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RESEARCH ARTICLE

A conserved transcriptional network regulates lamina development in the *Drosophila* visual system

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ABSTRACT

The visual system of insects is a multilayered structure composed externally by the compound eye and internally by the three ganglia of the optic lobe: lamina, medulla and the lobula complex. The differentiation of lamina neurons depends heavily on Hedgehog (Hh) signaling, which is delivered by the incoming photoreceptor axons, and occurs in a wave-like fashion. Despite the primary role of lamina neurons in visual perception, it is still unclear how these neurons are specified from neuroepithelial (NE) progenitors. Here we show that a *homothorax* (*hth*)-*eyes absent* (*eya*)-*sine oculis* (*so*)-*dachshund* (*dac*) gene regulatory cassette is involved in this specification. Lamina neurons differentiate from NE progenitors that express *hth*, *eya* and *so*. One of the first events in the differentiation of lamina neurons is the upregulation of *dac* expression in response to Hh signaling. We show that this *dac* upregulation, which marks the transition from NE progenitors into lamina precursors, also requires Eya/So, the expression of which is locked in by mutual feedback. *dac* expression is crucial for lamina differentiation because it ensures repression of *hth*, a negative regulator of *single-minded*, and thus *dac* allows further lamina neuron differentiation. Therefore, the specification of lamina neurons is controlled by coupling the cell-autonomous *hth-eya-so-dac* regulatory cassette to Hh signaling.

KEY WORDS: Lamina precursor, *Drosophila*, Homothorax, Eyes absent, Sine oculis, Gene network, Dachshund

INTRODUCTION

The insect visual system comprises two separate structures: the compound eye (often called 'retina'), which contains the photoreceptors, and the underlying optic lobes (OLs), which are part of the brain hemispheres (Meinertzhagen and Hanson, 1993; Sanes and Zipursky, 2010). Despite their functional importance as primary visual processing centers, our knowledge of the genetic mechanisms that control the specification and early development of the *Drosophila* OLs is poor compared with the eye. In addition, knowledge gained in *Drosophila* might be of general importance for understanding the mechanisms that specify the vertebrate visual neuroepithelium (reviewed by Erclik et al., 2009; Sanes and Zipursky, 2010).

The compound eye is formed by ~800 ommatidia, or unit eyes. Each comprises six outer (R1-6) and two inner (R7 and R8) photoreceptor (PR) neurons, together with pigment and lens-secreting cone cells. Outer PRs are in charge of motion and brightness detection and spatial vision, whereas inner PRs function as color, UV and polarized light sensors (Hardie, 1985). According to their differentiated function, R1-6 innervate the first OL neuropil, which is the lamina, whereas R7 and R8 axons run across the lamina to innervate the neuropil beneath, which is the medulla. Next, the lobula and lobula plate (together called the 'lobula complex') receive signals from medulla neurons and send projections to the higher-order visual centers of the brain (Meinertzhagen and Hanson, 1993; Sanes and Zipursky, 2010). This tetralayered organization is conserved within insects and shared with malacostracan crustaceans (Strausfeld, 2009).

The lamina, medulla and lobula complex are derived from the embryonic OL primordium (OP) (Green et al., 1993), which segregates into the outer and inner OL anlagen. After embryonic invagination, and during larval life, these two anlagen proliferate extensively and are termed the outer and inner proliferative centers (OPC and IPC), respectively. The OPC-derived neurons have their cell bodies in the lamina and medulla, whereas the IPC-derived neurons have their cell bodies mostly in the lobula complex (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993).

During late third larval stage (late L3) the OPC neuroepithelium is characterized by densely packed cells expressing DE-cadherin (DE-cad; Shotgun – FlyBase) that give rise to medulla neuroblasts medially and to lamina neurons laterally (Egger et al., 2007). Neuroepithelial and lamina cells are separated by an indentation called the lamina furrow (LF) (Selleck and Steller, 1991; Meinertzhagen and Hanson, 1993). Lamina cells are characterized by the expression of the transcription factor *dachshund* (*dac*) (Mardon et al., 1994; Huang and Kunes, 1996). As cells exit the LF, they are contacted by incoming retinal PR axons in what is termed the preassembly domain (Umetsu et al., 2006). Hedgehog (Hh), delivered by PR axons, triggers the lamina differentiation program along with a final cell division (Selleck and Steller, 1991; Huang and Kunes, 1996; Huang et al., 1998). Next, lamina cells and PR axons reorganize and assemble into lamina columns (Meinertzhagen and Hanson, 1993), a process that requires the expression of the bHLH-PAS transcription factor *single-minded* (*sim*) (Umetsu et al., 2006).

In recent years, the mechanisms of specification and early development of the eye primordium have been extensively studied [reviewed by Amore and Casares (2010)], in contrast to other components of the visual system. Eye specification is dependent on the expression of two Pax6 genes, *twin of eyeless* (*toy*) and *eyeless* (*ey*), with *toy* being required for the initiation of *ey* expression (Halder et al., 1995; Czerny et al., 1999). Then, *ey*-expressing cells are maintained as proliferative and undifferentiated progenitors as long as they express the TALE class homeodomain gene *homothorax* (*hth*) (Bessa et al., 2002; Peng et al., 2009; Lopes and Casares, 2010). Repression of *hth* by Decapentaplegic (Dpp; *Drosophila* BMP2/4) allows the upregulation of a class of genes collectively known as retinal determination (RD) genes (Bessa et al., 2002; Lopes and Casares, 2010). These include *sine oculis*

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(*so*), a Six2-type homeodomain transcription factor, its partner *eyes absent* (*eya*), and the transcription factor *dac* (Silver and Rebay, 2005). The RD genes, which also include the Zn-finger genes *teashirt* (*tsh*) and *tiptop* (*tio*), are knitted together in the RD gene regulatory network that, through extensive feedbacks, locks in the eye fate [reviewed by Kumar (2010)].

RD genes are also expressed in the OLs. However, their putative role during development has remained elusive. The RD genes *so* and *eya* are expressed in the embryonic OP, where they are required for its invagination (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Bonini et al., 1998; Daniel et al., 1999). After OP invagination, transcription of *so* and *eya* is reinitiated only during late larval stages (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Bonini et al., 1998). In addition to small or absent eyes, *so* and *eya* alleles result in reduced OLs, the lamina being specially affected (Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O'Tousa, 1994). However, experiments (Fischbach and Technau, 1984) using *so* chimeras have indicated that the lamina and medulla reduction seen in *so* flies is likely to be the consequence of a lack of innervation from the reduced eyes, raising the possibility that the OL expression of at least *so* has no major relevance for OL development. However, a cell-autonomous requirement for *so* or *eya* has not been tested to date. As mentioned above, the expression of *dac*, another RD gene, is detected in lamina cells, where it is required for the ensuing differentiation of the lamina (Huang and Kunes, 1996; Chotard et al., 2005). In the compound eye *dac* expression requires *eya* and *so* input (Chen et al., 1997; Pignoni et al., 1997), but it has not been established whether this is also the case during lamina development.

