Retinal determination genes coordinate neuroepithelial specification and neurogenesis modes in the *Drosophila* optic lobe

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ABSTRACT

Differences in neuroepithelial patterning and neurogenesis modes contribute to area-specific diversifications of neural circuits. In the *Drosophila* visual system, two neuroepithelia, the outer (OPC) and inner (IPC) proliferation centers, generate neuron subtypes for four ganglia in several ways. Whereas neuroepithelial cells in the medial OPC directly convert into neuroblasts, in an IPC subdomain they generate migratory progenitors by epithelial-mesenchymal transition that mature into neuroblasts in a second proliferative zone. The molecular mechanisms that regulate the identity of these neuroepithelia, including their neurogenesis modes, remain poorly understood. Analysis of Polycomblike revealed that loss of Polycomb group-mediated repression of the Hox gene *Abd-B* caused the transformation of OPC to IPC neuroepithelial identity. This suggests that the neuroepithelial default state is IPC-like, whereas OPC identity is derived. Ectopic Abd-B blocks expression of the highly conserved retinal determination gene network members *Eya*, *So* and *Hth*. These factors are essential for OPC specification and neurogenesis control. Finally, *eya* and *so* are also sufficient to confer OPC-like identity, and, in parallel with *hth*, the OPC-specific neurogenesis mode on the IPC.

KEY WORDS: Visual system, neuroepithelial specification, Neurogenesis, Polycomblike, Retinal determination genes, *Drosophila*

INTRODUCTION

Mature brains are built of interconnected neural circuits, development of which commonly begins with simple neuroectodermal or neuroepithelial (NE) sheets. In an initial proliferative phase, NE cells expand by symmetric cell divisions and become patterned into discrete territories. During a subsequent neurogenic phase, NE cells convert into neural stem cells (NSCs), which undergo asymmetric self-renewing divisions to produce diverse neuronal and glial subtypes (Paridaen and Huttner, 2014). Recent studies in vertebrates uncovered additional cortical neural stem and progenitor cell types with distinct morphologies, division modes and cellular behaviors that contribute to area- and species-specific differences in neurogenesis (Taverna et al., 2014). Similarly, in *Drosophila*, several neurogenesis modes have been identified in the embryonic and postembryonic brain and ventral nerve cord (VNC) (Yasugi and Nishimura, 2015). However, the molecular mechanisms that potentially could link region-specific patterning and neurogenesis modes remain poorly understood.

In the *Drosophila* visual system, photoreceptors (R-cells R1-R8) within the compound eye extend axons into the optic lobe consisting of four ganglia: the lamina, medulla, lobula plate and lobula (Fig. 1A). R-cell axons and highly diverse sets of target neuron subtypes are interconnected in a complex retinotopic map dedicated to processing visual information (Hadjieconomou et al., 2011). In contrast to other fly brain areas, these higher-order neurons are generated by two persisting postembryonic neuroepithelia, called the outer and inner proliferation centers (OPC and IPC; Fig. 1B,C) (Apitz and Salecker, 2014; Hofbauer and Campos-Ortega, 1990; White and Kankel, 1978). The IPC primarily gives rise to neurons associated with the lamina and medulla, and the IPC with the lobula plate and lobula (Hofbauer and Campos-Ortega, 1990). Both neuroepithelia are derived from the optic lobe placode, which arises during embryogenesis by invagination from the ectoderm and subsequently attaches to the lateral brain surface (Green et al., 1993). Already by the first instar larval stage, optic placode cells are partitioned into the two primordia (Apitz and Salecker, 2015). These initially expand by symmetric divisions into the two horseshoe-shaped OPC and IPC neuroepithelia (Egger et al., 2007; Nassif et al., 2003). During the late second instar larval stage, neurogenesis is initiated in the OPC, followed by the IPC (Hofbauer and Campos-Ortega, 1990). Recent studies uncovered that these neuroepithelia employ at least three distinct neurogenesis modes. First, NE cells at the lateral OPC edge give rise to lamina precursor cells (LPCs), which divide once to produce lamina neurons. Two R-cell axon-derived anterograde signals, Hedgehog (Hh) and the epidermal growth factor (EGF) homolog Spitz, promote the final division of LPCs and the generation and differentiation of lamina neurons, respectively (Huang and Kunes, 1996; Huang et al., 1998). Lamina neurogenesis further depends on the activity of the orphan nuclear receptor Taillless (Tll) (Guillermín et al., 2015) and retinal determination gene network (RDGN) members within LPCs (Pineiro et al., 2014). Second, NE cells at the medial OPC edge gradually convert into NSCs, called neuroblasts (Nbs), in a proneural wave, timely progression of which is controlled by several signaling pathways (Egger et al., 2010; Reddy et al., 2010; Yasugi et al., 2008, 2010). Nbs follow the common type I proliferation pattern by dividing asymmetrically to self-renew and produce ganglion mother cells (GMCs), which in a final division give rise to medulla neurons (Brand and Livesey, 2011; Egger et al., 2007). A series of temporal transcription factors – Homothorax (Hth), Eyeless (Ey), Sloppy paired (Slp), Dichate (D) and Tll – controls subtype diversification of medulla Nb progeny (Li et al., 2013; Suzuki et al., 2013). Third, in a subdomain of the IPC, the proximal IPC (p-IPC), NE cells convert into progenitors by a mechanism that shares characteristics with epithelial-mesenchymal transition (EMT) (Apitz and Salecker, 2015). These progenitors
migrate to a secondary proliferation zone, the distal IPC (d-IPC), where they mature into Nbs. Cross-regulatory interactions between D and Tll mediate a switch in Nb competence to generate two neuron populations: distal cells and lobula plate neurons (Apitz and Salecker, 2015). How the OPC and IPC are specified as distinct neuroepithelia and how this correlates with the control of their characteristic neurogenesis modes is currently unexplored.

