing that loss of PcG activity in the developing wing disc causes ectopic expression of *Ubx* (Beuchle et al., 2001), resulting in a wing-to-haltere transformation (Tiong and Russell, 1990).

But is *Antp* activation due only to the loss of PcG silencing? Because gene expression is controlled at both epigenetic and transcription factor levels, removal of an epigenetic silencer is not sufficient to switch the transcriptional state of a gene from off to on. Transcriptional activators must also be present at the locus. Here, we demonstrate that Tsh, which is normally present in the progenitors

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of the eye disc, is required to activate *Antp* in the Pc knockdown discs (Fig. 3). We propose that, in normal eye development, the ability of Tsh to activate *Antp* expression (and to induce an eye-to-wing transformation) is inhibited by the presence of both the RD network and the PcG-silencing machinery. In summary, the similarity of GRNs between the eye and wing contributes to this tissue fate change.

Distinct Polycomb complexes cooperate to maintain eye fate

Although losing Pc (PRC1) induces the eye-to-wing transformation, knocking down E(z) (PRC2) does not induce this fate change (Fig. 4). This discrepancy could be due to the difference in gene targets between PRC1 and PRC2, as recent studies have shown that PRC1 regulates a large set of proliferation and signaling-associated genes, including the JAK/STAT and Notch signaling pathways, independently of PRC2 (Classen et al., 2009; Loubiere et al., 2016; Martinez et al., 2009). The failure to induce over-proliferation in PRC2 knockdowns is the most likely explanation for why eye-to-wing transformations are observed only in PRC1 knockdowns. It has also been shown that when *Antp* is overexpressed in the developing eye disc, ectopic wings form within the head epidermis only when Notch signaling is co-activated, suggesting that increased cell proliferation is necessary for the fate switch (Kurata et al., 2000).

Unlike removal of Pc, individual knockdown of either PhoRC member (Pho or Sfmbt) fails to alter the fate of the eye disc, despite the fact that PhoRC recruits PRC1 and PRC2. Although PhoRC brings PRC1 and PRC2 to the PREs to initiate gene silencing (Frey et al., 2016), it might be possible that the spreading of PRC1 and PRC2 along the genome, via sterile alpha motif (SAM) interaction domains, occurs independently of PhoRC. We conducted time-course experiments to investigate the requirement of Pc and Sfmbt/Pax6 at various developmental stages. In our model (Fig. 8), PhoRC initially recruits PRC1 to the PREs within non-retinal genes such as *Antp* during early embryogenesis. As development proceeds, PRC1 and PRC2 spread along the entire gene locus, independently of PhoRC. Members of the PRC1 and PRC2 complexes contain SAM domains. These have been shown to be capable of facilitating the polymerization of protein chains (Webber et al., 2013). Finally, after local nucleosomes within non-retinal genes have been compacted into the Pc bodies (Bantignies et al., 2011), we propose that the transcription of inactive genes is completely silenced. This is supported by genome-wide chromatin immunoprecipitation (ChIP) analysis showing that high levels of H3K27me3 (tri-methylation marks at the H3K27 position) are found on the *Antp* gene locus (Classen et al., 2009). At this stage, newly synthesized PCGs are no longer needed to prevent changes in the fate of eye progenitor cells (Fig. 8).

Our model results contrast with those of several recent studies showing that PREs are required for gene silencing in the developing wing disc as late as the third larval instar (Coleman and Struhl, 2017; Laprell et al., 2017). However, as we have shown with the *Sfmbt* loss-of-function mutant, responses to loss of PcG activity show tissue specificity in various tissue types (Klymenko et al., 2006). Previous studies demonstrate that the wing-to-haltere transformation could be triggered in *Pc* mutant clones in the wing disc induced at as late as 108 h AEL, but the second-to-first-leg