Here, we investigate the specification mechanisms of the lamina, as the first neuropil of the OLs, focusing on the expression and function of RD genes. We show that the expression of the RD genes *eya* and *so* is mutually dependent. These genes are required cell-autonomously for *dac* expression, which additionally requires the Hh signaling provided by incoming retinal PR axons. *dac* is instrumental for the differentiation of lamina neurons as it is required to repress *hth*, which would otherwise impair *sim* expression. In addition, we identify a role for *hth* in the neuroepithelium (NE), where it is normally co-expressed with *eya/so. hth* is required for normal NE growth and to control the extent and levels of expression of *eya*. Therefore, an *hth-eya-so-dac* core network is shared by the lamina and the compound eye. However, major differences exist in the roles played by these genes, including a role of *dac* in lamina differentiation as a necessary *hth* repressor.

RESULTS

Changes in the expression of RD genes accompany the specification of the lamina

The development of the lamina and of the compound eye show a number of important similarities. The differentiation of lamina neurons is triggered by Hh. Differentiation progresses as a fan-shaped wave marked by an indentation, the LF (Selleck and Steller, 1991), which moves from lateral to medial as incoming axons are sent in by newly formed rows of ommatidia (Umetsu et al., 2006). This is reminiscent of the Hh-driven movement of the morphogenetic furrow in the eye (Wolff and Ready, 1991). In addition, expression of the RD genes *dac*, *eya* and *so* during L3 lamina development has been reported (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Bonini et al., 1998). Therefore, and in order to analyze these similarities in more detail, we first mapped the expression domains of *eya*, *so*, *dac*, *sim* and *hth* in the developing OL.

During L3, NE progenitors, which express high levels of DE-cad (Egger et al., 2007), give rise to *dac*-expressing lamina cells laterally and to medulla neuroblasts medially (Fig. 1A-D). The expression of *lethal of scute* [*l(1)sc*] in a narrow band, two to three cells in width, marks the transition zone after which medulla neuroblasts are generated medially (Yasugi et al., 2008) (Fig. 1E), while the LF marks the transition between the NE progenitor cells and the lamina (Hofbauer and Campos-Ortega, 1990; Huang and Kunes, 1996). Therefore, *l(1)sc* expression medially and the LF laterally define the NE progenitor domain (Fig. 1E,G). The onset of *dac* expression starts posterior to the LF, in its posterior slope, and is maintained throughout lamina development (Huang and Kunes, 1996; Chotard et al., 2005; Umetsu et al., 2006). Differentiating lamina cells expressing *sim* are located posterior to the LF (Umetsu et al., 2006). Expression of *sim* is detected weakly and uniformly in the preassembly domain, but clear nuclear staining can be seen only in the assembly domain (Umetsu et al., 2006) (Fig. 1C,D). *hth* expression is detected in medulla neuroblasts and neurons, in the NE progenitors (Reddy et al., 2010; Hasegawa et al., 2011; Morante et al., 2011; Li et al., 2013; Suzuki et al., 2013) and also within the posterior slope of the LF (Fig. 1D,E,F). However, *hth* expression is absent in more internal lamina regions (i.e. the preassembly and assembly domains). Interestingly, the expression of *hth* and *sim* abut each other within the lamina (Fig. 1D). *eya* expression does not extend into the medulla, being detected in the transition zone, overlapping *l(1)sc*, the NE progenitors and through the furrow into the lamina (Fig. 1E). *eya* levels increase just posterior to the furrow, and these levels are sustained throughout the lamina. The expression of *so* follows exactly that of *eya* (supplementary material Fig. S1).

Thus, three domains can be distinguished along the lamina differentiation path: (1) NE progenitor cells that express *hth* and low levels of *eya/so* anterior to the LF; (2) lamina precursor cells (LPCs), located in the posterior slope of the furrow, are characterized by the expression of *hth* and high levels of *eya/so* and *dac*; and (3) the preassembly and assembly domains of the lamina, where *hth* is no longer expressed and which accumulate increasing Sim. These patterns are summarized in Fig. 1G.

Mutual feedback of *eya* and *so*

The similar expression of *eya* and *so* suggested that these genes might regulate the expression of each other. To test this, we induced clones expressing RNAi transgenes specific for either *eya* or *so*. In these clones, the levels of the targeted proteins, as detected by specific antibodies, were reduced to background levels, indicating severe gene expression loss (Fig. 2). When *eya* was knocked down So expression was lost within the clones (Fig. 2A), and when *so* was knocked down Eya expression was strongly reduced (Fig. 2B). Therefore, the expression of *eya* and *so* is mutually dependent, although this dependence for *eya* might be only partial. A feedback between *eya* and *so* has also been described during eye and ocelli development (Pignoni et al., 1997; Brockmann et al., 2011).

eya and *so* are required for *dac* expression

dac expression is known to lie downstream of *eya* and *so* in the compound eye (Chen et al., 1997). We tested whether this was also the case in the lamina by inducing clones in which *eya* or *so* function had been lost (eya^{E8}) or knocked down (*so* RNAi). In both experiments, *dac* expression was not activated within the clones and the effects were cell-autonomous (Fig. 3A,B). Interestingly, loss of *eya* or *so* resulted in *hth* expression beyond its normal limit, raising the possibility that *dac* loss was due to its repression by *hth.* To distinguish between a direct activating role by Eya/So versus a repression by *hth*, we compared the effects on *dac* of removing *so* alone or together with *hth. dac* expression was cell-autonomously

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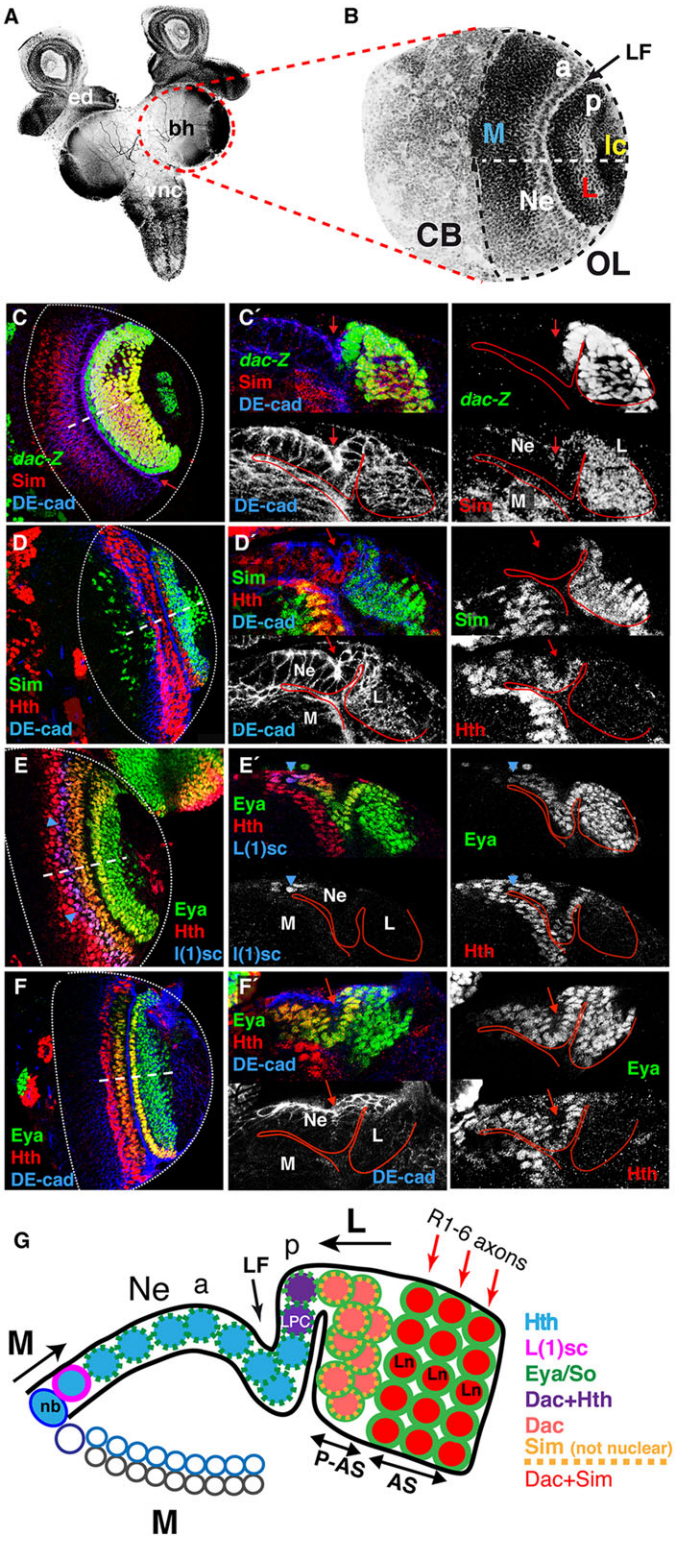