To gain insights into the underlying mechanisms, we conducted a forward genetic mosaic screen for mutants that affected OPC and IPC development. We isolated a new allele of the epigenetic regulator Polycomblike (Pcl) that caused the formation of conspicuous ectopic NE cell clusters within the lobula plate area. Detailed analysis of observed phenotypes revealed that large clusters originated from the OPC and adopted IPC characteristics. Pcl is required for maintaining OPC identity by preventing ectopic expression of the Hox gene Abdominal-B (Abd-B) and thus interference with area-specific determinants. Our search for these factors uncovered that the optic lobe NE default state is IPC-like, whereas the RDGN members Eyes absent (Eya), Sine oculis (So) and Hth (Kumar, 2010) confer OPC identity to NE cells and concomitantly mediate coordinated Nb generation by direct conversion.

**RESULTS**

**A genetic screen for determinants regulating OPC and IPC development**

To uncover the molecular pathways that control NE patterning in the OPC and IPC, we performed an ethyl methane sulfonate (EMS)-based forward genetic mosaic screen for chromosome 2R using the ELF system (Fig. 1D; for details see supplementary Materials and Methods). This approach relies on three transgenes, ey3.5-Gal80, lama-Gal4 and UAS-FLP, to generate homozygous mutant somatic clones in the optic lobe, while leaving wild-type activity in the eye (Bazigou et al., 2007; Chotard et al., 2005). Optic lobes were initially screened for R-cell projection pattern defects as a sensitive readout for patterning errors. Subsequently, optic lobes of mosaic animals that exhibited phenotypes were labeled with an antibody against the cell adhesion molecule Fasciclin 3 (Fas3) to distinguish the IPC and its offspring from the OPC (Apitz and Salecker, 2015; Hayden et al., 2007; Taylor et al., 2004). One mutant, 3-78, as well as a derived line, 3-78*38, in which second site lethal mutations were identified by deficiency mapping and removed by meiotic recombination, displayed two remarkable phenotypes in ELF mosaics. Large ectopic Fas3-positive NE cell clusters formed in the lobula plate area during postembryonic development and persisted into adulthood. Moreover, small Fas3-positive clusters accumulated in close vicinity of OPC and p-IPC NE cells (Fig. 1E-K, Fig. S1A-D).

Complementation assays and sequence analyses (Fig. 1L) identified the affected gene as a novel mutant allele of Pcl (Lonie et al., 1994), which we named Pcl3-78*38. Pcl and its vertebrate homolog PHF1 belong to the highly conserved Polycomb group (PCG) family of chromatin-modifying proteins. These form two functionally distinct Polycomb repressive complexes, PRC1 and PRC2 (Muller and Verrijzer, 2009). Pcl joins the PRC2 complex to mediate high levels of histone 3 lysine 27 trimethylation and, thus, effective target gene silencing (Nekrasov et al., 2007; Sarma et al., 2008). Pcl3-78*38 carries a G-to-A base pair substitution in the open reading frame, resulting in a premature stop codon at amino acid position 536 (Fig. 1L). This truncates the second of two plant homeodomain (PHD) fingers, abolishing the interaction of Pcl with PRC2 (O’Connell et al., 2001). Another allele, Pcl27T7a (Gaytan de Ayala Alonso et al., 2007), caused similar Fas3-positive cell clusters in larval and adult optic lobes (Fig. S1E,F), confirming Pcl as the responsible gene. Finally, expression of a reporter transgene for the IPC-specific NE cell marker hairy (h-lacZ; Southall et al., 2013) provided additional evidence that Pcl3-78*38 mutant clusters consisted of NE cells with IPC identity (Fig. 1M,N).