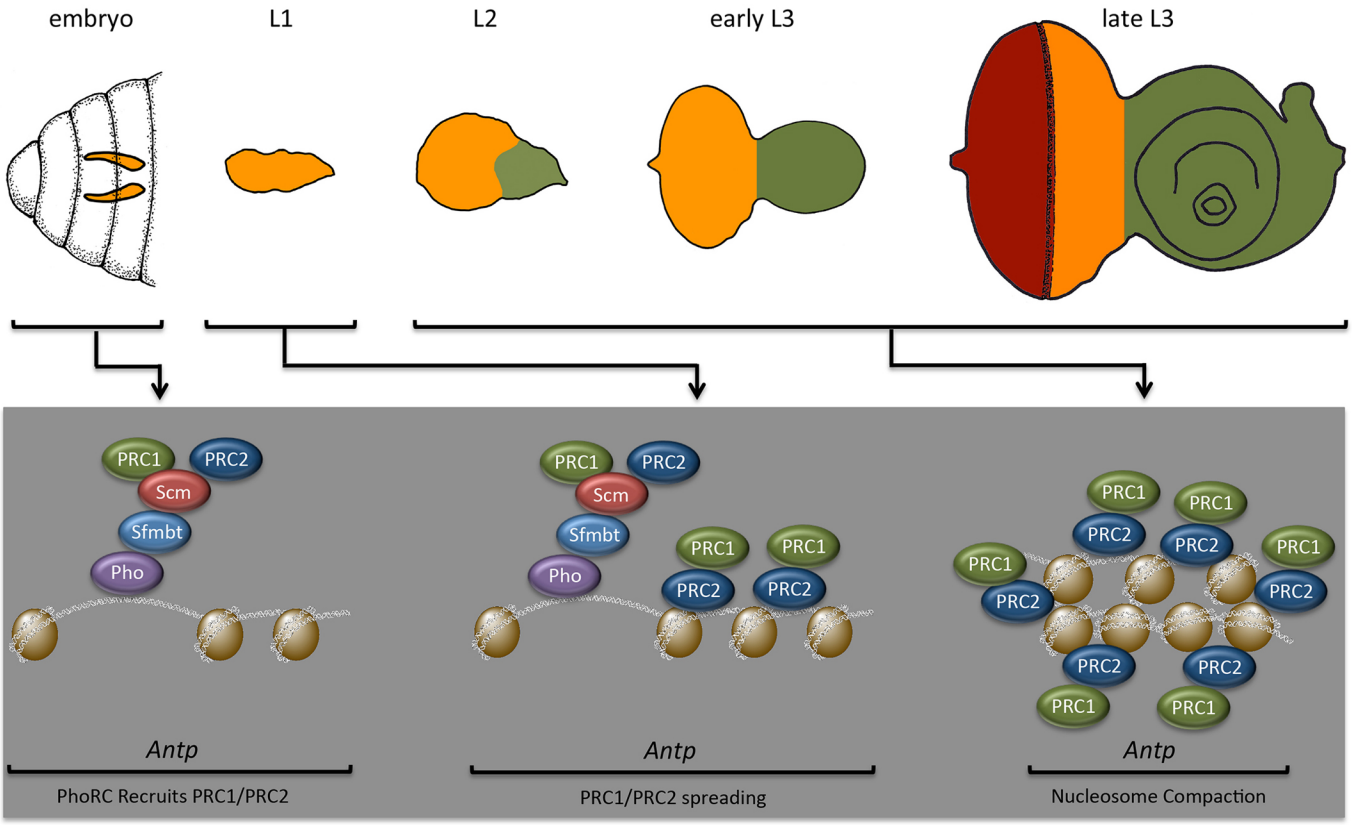

Fig. 8. Model for the repression of wing selector genes in the eye-antennal disc. PcG proteins establish and maintain eye fate within progenitors (orange region) of the eye disc. Based on our findings, we propose that PhoRC initially recruits PRC1 and PRC2 to a PRE site within the *Antp* locus during early embryogenesis. During late embryogenesis and the first larval instar (L1) stage, the PRC1/PRC2 complexes spread along the entire *Antp* locus, possibly through SAM domain-mediated polymerization. From the second larval instar (L2) stage onwards, PRC1 and PRC2 remain and compact chromatin, thereby permanently silencing the *Antp* locus.

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transformation could no longer be triggered if *Pc* mutant clones are induced in the leg disc later than 24 h AEL (Tiong and Russell, 1990). We propose that the resistance of the eye disc to loss of Sfmbt could be due to the presence of the RD network. Thus, reduction in levels of Pax6 might cause a delay in the differentiation of eye progenitor cells, thereby keeping these cells in an earlier and developmentally more pluripotent state. As a result, the concomitant loss of Pax6 and PhoRC during late embryogenesis results in the reprogramming of the eye into a wing. However, knocking down other RD genes, such as *eya* or *so*, is not sufficient to cause this PcG loss-dependent tissue transformation. *eya* and *so* are essential for photoreceptor differentiation, but are activated by Pax6 in later development (from the early second instar). Our results indicate that simply blocking the eye development at any stage cannot trigger the reprogramming of the eye to a wing, indicating that cells in the eye field have already committed to the fate of the head segment.

In conclusion, our finding that PcG complexes function to prevent the eye imaginal disc from adopting a wing fate provides a potential way to understand transdetermination events. The path that the eye-antennal disc takes is not straight. Instead, it looks much like a creode that was described for a cell by C. H. Waddington (Waddington, 1957). Early in development, the progenitor cells in the eye disc have many paths that they can take, including all the fates that are found within the eye-antennal disc and some that are normally reserved for other discs. A combination of transcription factors and epigenetic enzymes work to push the eye disc down the desired path. Alterations to the levels of these factors can drive the eye towards alternative fates such as those of the antenna, leg, head epidermis and wing. These findings coupled with further research on the epigenetic regulation of disc development will provide a clearer understanding of organ growth, tissue specification, pattern formation and regeneration.