Fig. 1. Expression of *hth*, *eya*, *dac* and *sim* in the OL during the progression from NE progenitors to lamina neurons. Except in A, OLs are oriented medial (proximal) to the left, lateral (distal) to the right in this and all other figures. In B (black dashed line) and in C-E (white dotted line) the OL is outlined. (A) CNS of a late L3 larva counterstained with the DNA marker DAPI. The CNS comprises the ventral nerve cord (vnc) and the brain hemispheres (bh). The eye discs (ed) lie directly on top of the brain hemispheres. (B) Magnified surface view of the right brain hemisphere. CB, central brain; M, medulla; Ne, neuroepithelium; L, lamina; lc, lobula complex; a, anterior to the lamina furrow (LF, arrowed); p, posterior to the LF. The white dashed lines in C-F indicate the approximate positions of the transverse sections shown in C′-F′. Surface views (C-F) and confocal *z*-sections (C′-F′) of late L3 OLs, stained as indicated (single channels and merges are shown). In C, the OL is from a *dac-Z* larva. Anti-β-galactosidase is used to follow *dac* transcription. The blue arrowheads (E,E′) point to *l(1)sc*-expressing cells. Red arrows indicate the LF. (G) Summary of expression patterns on a schematic section through the medulla and lamina. The arrows marked by M and L indicate the movement of the medulla and lamina differentiation waves, respectively. The green dotted outline indicates low expression levels of Eya and So. nb, medulla neuroblasts; Ln, lamina neurons; LPC, lamina precursor cell; P-AS, preassembly domain; AS, assembly domain. Red arrows indicate R1-6 incoming axons.

lost in both *so-RNAi* clones (in which *hth* is maintained) as in *so-RNAi+hth-RNAi* clones (Fig. 3B,C). Therefore, *dac* regulation requires the positive input of the RD genes and does not seem to be negatively regulated by *hth* (see below). To test this specifically, we induced *hth-RNAi* clones and checked for changes in Dac levels. We compared the Dac immunofluorescence signal within and outside *hth-RNAi* clones (Fig. 3D) and found no difference (ten clones from seven OLs were analyzed). Therefore, *hth* is not acting as a *dac* repressor behind the LF. In addition, the fact that *dac* expression did not extend medially into the *eya/so*-expressing domain in *hth*$^{-}$ clones indicated that these RD genes are not sufficient to induce *dac* expression. This is consistent with the *hh* pathway being additionally required for *dac* expression (Huang and Kunes, 1996).

Previous work by Pappu and co-workers identified two enhancers in the *dac* gene (Pappu et al., 2005), one of which, dac3EE, was noted to drive strong reporter gene expression in the lamina. We confirmed very strong expression of this enhancer in the lamina, plus weaker expression in the lobula (Fig. 3E; data not shown). *eya* knockdown clones, in which *dac* expression was lost, also lost dac3EE-Z activity (Fig. 3E). This suggests that *eya* acts via dac3EE to regulate *dac* lamina expression.

dac is required in the lamina to repress *hth* downstream of the *hh* pathway

The expression of *dac* builds up in the posterior slope of the LF, preceding the shutting off of *hth* in the lamina preassembly domain. This observation raised the possibility of *dac* being involved in *hth* repression. In fact, blocking the Hh signaling pathway by removing the Hh signal transducer *smoothened* (in *smo*^{3}clones) results not only in a loss of *dac* (Fig. 4A) (Huang and Kunes, 1996) but also in an expansion of *hth* expression into the lamina (Fig. 4B). Interestingly, the expression of *eya* does not depend on *hh*, as its expression remains unaltered in *smo*$^{-}$ clones spanning the lamina (Fig. 4B) in spite of *hth* expression. In test if *hth* repression is mediated by *dac*, we examined the effect of removing *dac* on *hth* expression. In *dac*$^{-}$ clones, *hth* is upregulated in internal regions of the lamina whereas *eya* expression is unaffected (Fig. 4E), suggesting that Eya/So cannot repress *hth* in the absence of Dac. To prove that this effect was not due to a regulatory feedback of *dac* on the Hh signaling pathway, we checked *dac* requirement for Hh signaling activity. Whereas in *smo*$^{-}$ clones the expression of the downstream signaling component *cubitus interruptus* (*ci*) (Motzny and Holmgren, 1995; Alexandre et al., 1996) is reduced (Fig. 4C), in *dac*$^{-}$ clones *ci* expression was unaltered (Fig. 4D), indicating that *dac* is not generally required for Hh signal transduction. Altogether, these results indicate that *dac* is required downstream of *eya/so* and the *hh* pathway to repress *hth*. As *smo*$^{-}$ cells cannot differentiate as lamina neurons, these *smo*$^{-}$, *eya*-expressing, *dac*-nonexpressing cells are likely to remain in a lamina precursor state. When we performed the converse experiment and expressed *dac*

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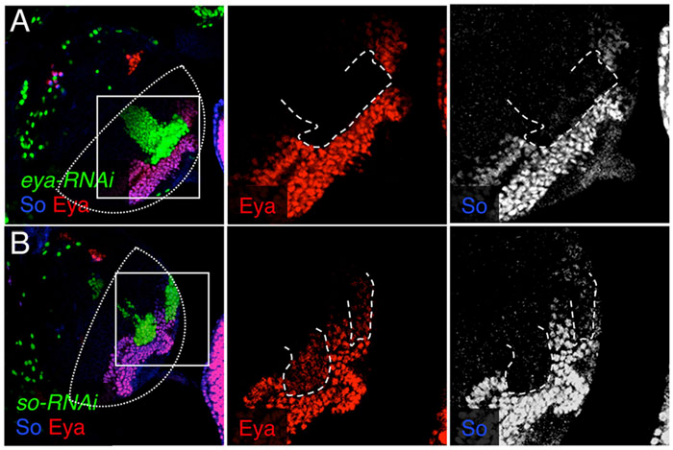

Fig. 2. Mutual dependence of *eya* and *so* expression. GFP-marked clones expressing *eya-RNAi* (A) or *so-RNAi* (B). OLs are outlined (white dotted line). Single channels are shown at higher magnification for the boxed regions, where clones are outlined (white dashed line). (A) RNAi-mediated *eya* knockdown efficiently reduces Eya signal to background levels. In these clones So signal is absent. *eya-RNAi* clones extend to, but do not enter, the lamina. *N*=10. (B) RNAi-mediated *so* knockdown also reduces So signal to background levels. In these clones Eya is still expressed, although at reduced levels.

ectopically in the NE, *hth* expression was downregulated cell-autonomously (Fig. 4F), indicating that *dac* is not only required but also sufficient to repress *hth*.