**PcG members are required for the acquisition of OPC identity**

The expression of Fas3 and h-lacZ within large ectopic cell clusters in the lobula plate area suggested that these originated from the p-IPC. However, two observations support the notion that these clusters are derived from the OPC. First, 3D analysis of samples, stained with the cell polarity marker atypical protein kinase C (aPKC) to facilitate tracing of epithelial membranes, revealed that ELF system-induced clusters were continuous with the OPC neuroepithelium (Fig. 2A-C). Ectopic clusters formed immediately adjacent to the dorsal and ventral Decapentaplegic (Dpp)-expressing OPC subdomains (Fig. 2D; Kaphingst and Kunes, 1994). Second, to induce clone formation solely in the IPC, but not the IPC, we utilized eyeless (ey)-FLP (Newsome et al., 2000) as recombine source (Fig. 2E). These Pcl3-78*38 clones formed large h-lacZ-positive NE cell clusters (Fig. 2F) at similar positions as those generated by the ELF system.

To test whether large clusters arose because of increased proliferation, optic lobes were labeled with the mitotic marker phoshoHistone 3 (PH3). Mutant clusters were discernible from the early third instar larval stage onwards (Fig. 2G-I). Comparing Pcl3-78*38 mutant ectopic clusters with wild-type OPC NE cells in wandering third instar larvae, we observed a slight nonsignificant decrease in mitotic activity. Moreover, the average number of mitotic cells in ectopic clusters in early and wandering third instar larvae remained constant (Fig. 2J,K; Fig. S1G; for details, see supplementary Materials and Methods). Abundance of staining with the Nb-specific markers Miranda (Mira) and Deadpan (Dpn) around Pcl3-78*38 mutant clusters indicated that these did not generate Nbs (Fig. 2L,M). Finally, using mosaic analysis with a repressible cell marker (MARCM; Lee and Luo, 1999) we found that, similar to Pcl3-78*38, OPC NE cells mutant for the PRC1 component Sex combs extra (Sce) ectopically expressed Fas3 (Fig. 2N,O). This observation indicates that Pcl functions within the context of PRC1 and PRC2. Thus, impaired Pcl function leads to the formation of large clusters not by affecting proliferation, but by altering the identity of OPC NE cells, including their ability to generate Nbs.

**PcG members repress Abdominal-B in the OPC**

One key function of PcG proteins is to silence Hox genes (Lanuzolo and Orlando, 2012). These encode evolutionarily conserved homeodomain-containing transcription factors essential for patterning tissues along the anterior-posterior body axis (Peter and Davidson, 2011). Hox genes have to be tightly regulated in the areas where they are not normally active, because ectopic posterior homeotic genes can repress their anterior counterparts or interfere with the activity of signaling pathways and endogenous determinants (Gehring et al., 2009). Examining Abdominal-B (Abd-B), Sex combs reduced (Scr) and Ultrabithorax (Ubx) we found that, similar to Pcl3-78*38 OPC NE cells mutant for the PRC1 component Sex combs extra (Sce) ectopically expressed Fas3 (Fig. 2N,O). This observation indicates that Pcl functions within the context of PRC1 and PRC2. Thus, impaired Pcl function leads to the formation of large clusters not by affecting proliferation, but by altering the identity of OPC NE cells, including their ability to generate Nbs.
sufficient to induce their formation (Fig. 3L,M). Together, this indicates that in Pcl clones, ectopic Abd-B mediates the change of NE cells from an OPC fate to an IPC-like fate.

**Ectopic Abd-B interferes with Decapentaplegic-dependent EMT and progenitor differentiation in the IPC**

Loss of Pcl causes the formation of large ectopic OPC-derived clusters, as well as small clusters adjacent to the p-IPC (Fig. 1K). We previously had observed a similar phenotype upon removal of the BMP type I receptor *thickveins (tkv)*, consistent with a requirement of the TGFβ family member Dpp for EMT in IPC subdomains (Apitz and Salecker, 2015). We therefore tested whether loss of Pcl or ectopic Abd-B could interfere with Dpp signaling. Consistent with known regulatory interactions between ectopic Hox gene expression and Dpp signaling in wing imaginal discs (Crickmore and Mann, 2006), PclΔ78–38 clones failed to express dpp-lacZ at the anterior-posterior boundary (Fig. 4A,B). Similarly, small PclΔ78–56 clusters adjacent to the p-IPC did not express dpp-lacZ (Fig. 4C,D), but upregulated Abd-B (Fig. 4E). Consistent with this, Abd-B overexpression was sufficient to