MATERIALS AND METHODS

Fly stocks and clonal analysis

The following fly stocks were used: (1) *DE-GAL4* (Georg Halder, Katholic University, Leuven, Belgium); (2) *UAS-Pc RNAi* [Bloomington Drosophila Stock Center (BDSC), Indiana University, Bloomington, USA]; (3) *UAS-tsh RNAi* (BDSC); (4) *ey-GAL4* (BDSC); (5) *Sfmbt*^{1} (Jurg Muller, Max Planck Institute of Biochemistry, Martinsried, Germany); (6) *UAS-ey RNAi* (BDSC); (7) *UAS-toy RNAi* (BDSC); (8) *UAS-Sfmbt RNAi* (BDSC); (9) *FRT40A Ubi GFP/CyO* (BDSC); (10) *ey-FLP* (BDSC); (11) *eya*^{2} (Nancy Bonini, University of Pennsylvania, Philadelphia, USA); (12) *UAS-GFP* (BDSC); (13) *tub-GAL80*^{ts} (BDSC); (14) *dilp8-GFP* (BDSC); (15) *UAS-E(z) RNAi* (BDSC); (16) *UAS-ph-d RNAi* (BDSC); (17) *UAS-ph-p RNAi* (BDSC); (18) *UAS-pho RNAi* (BDSC); and (19) *ey*^{2} (BDSC). All crosses were conducted at 25°C except for time-course experiments, which were conducted at 18°C and/or 30°C.

Antibodies and microscopy

The following primary antibodies were used: (1) rabbit anti-Vg (1:50, Sean Carroll, University of Wisconsin, Madison, Wisconsin, USA); (2) mouse anti-Eya [1:4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, USA]; (3) rat anti-Ci (1:100, Robert Holmgren, Northwestern University, Evanston, USA); (4) mouse anti-En (1:100, DSHB); (5) mouse anti-Antp 8C11 (1:100, DSHB); (6) rabbit anti-Pc (1:100, Vincenzo Pirrotta, Rutgers University, Piscataway, USA); (7) mouse anti-Ubx (1:500, DSHB); (8) mouse anti-Ey (1:100, DSHB); (9) rat anti-Elav (1:100, DSHB); (10) guinea pig anti-Toy (1:500, Henry Sun, Academia Sinica, Taipei, Taiwan); (11) mouse anti-Dll (1:500, Dianne Duncan, Washington University, St. Louis, Missouri, USA); (12) mouse anti-Cut (1:100, DSHB); (13) mouse anti-Wg (1:800, DSHB); (14) mouse anti-Scr (1:100, DSHB); (15) mouse anti-AbdB (1:50, DHSB); (16) rabbit anti-Dfd (1:80, Thom Kaufman, Indiana University, Bloomington, USA); (17) rabbit anti-Pb (1:50, Thom Kaufman); (18) rabbit anti-Lab (1:50, Thom Kaufman); and (19) chicken anti-β-gal (1:800, Abcam). Fluorophore-conjugated secondary antibodies and phalloidin-fluorophore conjugates were obtained from Molecular Probes (Invitrogen). Imaginal discs were prepared as previously described (Spratford and Kumar, 2014) and photographed using either a Zeiss Axioplan II compound microscope or a Leica SP5 Scanning Confocal microscope. Adult flies were viewed with a Zeiss Discovery light microscope and a Leica M205FA Stereo microscope.

Quantification of eye-to-wing transformations and the size of eye fields

An eye-to-wing transformation was scored as such if we were able to identify a new wing pouch. Adobe Photoshop CC 2015 was used to outline and measure the area of the eye field. Statistical significance was calculated using one-way ANOVA with GraphPad Prism.

Time-course experiments and clonal analysis

tub-Gal80^{ts}*; DE-GAL4>Pc RNAi*, *tub-Gal80*^{ts}*; DE-GAL4>Sfmbt RNAi+ey* RNAi or *tub-Gal80*^{ts}*; DE-GAL4>Sfmbt RNAi+toy* RNAi eggs were collected for 2 h at 25°C and then kept at 18°C (permissive temperature) for various time periods before being shifted to 30°C (non-permissive temperature). Eye-antennal discs were dissected either at the wandering third instar larval stage or at defined time points after shifts in temperature. *Sfmbt*^{1} loss-of-function clones were induced in eye-antennal discs of *ey-FLP; FRT40A Ubi-GFP/FRT40A Sfmbt*^{1} flies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.Z., J.P.K.; Formal analysis: J.Z., J.P.K.; Investigation: J.Z., A.J.O., L.W., K.B.; Writing - original draft: J.Z., J.P.K.; Writing - review & editing: J.Z., A.J.O., J.P.K.; Supervision: J.Z., J.P.K.; Project administration: J.Z., J.P.K.; Funding acquisition: J.Z., J.P.K.

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Supplementary information

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