Molecularly, Dac proteins have been shown to work, at least in some developmental contexts, in a complex with So/Six and Eya proteins (Chen et al., 1997; Pignoni et al., 1997; Ikeda et al., 2002). In order to gain insight into whether such a complex might be involved in *sim* activation and *hth* repression in the developing lamina, we examined the expression of both genes in clones expressing *dac* but lacking *eya* (and, therefore, also *so*) using the MARCM technique. eya^{-} dac^{+} clones, as is also the case for clones mutant for *eya* only, abutted the lamina. In these clones (*N*=5), Hth expression was maintained at levels similar to those in adjacent, non-mutant cells (supplementary material Fig. S2A). Only in two cases (out of more than 40 clones examined) the eya^{-} dac^{+} clone clearly spanned the preassembly domain, allowing the analysis of clones in the Sim-expressing region. In one case, Sim expression was reduced within the clone (supplementary material Fig. S2B,C), whereas no noticeable alteration in Sim levels was observed in the second clone (supplementary material Fig. S2D,E). eya^{-} dac^{+} clones falling entirely within the lamina were extremely rare and small (1-3 cells). In one such example, Sim expression was absent from the eya^{-} dac^{+} cells (supplementary material Fig. S2D,E, inset). These results suggest that Dac is unable to regulate *hth* in the absence of its partner Eya; with the limited evidence at hand, this might also be the case for *sim*. The low recovery of eya^{-} dac^{+} cells in the OLs might be due to high apoptosis rates induced by *dac*, as we detect increased levels of the apoptosis marker activated Caspase 3 in OLs from brains containing dac^{+} clones (data not shown).

hth maintenance in lamina cells prevents *sim* upregulation

Finally, we examined the impact of misregulation of *hth*, *eya/so* and *dac* on *sim* expression, as this gene marks further differentiation steps in the lamina (Umetsu et al., 2006). In so^{-} clones, *sim* expression is reduced (Fig. 5A). However, in these clones *hth* is derepressed (Fig. 5A and see Fig. 3B). Therefore, the loss of *sim* could be due to either the loss of a positive input (RD) or the

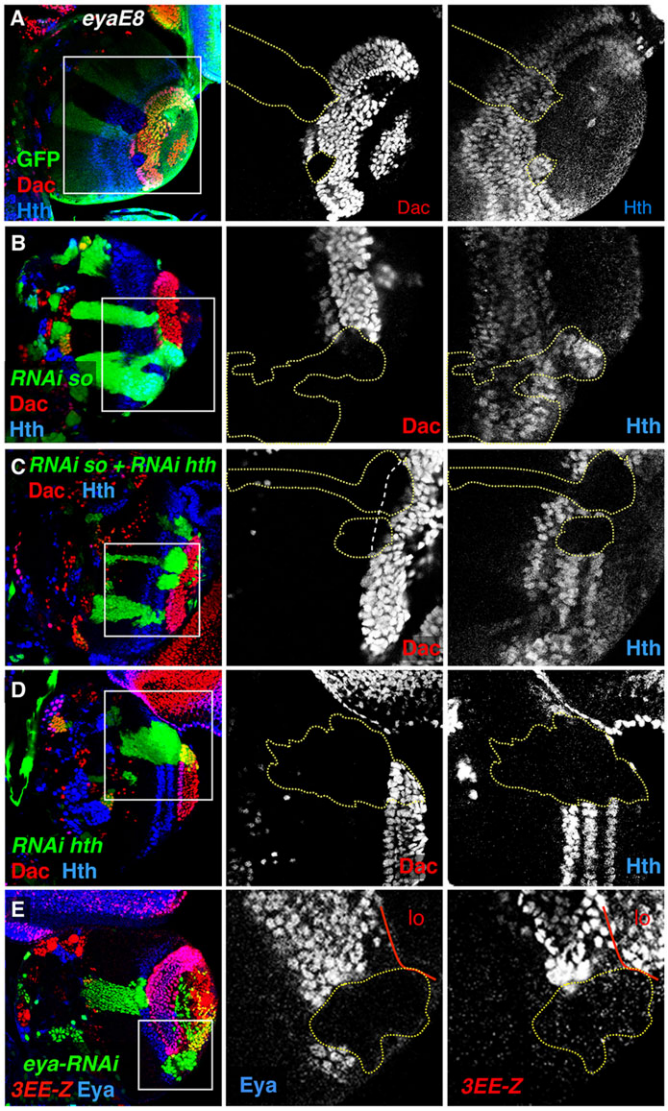

Fig. 3. *eya* and *so* regulate *dac* expression in the lamina, probably through the *dac3EE* enhancer. (A-E) (Left) Surface view of late L3 OLs containing clones. (Middle and right) Higher magnifications of the boxed regions as individual channels. Clones are outlined (dotted yellow line). (A) *eya* mutant cells fail to upregulate Dac and maintain Hth expression. eya^{E8} mutant cells, marked by the absence of GFP, are stained for Eya and Hth. *N*=5. (B,C) So is required for Dac expression. (B) *so-RNAi* cells and (C) *so-RNAi+hth-RNAi* clones stained for Hth and Dac. Clones are positively labeled with GFP. (B) In *so* mutant cells expression of Hth is maintained. *N*=3. (C) Failure to upregulate Dac expression in *so* mutant cells is not due to the maintenance of Hth. (D) Dac expression is not affected in *hth-RNAi* clones. *N*=6. (E) GFP-marked *eya-RNAi* clone in a *dac3EE* background, stained with anti-β-galactosidase (3EE-Z) and for Eya. Within the clone, enhancer expression is lost. *N*=3. The solid red line (E) separates the lamina from the prospective lobula (lo), where *dac3EE-Z* is also expressed.

presence of a repressor (Hth). To distinguish between these two possibilities, we simultaneously knocked down *so* and *hth*. In so^{-} hth^{-} clones, *sim* expression is still reduced or absent (Fig. 5B), pointing to the need for a positive *sim* regulator. Since *eya* and *so* are required for *dac* expression, we next checked the effect of only removing *dac* (which does not affect the expression of its upstream regulators *eya/so*; Fig. 4E). Again, *sim* expression was reduced cell-autonomously in dac^{-} clones (Fig. 5C). However, and as we showed previously, loss of *dac* is accompanied by *hth* upregulation (Fig. 5B,