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**Fig. 1. Identification of Pcl in a screen for genes controlling NE patterning.** (A,B) Schematic of adult (A) and third instar larval (B) *Drosophila* optic lobes in horizontal and lateral views. The outer and inner proliferation centers (OPC, IPC) and progeny are shown in magenta and green, respectively. (C) In third instar larval optic lobes, horizontal and lateral views. The outer and inner proliferation centers (OPC, IPC) and progeny are shown in magenta and green, respectively. (C) In third instar larval optic lobes, horizontal and lateral views. The outer and inner proliferation centers (OPC, IPC) and progeny are shown in magenta and green, respectively. (C) In third instar larval optic lobes, horizontal and lateral views. The outer and inner proliferation centers (OPC, IPC) and progeny are shown in magenta and green, respectively. (C) In third instar larval optic lobes, horizontal and lateral views. The outer and inner proliferation centers (OPC, IPC) and progeny are shown in magenta and green, respectively.
ectopic Abd-B maturing into Nbs by a mechanism that is sensitive to loss of Hh. Hence, migratory progenitors within the IPC are prevented from giving rise to Nbs in the presence of ectopic Abd-B (Fig. 4H,I; Apitz and Salecker, 2015). Again, ectopic Abd-B differentiated into Ase-expressing Nbs within cell streams of p-IPC cells (Fig. 4G). The contribution of ectopic Abd-B and Hh interference with Dpp-mediated EMT in a subset of IPC cells was confirmed immunohistochemically (Bonini et al., 1993; Pineiro et al., 2014; Southall et al., 2013). However, their expression had not been compared between the OPC and IPC, and their function in the optic lobe (Cheyette et al., 1994; Daniel et al., 1999; Pineiro Kumar, 2010). Some members are also expressed and required in genes that distinguish the OPC from the IPC. Hence, our previous data can therefore serve as powerful genetic tools to uncover the genes that distinguish the OPC from the IPC.

**RDGN members are specifically expressed in the OPC**

In eye-antenna disc epithelia, the RDGN bestows retinal identity (Kumar, 2010). Some members are also expressed and required in the optic lobe (Chayote et al., 1994; Daniel et al., 1999; Pineiro et al., 2014; Southall et al., 2013). However, their expression had not been compared between the OPC and IPC, and their function in the medial OPC is not well understood. Using antisera or genetic markers, we detected the transcription factor and protein phosphatase Eya (Bonini et al., 1993), the SIX protein So

repress dpp-lacZ in p-IPC subdomains (Fig. 4F). Thus, loss of Pcl and ectopic Abd-B interfere with Dpp-mediated EMT in a subset of p-IPC cells (Fig. 4G).

Furthermore, we had observed in MARCM experiments that migratory progenitors in the IPC that lacked Pcl prematurely differentiated into Asense (Ase)-expressing Nbs within cell streams of p-IPC cells (Fig. 4H,I; Apitz and Salecker, 2015). Again, ectopic Abd-B expression was sufficient to induce a similar phenotype (Fig. 4J). Hence, migratory progenitors within the IPC are prevented from maturing into Nbs by a mechanism that is sensitive to loss of Pcl and ectopic Abd-B. Defects observed following Pcl loss are primarily caused by ectopic Abd-B activity. Genetic manipulations of Pcl and Abd-B can therefore serve as powerful genetic tools to uncover the genes that distinguish the OPC from the IPC.
Cross-regulatory interactions are a hallmark of RDGN function in the developing eye epithelium (Kumar, 2010). Therefore, we assessed RDGN member expression in OPC NE cells in eya^{clift} and so^{3} clones, as well as hth, eya and so single or double knockdown experiments. Knockdown was achieved using validated UAS-RNA interference (RNAi) transgenes in combination with esg^{M676-Gal4} and ey^{3.5-Gal80} transgenes (Fig. S3E–M'). These experiments revealed that in the OPC NE domain dedicated to generating medulla neurons, so expression depends on eya expression and vice versa (Fig. 5H–N).