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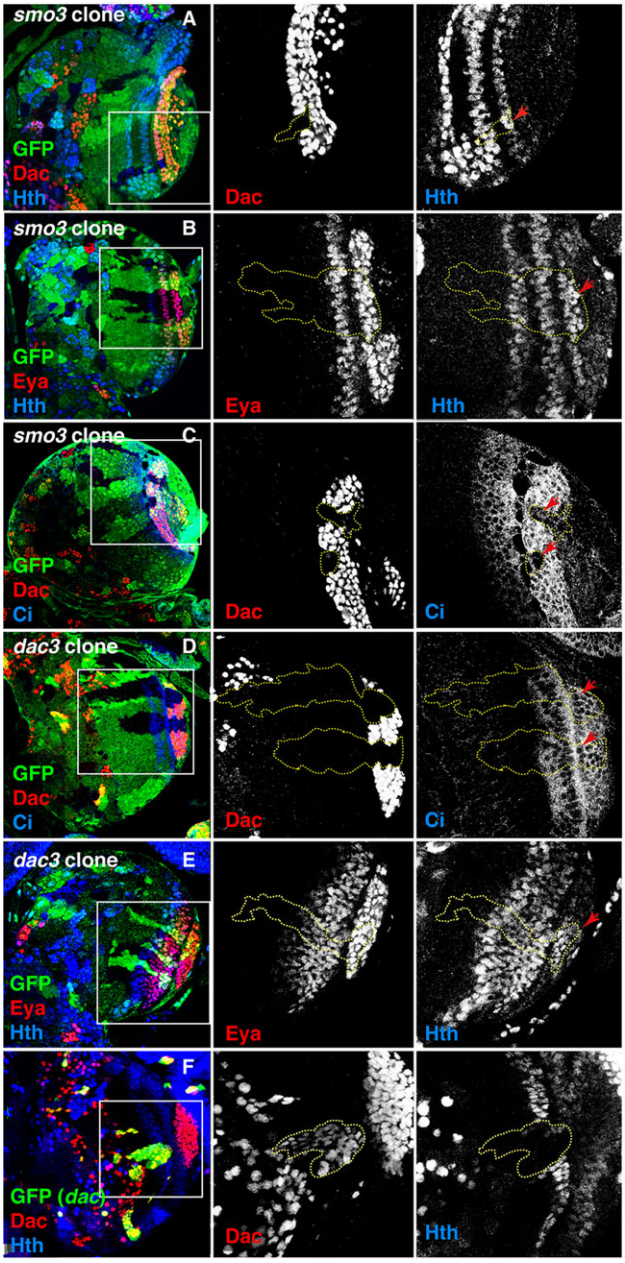

Fig. 4. *dac* represses *hth* downstream of the *hh* pathway. (Left) Surface view of late L3 OLs containing clones. (Middle and right) Higher magnifications of the boxed regions as individual channels. Clonal tissue is outlined (dotted yellow line) and indicated by red arrows. (A-E) smo^3 (A-C) and dac^3 (D,E) mutant clones (marked by the absence of GFP) were stained for Dac and Hth (A), Eya and Hth (B,E) and Dac and Ci (C,D). (A,B) In smo^3 clones, Dac is not expressed (A, *N*=4) and Hth is upregulated and at times extended (B). (B,E) Levels of Eya remain unaltered in both smo^3 (B, *N*=7) and dac^3 (E, *N*=3) mutant clones. (C) In the OL, the expression of *ci* depends on Hh signaling, as Ci signal is reduced in smo^3 clones. *N*=2. (D) This is not the case in *dac* clones, suggesting that in the absence of *dac*, Hh signaling is still active. *N*=2. (E) Loss of *dac* results in Hth extension into the lamina. *N*=2. (F) GFP-marked *dac*-expressing clone stained for Dac and Hth. In gain-of-function *dac* clones *hth* is repressed. *N*=2.

see also Fig. 4E). In order to determine if *hth* was directly responsible for *sim* repression in dac^- cells, we induced *hth*-expressing clones in the lamina (Fig. 5D,E). In these clones *sim* is repressed (Fig. 5D) without detectable changes in *dac* expression (Fig. 5E).

We conclude that the RD genes are required for full *sim* expression, acting both as *sim* activators and *hth* repressors. A crucial role in this regulation is played by *dac*, which is necessary to repress *hth*.

hth is required in the NE to promote growth and to modulate the extent and levels of *eya* expression

Our results so far indicate that the repression of the transcription factor *hth* within the lamina is necessary to allow its proper differentiation. However, *hth* is expressed at earlier stages in the NE progenitors, where it overlaps with *eya* and *so* (Fig. 1E,F; supplementary material Fig. S1). To establish whether *hth* plays any positive role in the NE, we first analyzed the impact of loss of *hth* on tissue growth. hth^- clones were recovered throughout the OL, although they were 30-40% smaller than their wild-type twin clones (Fig. 6A,B), suggesting a role of *hth* in proliferation. Next, we investigated whether *eya* expression would be affected by removing *hth*. Owing to the smaller size of hth^- clones, we induced *hth* loss-of-function mosaics using the Minute technique in order to recover larger clones and make the analysis of the effects of *hth* removal on *eya* expression easier. *hth,* M^+ clones showed a slight expansion of *eya* expression (Fig. 6C). In addition, when the intensity of the *eya* signal was compared between *hth* mutant and adjacent control tissue, we observed an increase of ~20% in *eya* levels in the hth^- cells (12 *hth-RNAi* clones from seven OLs analyzed).

These results indicate that *hth* modulates the extent and levels of *eya* expression, suggesting that the levels of *hth* might be subject to tight regulation. Indeed, this does seem to be the case, as clones overexpressing *hth* block *eya* expression in the NE (Fig. 6D). We noted, however, that this repression was only detectable in the NE. Neither in smo^- nor in *dac*-mutant clones, where *hth* expands a few rows into the lamina, did we observe any significant change in *eya* expression posterior to the LF. This might indicate that another, as yet unidentified, factor aids *hth* in modulating *eya* expression in the NE. Alternatively, *dac*, the expression of which is turned on just after the LF, could enhance *eya* expression, making it insensitive to *hth*. Loss of *hth*, in *hth-RNAi* clones, does not result in premature Sim expression (Fig. 5F), consistent with the requirement of multiple inputs for *sim* activation.

DISCUSSION

In this study we have uncovered a gene regulatory network that operates cell-autonomously during the specification of lamina neurons in the OLs. The regulatory model that emerges from our work is summarized in Fig. 7.

We have shown that the expression of the RD genes *eya* and *so* is required cell-autonomously for the specification of lamina neurons through the activation of at least *dac* and *sim*. Their expression is initiated in the NE cells at low levels, but this expression increases after the LF. The fact that *eya* and *so* positively regulate each other would, above a certain expression threshold, cause *eya* and *so* to lock in their transcription to maximal levels. *hth* might regulate that threshold, since *hth* acts *in vivo* as a repressor of *eya* in NE progenitors. Regulation of *eya* expression might be further required for the spatial segregation of cells in different states along the lamina differentiation pathway. Thus, eya^- clones are seldom recovered in internal regions of the lamina (Fig. 3A), which might be indicative of a segregation of the *eya*-mutant tissue or the elimination of these cells from within the lamina. Something similar happens with *smo* and *sim* mutant clones, which do not appear in internal regions of the lamina either (Umetsu et al., 2006). In addition, *hth* is required to