**RDGN members contribute to medial OPC development**

Based on the observed expression pattern, we next examined the role of eya, so and hth in the OPC neuroepithelium. Previous studies had shown that Notch (N) signaling maintains the NE state of the OPC, and that downregulation, in part mediated by the proneural factor Lethal of scute [L^{sc}; L(1)sc], is required for the timely conversion of medial NE cells into medulla Nbs (Egger et al., 2010; Ngo et al., 2010; Orihara-Ono et al., 2011; Reddy et al., 2010; Wang et al., 2011; Yasugi et al., 2010) (Fig. 6A). We observed that knockdown of eya and so specifically in the optic lobe induced phenotypes consistent with previously reported defects linked to N signaling (Egger et al., 2010). Compared with wild type, N expression appeared reduced and diffuse in OPC NE cells (Fig. 6B,C). Labeling optic lobes with the Nb/GMC-specific marker Ase showed that clusters of Nbs/GMCs were

![Diagram](image-url)
mispositioned in the medulla cortex, or areas containing these cell types were expanded within gaps in the OPC neuroepithelium (Fig. 6D,E). In controls, Lsc was expressed in one or two NE cells that converted next into Nbs (Fig. 6A,F) (Yasugi et al., 2008), but knockdown of eya and so increased the number of cells expressing this factor (Fig. 6G,H). As newborn Nbs mature, they successively express a series of temporal transcription factors (Hth, Ey, Stp, D and Tll) that control medulla neuron subtype differentiation in a birth order-dependent manner (Li et al., 2013; Suzuki et al., 2013) (Fig. 6F).Despite knockdown of eya and so, Nbs were able to express these markers (Fig. 6I-L; data not shown).

By contrast, knockdown of hth, which is expressed in OPC NE cells and Nbs, did not affect N expression (Fig. 6M), but reduced the volume of Nbs and GMCs in the OPC by ~37% (Fig. 6N-P). Consistently, Ey-positive Nbs and their progeny were located in closer proximity to OPC NE cells (Fig. 6Q,R). This suggests that hth is required in OPC NE cells to provide input into the number of generated Nbs that can progress through the temporal cascade. Hence, eya, so and hth contribute to the specification of the OPC and its distinct neurogenesis mode by direct conversion of NE cells to Nbs.

**Loss of Pcl and ectopic Abd-B affect OPC-specific expression of RDGN members**

Subsequently, we tested our hypothesis that loss of Pcl and ectopic Abd-B could cause the formation of large OPC-derived ectopic clusters with IPC-like identity by interfering with the expression of OPC-specific RDGN members. We observed that, in addition to defects within the OPC crescent, *eya* and *so* expressed in these ectopic clusters (Fig. 7A,B). Clusters failed to express Lsc (Fig. 7C), suggesting that the conversion into Nbs was blocked. However, they did not upregulate Fas3, indicating that the switch to an IPC identity was partial (Fig. 7A,B). Also, hth-deficient MARCM clones did not express Fas3 (Fig. 7D), excluding Hth as the sole determinant responsible for Fas3 suppression. To determine whether so, eya and hth act redundantly, we simultaneously knocked down their expression. Whereas optic lobe development was impaired, NE cells did not express ectopic Fas3 (Fig. 7E). Finally, we assessed a possible contribution of Optix (Fig. 7F; Fig. 5A,B). Although the simultaneous knockdown of eya, hth and Optix affected all four genes (because so depends on eya), it did not result in ectopic Fas3 expression (Fig. 7G). This indicates that RDGN members alone do not confer full OPC identity.

Next, we examined the effects of loss of Pcl and ectopic Abd-B on RDGN member expression. In *Pcl* d-IPC mosaics, Eya and Hth were decreased in OPC NE cells and in large ectopic cell clusters (Fig. 7H-K). Consistent with this, overexpression of Abd-B in the OPC using the hs-FLPout approach led to upregulation of Fas3 and downregulation of Eya and Hth (Fig. 7M-O). By contrast, the homeodomain-containing transcription factor Cut (Blochlinger et al., 1990), which is expressed in the OPC and IPC (Fig. S4C,D),

**Fig. 4. Ectopic Abd-B affects Dpp-dependent EMT and progenitor differentiation in the IPC.** (A–B) Unlike in controls (A,A'), *dpp-lacZ* expression (blue) at the anterior-posterior boundary (arrow) was repressed in Pcl3-78*38* ELF mosaic wing imaginal discs (B,B, asterisk). (C–E) Unlike in controls (C), Pcl3-78*38* ELF mosaics (D) showed small Fas3-positive (red) clusters (arrowheads) close to the p-IPC (dashed line). These were *dpp-lacZ* negative (blue, D') and Abd-B positive (red, E, inset). (F) *hs-FLPout* clones (green) expressing ectopic Abd-B formed small clusters (arrowheads) adjacent to p-IPC (dashed line) subdomains and downregulated *dpp-lacZ* (blue; inset, white; arrowheads). (G) Model of Pcl function in p-IPC subdomains. (H–J) Progenitor cell streams between the p-IPC and d-IPC in *esg*MN766-Gal4, UAS-cd8GFP (green) animals did not express Ase (blue) (H,H'). Pcl3-78*38* mutant progenitors (green) generated by MARCM ectopically expressed Ase (blue, arrows) (I,I'). Ectopic expression of Abd-B using the *hs-FLPout* approach had the same effect (J,J', arrows). For genotypes and sample numbers, see Table S1. Scale bars: 50 µm.
subsequently mature into Nbs in the d-IPC. Whereas OPC, the p-IPC gives rise to migratory progenitors, which eya co-regulated, we monitored the effects of ectopically expressing Finally, to assess whether NE specification and neurogenesis are remained unaffected (Fig. 7L). Hence, loss of Pctl and ectopic Abd-B specifically affected the expression of OPC-specific RDGN members. We propose that interference with these and additional determinants transform the OPC into an undifferentiated IPC-like neuroepithelium (Fig. 7P).

eya and so are sufficient to induce OPC-like identity

Finally, to assess whether NE specification and neurogenesis are co-regulated, we monitored the effects of ectopically expressing eya, so and hth in the p-IPC neuroepithelium. In contrast to the OPC, the p-IPC gives rise to migratory progenitors, which subsequently mature into Nbs in the d-IPC. Whereas eya or so alone had only mild effects (Fig. S4E-H), co-expression of eya and so reduced Fas3 levels in p-IPC NE cells and, strikingly, induced the direct conversion of p-IPC NE cells into Dpn- and Mira-positive Nbs (Fig. 8A,B; Fig. S4I). Although ectopic Hth did not affect Fas3 levels (Fig. 8C), it triggered the conversion of p-IPC NE cells into Nbs (Fig. 8D; Fig. S4J). The proneural gene l′sc, the Notch target gene E(spl)m5 HLI and the EGF receptor target gene pointed (pnt) serve as proneural wave markers in the OPC (Yasugi et al., 2010). We observed that these genes were expressed in OPC and p-IPC neuroepithelia (Apitz and Salecker, 2015; Fig. S4K,L) and therefore could not be used as OPC-specific readouts. However, eya and so overexpression induced ectopic Hth (Fig. 8E), which also constitutes the first temporal marker in the medulla (Li et al., 2013; Suzuki et al., 2013). Upon ectopic activation of Eya and So but not Hth, Nbs in the p-IPC, as well as Nbs and Elav-positive neuronal progeny in the d-IPC, expressed the second OPC-specific temporal marker Ey (Fig. 8F,G; Fig. S4M). Consistent with a progression through the OPC-specific temporal series, more Hth- than Ey-positive cells (Hth: 23.67±5.06 95% confidence interval; Ey: 4±1.4 95% confidence interval; n=12 optical sections from four optic lobes each) were found in the proximity of transformed OPC-like NE cells (unpaired, two-tailed Student’s t-test, Welch corrected, P=1.42×10⁻⁶). Conversely, the d-IPC specific factors Atonal (Ato) and Dac failed to be expressed (Fig. 8H-K). Collectively, these findings show that eya and so play an instructive role in inducing OPC identity and in concert with hth promote Nb formation by direct transformation of NE cells, thus imposing an OPC-like mode of neurogenesis on the IPC (Fig. 8L).
DISCUSSION

Three roles of Pcl in optic lobe development

Chromatin-regulating proteins play pivotal context-dependent roles during neural development (Ronan et al., 2013). Despite the insights into the molecular function of Pcl within PRC2 (Nekrasov et al., 2007), our understanding of its in vivo roles in the CNS remained limited. Our findings uncovered central roles of Pcl in NE specification, EMT of migratory progenitors and their timely maturation into Nbs. Pcl does not regulate proliferation, and thus differs from some PcG members, such as the PRC1 components Posterior sex combs and Suppressor of zeste 2, loss of which causes tumor-like growth in imaginal discs (Classen et al., 2009) and the optic lobe (H.A. and I.S., unpublished). These roles are also distinct from those reported for other PcG genes in the central brain and VNC, as these regulate Nb survival and maintenance of neuronal identity in these contexts (Bello et al., 2007; Wang et al., 2006). Pcl mutant cells in the optic lobe specifically upregulated Abd-B, and loss of Pcl and overexpression of Abd-B caused qualitatively similar defects. This is consistent with the known posterior prevalence of Hox genes, whereby posterior genes are epistatic to anterior ones (Gehring et al., 2009). Thus, Pcl function in the optic lobe probably cannot be uncoupled from the fundamental developmental role of PcG members in silencing ectopic Hox expression. Although the mechanisms underlying Hox-mediated defects remain to be investigated, previous studies suggest that this may involve transcriptional repression (Vachon et al., 1992) or competitive protein-protein interactions (Gehring et al., 2009; Plaza et al., 2008, 2001).