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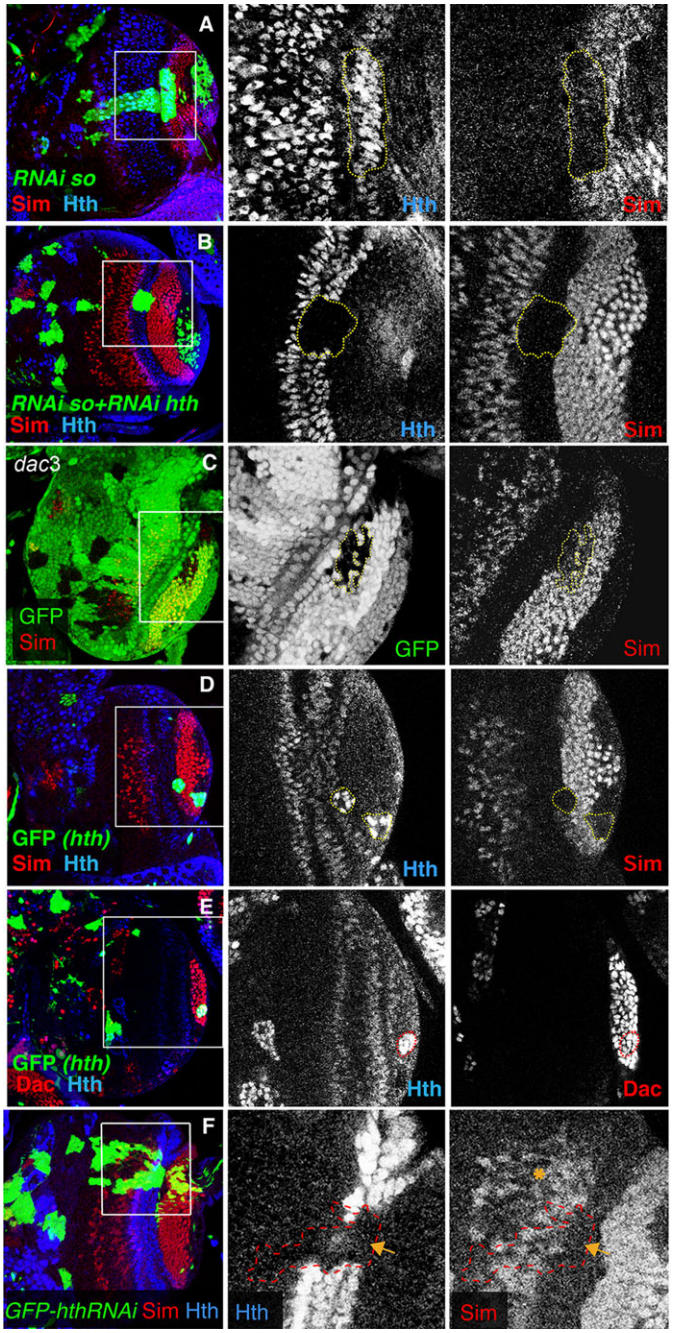

Fig. 5. *sim* expression depends on a direct regulatory input from RD genes and on the repression of Hth. (A-E) (Left) Lateral views of late L3 OLs. (Middle and right) Higher magnifications of the boxed regions as individual channels. (A,B) Sim expression depends on a positive input from the RD. *so-RNAi* (A, *N*=5) and *so-RNAi+hth-RNAi* (B, *N*=4) clones positively marked by GFP expression were stained for Hth and Sim. In both situations, the expression of Sim is lost cell-autonomously. (C) In dac^3 clones (marked by the absence of GFP) Sim expression is reduced, but not totally lost. *N*>10. (D,E) Ectopic expression of *hth* in clones within the lamina. Clones are positively marked by GFP and were stained for Hth and Sim (D, *N*=11) and Hth and Dac (E, *N*=5). The expression of Sim is lost cell-autonomously, whereas Dac expression remains unaltered. (F) Flip-out *hth-RNAi* clones, marked positively by GFP, and stained for Hth and Sim. Loss of Hth does not cause Sim expression changes. *N*=6. The clone is outlined (red dashed line). The orange arrow points to the LPC domain. The asterisk marks some scattered Sim-positive cells that lie beneath the NE in the developing medulla (see also Fig. 1C,D for Sim expression in transverse sections of the OLs).

sustain normal NE cell proliferation, as *hth*-mutant clones grow, on average, 30% less than wild-type clones.

The recruitment of lamina precursors from NE progenitors is driven by incoming waves of R1-6 axons, thereby coupling it to retinal differentiation (Selleck and Steller, 1991; Huang and Kunes, 1996). Axon-delivered Hh activates its downstream pathway and, as a consequence, *dac* expression is upregulated in *eya/so*-expressing cells (Huang and Kunes, 1996; Chotard et al., 2005). We show that the net result of this activation is an efficient repression of *hth* to allow the full expression of *sim* and, thereby, normal lamina differentiation. Dac, and its vertebrate Dach homologs, have been shown to form a protein complex with So/Six and Eya family proteins (Chen et al., 1997; Pignoni et al., 1997; Ikeda et al., 2002) and to synergize with them in ectopic eye induction assays (Chen et al., 1997; Pignoni et al., 1997). In addition, Dac possesses a DNA-binding domain (Kim et al., 2002). The fact that ectopic *dac* expression does not seem capable of repressing *hth* without *eya* suggests that this function would require the formation of a trimeric complex with So and Eya. *hth*, *eya*, *so* and *dac* are transiently co-expressed in some cells of the posterior slope of the LF, before *hth* is shut off. This posterior slope might represent a transition zone in which these regulatory processes are taking place (Fig. 1G). Regarding the activation of *sim* expression, we have not been able to clearly determine whether it can be carried out by Dac alone or by a So-Eya-Dac complex, although the limited evidence that we have gathered is compatible with Dac requiring Eya/So cooperation.

In the compound eye, progenitors are characterized by *hth* expression, whereas the precursor population (contained in the so-called pre-proneural domain) expresses high levels of *eya*, *so* and *dac*, and no *hth* (Bessa et al., 2002). However, detailed inspection of the eye progenitor domain shows that the *hth*-expressing cells also co-express RD genes such as *eya*, although at low levels (supplementary material Fig. S3), as we have shown to be the case in NE progenitors in the OL. Therefore, eye and lamina derive from progenitors that show many similarities. However, there are several profound differences. Neither *ey* nor *toy*, the two Pax6 genes positioned at the top of the genetic hierarchy of eye development, is expressed during lamina development (Callaerts et al., 2001; Morante et al., 2011; Southall et al., 2013) (supplementary material Fig. S4), and all available information on *ey* function, including that from *ey* mutants (Callaerts et al., 2001), expression of dominant-negative *ey* forms (Morante et al., 2011) or RNAi-mediated *ey* and *toy* knockdowns (our unpublished data), is compatible with these genes not playing a direct role in lamina development, at least during larval stages. Also, neither *tsh* nor its paralog *tio* is expressed in the OPC NE (Southall et al., 2013; data not shown), although cells of the OPC NE are ready to respond to *tsh* expression by proliferating and blocking lamina differentiation (supplementary material Fig. S5). Since, in the eye, *tsh* and *hth* have been shown to directly interact with Yki to activate Hippo-regulated target genes (Peng et al., 2009), and since the proliferation in the OPC is controlled by the Hippo pathway (Reddy et al., 2010), providing *tsh* is likely to engage *hth* and *yki* in maintaining the progenitor state of neuroepithelial cells as well. Another significant difference between lamina and eye development is that, during eye development, *hh* does not regulate *hth* expression, at least not directly, because blocking the *hh* pathway in smo^{-} clones does not affect the *hth* expression pattern (Firth and Baker, 2009; Lopes and Casares, 2010). In the eye, *hth* repression is carried out mostly by the BMP2 homolog Dpp (Lopes and Casares, 2010), which is itself an Hh target (Heberlein et al., 1993; Greenwood and Struhl, 1999; Fu and Baker, 2003). In the OLs, Dpp has been shown to play a different role: the specification of lamina glia (Yoshida et al., 2005).

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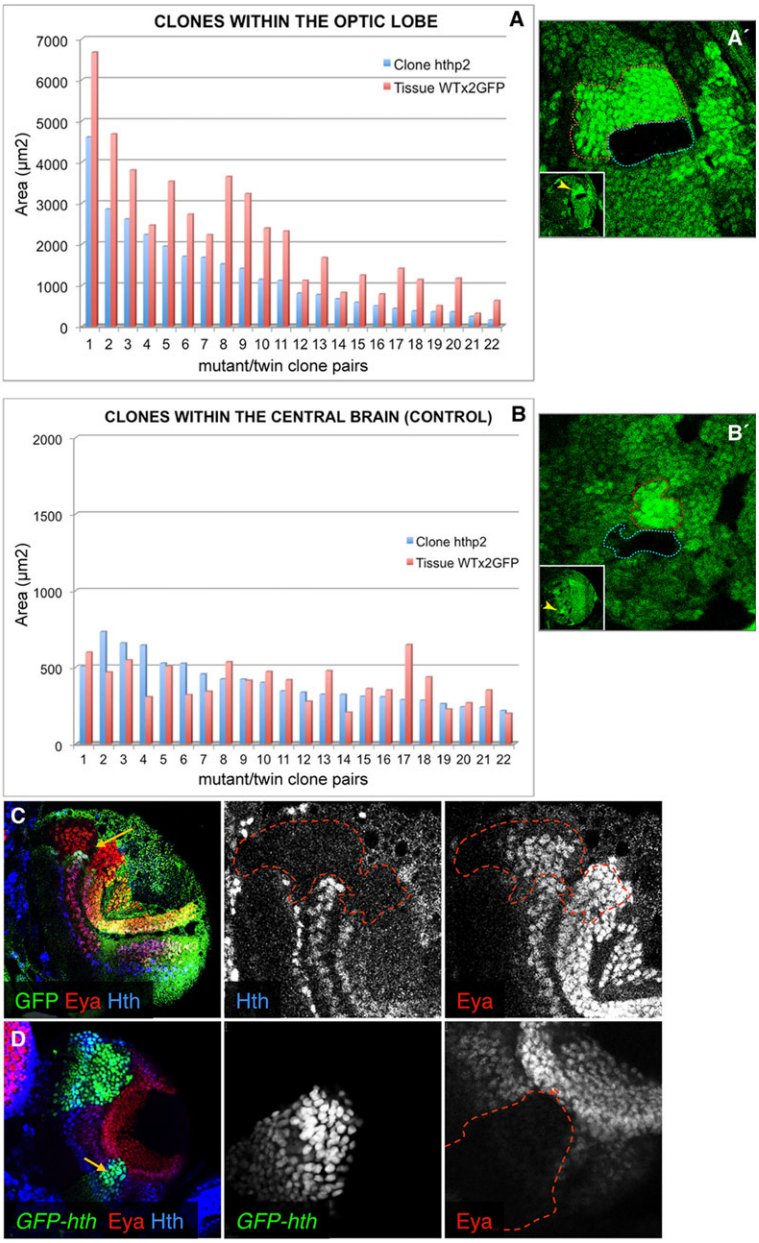

Fig. 6. Loss of *hth* reduces tissue growth in the OL NE and modulates the extent and levels of *eya* expression. (A,B) The clonal area of each pair of hth^{P2} and wild-type twin clones in the OPC of the OL (A) and in the adjacent central brain (CB) (B). On the *x*-axis, all pairs of mutant/twin clones are represented (*N*=22 pairs of mutant/twin clones in both the OPC and CB). The CB is used as an internal control because *hth* expression is not generalized here. In the OPC, the mutant clone area is always smaller than the adjacent twin area by ~30-40% ($P<5\times10^{-7}$), whereas there is no difference in the CB ($P>0.9$). For statistical analysis a non-parametric paired Mann–Whitney-Wilcoxon's test was applied. (A′,B′) Two representative mutant/twin clone pairs in each region are shown. Late L3 OLs were stained for GFP; the blue dotted line delimits the mutant clone and the red dotted line delimits the 2×GFP twin clone. For orientation, in the insets the arrowhead marks the position of the clone pair. Whereas in the CB the area of the hth^{P2} clone (marked by the absence of GFP) is similar to that of its twin clone (2×GFP: brightest GFP area), in the OPC the hth^{P2} clone is smaller. (C,D) Surface views of late L3 OLs. Arrows point to clones shown at higher magnification in the central and right panels as individual channels. Clones are outlined (dashed red line). (C) hth^{P2}, M^{+} clone (marked by the absence of GFP in green) stained for Hth and Eya. Within the clone, Hth immunoreactivity is lost and Eya expression extends medially. (D) Flip-out *GFP-hth* clones stained for Hth and Eya. Forced expression of *hth* results in Eya repression. *N*=5.

Perhaps the most striking difference, though, lies in the role played by *dac*. In the eye, *dac* is required for the initiation of retinal differentiation; however, once the differentiation wave is progressing, removal of *dac* has little effect on the process (Mardon et al., 1994). Accordingly, dac^{-} clones do not derepress *hth* (C. Bras-Pereira and F.C., unpublished). By contrast, *dac* is necessary for the correct differentiation of the lamina, where it is required for *hth* repression. Our study shows that *sim* expression is reduced in *dac*-mutant cells. In a previous study, Umetsu et al. (2006) found no effect on *sim* upon *dac* removal. We note that our clones were induced using a heat shock-inducible flipase (*hsFLP*), whereas the clones used by Umetsu and co-workers were induced with the *NP6099*-GAL4 line (Yoshida et al., 2005) driving UAS-*FLP*, which may cause a milder phenotype due to delayed timing of clone induction. This could have resulted in only a subtle reduction in Sim expression. The fact that in the absence of *dac* the expression of *sim* is reduced but not absent argues for the existence of a *dac*-independent *sim* activator, probably Hh (Fig. 7).

The use of the *hth-eya-so-dac* cassette seems to be evolutionarily conserved, as genes of the Pax, Six, Eya, Dach and, in some instances, Meis gene families have been shown to be co-expressed and functional during the development of many different organ types in vertebrates: from eyes, sensory placodes or brain regions to muscle, kidney or pancreas (Ikeda et al., 2002; Zhang et al., 2002, 2006; Li et al., 2003; Bessarab et al., 2004; Purcell et al., 2005; Bumsted-O'Brien et al., 2007; Kaiser et al., 2007; Erickson et al., 2010; Santos et al., 2011). Therefore, further study of the early development of the *Drosophila* lamina might shed light on the general mechanisms governed by this multipurpose genetic cassette in vertebrates.