The IPC reflects a neuroepithelial default state in the optic lobe

The ability of Pcl loss to interfere with the activity of local transcriptional networks and signaling pathways revealed that the NE default state in the optic lobe is IPC-like, whereas OPC NE identity is derived. Several lines of evidence support this notion: First, IPC NE cells express the cell adhesion molecule Fas3 from the first instar larval stage onwards (Apitz and Salecker, 2015; Hayden et al., 2007; Tayler et al., 2004), whereas the RDGN members Eya and Hth are specific to the OPC with similar developmental timing. Second, Pcl mutant clusters originating from the OPC ectopically express Fas3 and h-lacZ. Interestingly, Fas3 was first identified as the antigen of a monoclonal antibody with high specificity to undifferentiated epithelial cells of imaginal discs (Brower et al., 1980). Third, the RDGN members Eya, So and Hth contribute to establishing the OPC
as distinct neuroepithelium including the neurogenesis mode by direct NE-to-Nb conversion in the medulla. Eya and So together are sufficient to confer OPC-like identity on the p-IPC, which includes Fas3 downregulation. Fourth, ectopic Abd-B in Pcl mutant clones and in Abd-B gain-of-function experiments interfered with the expression of the OPC-specific genes eya, so and hth, and resulted in the formation of large NE cell clusters expressing IPC markers. Although our findings reveal effects for two neuroepithelia, they are consistent with the concept of neuronal homeosis and the relevance of recruiting determinants into new contexts to generate cellular diversity in the nervous system (Arlotta and Hobert, 2015).

Although the NE default state is IPC-like, full p-IPC differentiation and neurogenesis clearly depend on additional factors, such as Dpp signaling, which is required for EMT of migratory progenitors in p-IPC subdomains (Apitz and Salecker, 2015). Consistent with this, although large ectopic Pcl mutant NE cell clusters displayed a similar proliferation rate as wild-type OPC NE cells, they did not generate any neuronal progeny and persisted until adulthood. Two reasons may explain this lack of neurogenesis. Clusters are found in areas that may be spatially segregated from signals that promote neurogenesis in the IPC. Furthermore, ectopic Abd-B expression in the clusters may interfere with these signals, if they are present. This idea would be consistent with our observation that ectopic Abd-B affects Dpp signaling in the p-IPC, as well as maturation of progenitors into Nbs.

RDGN members control OPC specification and direct NE-to-Nb conversion

Our genetic analyses revealed central roles for eya and so in medial OPC development. They are necessary for OPC specification and maintenance, because their knockdown interfered with coordinated NE-to-Nb conversion by directly or indirectly impairing N signaling in the OPC. Moreover, eya and so are sufficient for OPC specification, because ectopic expression in the IPC suppressed Fas3, altered the neurogenesis mode to that of the medial OPC, and triggered the generation of Nbs expressing two temporal series markers, Hth and Ey. However, eya and so are not required for Hth or Ey induction, and thus may not act directly upstream of the temporal series of
transcription factors in medulla Nbs (Hasegawa et al., 2011; Li et al., 2013; Suzuki et al., 2013). Knockdown of hth reduced OPC Nb and GMC numbers without affecting N, and ectopic expression in the p-IPC triggered Nb formation by direct conversion. Therefore, in addition to neuron subtype specification (Hasegawa et al., 2011; Li et al., 2013; Suzuki et al., 2013), hth may independently influence the conversion and number of Nbs, potentially by also regulating OPC NE proliferation (Pineiro et al., 2014).eya, so and hth knockdown in different combinations did not lead to Fas3 upregulation in OPC NE cells, indicating that Eya, So and Hth function redundantly with additional factors. This does not include the Six family member Optix, as simultaneous knockdown of Optix, hth, eya, and consequently also so because of its dependence on eya, did not result in ectopic Fas3 expression in OPC NE cells.

Our findings provide additional evidence for the notion that in the Drosophila visual system, RDGN core components engage in versatile cross-regulatory interactions and subcircuits to control eye (Atkins et al., 2013; Silver and Rebay, 2005), lamina (Pineiro et al., 2014) and medulla development. For instance, in eye imaginal discs, anterior to the morphogenetic furrow, positive-feedback loops between eya, so and ey induce Dac and maintain Ey expression, whereas posterior to the furrow, So, upregulated by Eya, and Dac repress ey transcription (Atkins et al., 2013). Ey is not expressed in the lamina, but acts as a member of temporal transcription factors in medulla Nbs. Although eya and so can induce the ectopic formation of Ey-positive Nbs in the p-IPC, they are not required for Ey expression in medulla Nbs. In the lamina, eya and so cooperate with R-cell axon-derived Hh signaling to activate the core RDGN member Dac, which in turn represses hth (Chotard et al., 2005; Huang and Kunes, 1996; Pineiro et al., 2014). By contrast, in the medulla, eya and so do not interact with Dac, and are not essential for hth regulation, expression of which is maintained in NE cells and first-born medulla Nbs.

Eya and So play a central role in distinguishing the OPC from the IPC. Although cranial placodes and neural crest are vertebrate innovations (Northcutt, 2005), Eya and Six proteins could play an analogous role in vertebrates by delineating adjacent epithelial head territories that generate sensory placodes from those dedicated to neural crest and epidemis (Christophorou et al., 2009). Six and Eya have been discussed as a driving force for the formation of vertebrate
placodes by the acquisition of a novel function in NE patterning and proliferation in addition to its ancient function in neuronal differentiation to generate a larger density of specialized neurons (Schlosser et al., 2014). Interestingly, recent studies uncovered the existence of neurogenic proto-placodal ectoderm expressing the Six1/2 and Eya homologs in the tunicate Ciona intestinalis (Abitua et al., 2015), suggesting a conserved pre-vertebrate role of these two genes in regional patterning of epithelia with neurogenic potential. These and our findings support the notion that the ancestral gene regulatory cassette of Eya and So may have been re-employed several times to impart specific cellular properties, including neuroepithelial specification, during invertebrate and vertebrate evolution.

MATERIALS AND METHODS

Drosophila stocks and husbandry

Drosophila melanogaster crosses were maintained in standard medium at 25°C except for RNAi experiments, for which progeny were shifted to 29°C at 24 h after egg laying (AEL). Expression and functional studies were conducted using combinations of the Ga4/UAS, the FLP/FRT system-based ELF (Bazigou et al., 2007; Chotard et al., 2005), MARCM (Lee and Luo, 1999), FLPout (Ito et al., 1997; Struhl and Basler, 1993) and RNAi approaches. Pc1-3-78*38 was isolated in a forward genetic mosaic screen. Detailed descriptions of parental stocks and crosses, conditions for clone induction, the EMS screen and deficiency mapping, as well as full genotypes and numbers of samples shown in main and supplementary figures are provided in supplementary Materials and Methods and in Tables S1 and S2.

Molecular biology

Genomic DNA extraction, PCR and sequence analysis were performed following standard protocols. To determine the EMS-induced mutation in 3-78*38, the following primers were used to amplify genomic DNA collected with Zeiss/Bio-Rad Radiance 2100 and Leica TCS SP5 II confocal laser scanning microscopes and processed using Adobe Photoshop.

Immunolabeling and imaging

Brains were dissected in PBS, fixed for 1 h at room temperature in 2% paraformaldehyde in 0.1 M L-lysine (Sigma-Aldrich) containing 0.05 M sodium phosphate buffer (pH 7.4), and washed in PBS containing 0.5% Triton X-100 (Sigma-Aldrich) (for details of staining protocol, see Shimosako et al., 2014). Primary and secondary antibodies used in this study are described in supplementary Materials and Methods. Images were collected with Zeiss/Bio-Rad Radiance 2100 and Leica TCS SPS II confocal scanning microscopes and processed using Adobe Photoshop.

Quantification and statistics

Sample numbers for each experiment in this study are provided in Tables S1 and S2. Strategies to determine: (1) OPC volumes of wild-type and UAS-hthR-expressing animals, (2) the numbers of PH3-positive cells in wild-type OPC NE cells or Facs-positive Pcl1-3-78*38 mutant cell clusters, (3) the number of Pcl-deficient migratory progenitors that prematurely differentiate into Nbs, and (4) the numbers of Hth- and Ey-positive cells in eya/SO gain-of-function experiments are described in detail in supplementary Materials and Methods. Calculations of 95% confidence interval error bars and unpaired two-tailed Student’s t-test P values were performed using Microsoft Excel software [Confidence.T and T.Test (type 3, not assuming equal variance)]. Prism 6 GraphPad was used to perform Shapiro–Wilk and D’Agostino–Pearson omnibus normality tests and to present quantifications as scatter plots and bar graphs. P<0.05 was considered to be statistically significant; ***P<0.0001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.A. and I.S. conceived and designed the study. H.A. performed the experiments and analyzed the data. H.A. and I.S. prepared the manuscript.

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Supplementary information

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