MATERIALS AND METHODS

Genotypes and genetic manipulations

Larvae were raised at 25°C, unless otherwise indicated. $w^{1118}$was used as control strain. *P{PZ}dac P(ry*$^{+}$*)/Cyo; ry*^{506} was used as a reporter for *dac* expression. For targeted misexpression we used the UAS/GAL4 system

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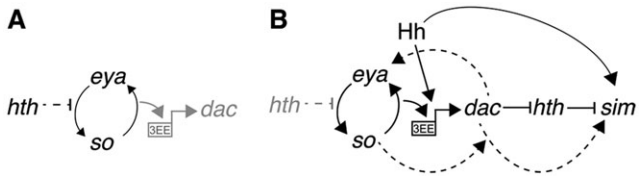

Fig. 7. Regulatory network of lamina specification. Regulatory relationships before (A) and after (B) the LF. Before the LF, NE cells co-express *hth* plus *eya* and *so*. The dashed repressive line (with T-bar) indicates a minor role for *hth* in regulating the extent and levels of *eya* (A). Behind the furrow, *dac* expression requires Eya and So cell-autonomously, as well as Hh delivered by retinal axons. This activation might be through the dac3EE enhancer (3EE). In addition, *dac* expression is required for tilting the equilibrium between *hth* and *eya*/*so* autoregulatory loop towards full *eya*/*so* activation and full *hth* repression (dashed connection from *dac* to *eya*) (B). Expression of Sim within the lamina requires Hh signaling (Umetsu et al., 2006), repression of Hth mediated by *dac* and, at the same time, the positive input of the RD genes (dashed arrow from the Eya/So autoregulatory loop into the *dac* to *sim* link). Whether this input is exerted by Dac is not known (dashed arrow from *dac* to *sim*; B).

(Brand and Perrimon, 1993). UAS strains used were: *UAS-eya-RNAi* (VDRC, 43911), *UAS-so-RNAi* (VDRC, 104386), *UAS-hth-RNAi* (VDRC, 12764), *UAS-GFP-hth* (Casares and Mann, 2000), *UAS-Flag-HA-eyaB2* and *UAS-Flag-HA-tsh* (both kindly provided by C. M. Luque, Universidad Autónoma, Madrid, Spain), *UAS-dac* and *UAS-GFP* (Bessa and Casares, 2005).

Clones of cells mutant for *hth* (hth^{P2}), *smo* (smo^{3}), *eya* (eya^{E8}) and *dac* (dac^{3}) were generated through mitotic recombination (Xu and Rubin, 1993). These alleles are described in FlyBase. Clones were induced by a 30 min heat shock at 37°C between 48 h and 72 h after egg-laying (AEL) in larvae of the following genotypes: *yw, hsFLP;; FRT82B* hth^{P2}*/FRT82B Ubi-GFP*, *yw, hsFLP;* eya^{E8} *FRT 40A/Ubi-GFP FRT40A*, *yw, hsFLP,* smo^{3} *FRT40/Ubi-GFP FRT40A* and *yw, hsFLP;* dac^{3} *FRT40/Ubi-GFP FRT40A*. In all these cases, mutant cells were marked by the absence of GFP. In order to give hth^{P2} mutant cells a growth advantage, hth^{P2} clones were induced using the Minute technique (Morata and Ripoll, 1975). Clones were induced in *yw, hsFLP; FRT82B* hth^{P2}*/FRT82B arm-lacZ, M(3)*w^{124} larvae by a 30 min heat shock at 37°C between 48 h and 72 h AEL. Clones were detected by the absence of β-galactosidase. The MARCM technique (Lee and Luo, 2001) was used to ectopically express Dac in the absence of *eya*. *yw, hsFLP, tub-Gal4,UAS-GFP; FRT40A,tub-Gal80/CyO* females were crossed to eya^{E8} *FRT40A/CyO; UAS-Dac/TM6B* males. Larvae were heat shocked between 48 h and 72 h AEL for 45 min at 37°C. Non-*Tb*, GFP-positive larvae were selected for analysis. Mutant tissue was positively marked with GFP.

Ectopic expression clones were generated randomly using the flip-out method (Struhl and Basler, 1993). *yw, hsFLP, act>y+>Gal4;; UAS-GFP/TM6B,Tb* females were crossed to males carrying the *UAS* transgenes (either homozygous or balanced over *TM6B, Tb*). Clones were induced between 48 h and 72 h AEL by a 10 min heat shock at 35.5°C. For *UAS-RNAi* lines only, after heat shock larvae were grown at 29°C to maximize transgene expression; otherwise, cultures were maintained at 25°C. To induce clones within the lamina region, heat shock was performed between 72 h and 96 h AEL. Non-*Tb* larvae were selected for dissection and analysis. Clones were positively marked with GFP.

Construction of the *dac3EE-Z* transgenic strain

The genomic region containing the dac3EE enhancer (Pappu et al., 2005) was PCR amplified, cloned into PCR8/GW/TOPO (Invitrogen) and transferred into *attB*-pRVV-*lacZ* vector (kindly provided by R. S. Mann, Columbia University, New York). The *attB* construct was inserted in the second chromosome at the 22A *attP* site via phi-C31-mediated transgenesis (Bischof et al., 2007). The primers used were: 5′-GATCCCAAAAG-GACATCTTCAA-3′ and 5′-TCGAATGCAATTTTAACAGAAAAA-3′. Standard genetic techniques were used to introduce the *dac3EE-Z* line into appropriate genetic backgrounds.

Immunohistochemistry

Eye imaginal discs and brains were dissected and fixed according to standard protocols. Primary antibodies used were: guinea-pig anti-Hth, 1/2000 (Casares and Mann, 1998); rabbit anti-β-galactosidase, 1/1000 (Cappel, 55976); rabbit anti-GFP, 1/1000 (Molecular Probes, A11122); guinea-pig anti-So, 1/1000 (Jemc and Rebay, 2007); rabbit anti-Toy, 1/50 (Jacobsson et al., 2009); rat anti-Ey, 1/100 (Halder et al., 1995); rat anti-L(1)sc, 1/100 (gift from M. D. Martín-Bermudo, CABD, Seville); and mouse anti-Sim, 1/50 (gift from A. Baonza, CBMSO, Madrid). Mouse anti-Eya (eya10H6; 1/100), rat anti-DE-cad (DCAD2; 1/100), rat anti-Elav (7EBA10; 1:1000), mouse anti-Dac (mAbdac 1-1; 1/100) and mouse anti-Ci (mAb2A1; 1/5) were from Developmental Studies Hybridoma Bank. Primary antibodies were incubated in PBS with 0.2% Triton X-100. Fluorescently labeled secondary antibodies (anti-mouse-488 and -568; anti-rabbit-488 and -568; anti-rat-488 and -647 and anti-guinea pig-567 and -647) were from Molecular Probes. Images were obtained on SPE or SP2 Leica confocal systems and processed with Adobe Photoshop.

Immunofluorescence signal measurements

In order to detect quantitative changes in gene expression in gain- or loss-of-function cell clones, we measured the immunofluorescence signal within the clones and in neighboring (control) areas as an expression level correlate. Analysis was performed with ImageJ (NIH). Each clone was compared with a neighboring patch of tissue of similar area.

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Competing interests

The authors declare no competing financial interests.

Author contributions

F.C. developed the concept of the study; C.P., C.S.L. and F.C. designed the experiments, which were carried out mostly by C.P.; F.C. prepared the manuscript with equal contributions from C.P. and C.S.L.

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Supplementary material

Supplementary material available online at
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