RESEARCH ARTICLE

Anteroposterior patterning of Drosophila ocelli requires an anti-repressor mechanism within the hh pathway mediated by the Six3 gene Optix

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ABSTRACT

In addition to compound eyes, most insects possess a set of three dorsal ocelli that develop at the vertices of a triangular cuticle patch, forming the ocellar complex. The wingless and hedgehog signaling pathways, together with the transcription factor encoded by orthodenticle, are known to play major roles in the specification and patterning of the ocellar complex. Specifically, hedgehog is responsible for the choice between ocellus and cuticle fates within the ocellar complex primordium. However, the interactions between signals and transcription factors known to date do not fully explain how this choice is controlled. We show that this binary choice depends on dynamic changes in the domains of hedgehog signaling. In this dynamics, the restricted expression of engrailed, a hedgehog signaling target, is key because it defines a domain within the complex where hedgehog transcription is maintained while the pathway activity is blocked. We further show that the Drosophila Six3, optix, is expressed in and required for the development of the anterior ocellar region specifically, limiting the ocellar expression domain of en. This finding confirms previous genetic evidence that the spatial allocation of the primordia of anterior and posterior ocelli is differentially regulated, which may apply to the patterning of the insect head in general.

KEY WORDS: Drosophila, Gene network, Head patterning, Ocelli, Optix

INTRODUCTION

The dorsal adult head of Drosophila derives from the dorsal-anterior region of the eye-antennal imaginal disc. In addition, this disc gives rise to the remaining head capsule, the eyes, the antennae and the maxillary palps (Younossi-Hartenstein, 1993; Domínguez Casares, 2005). The dorsal head is patterned by the dynamic interplay between orthodenticle [otd; also known as ocelliless (oc)], which encodes an Otx family transcription factor, and the wingless [wg; the fly Wnt1 homolog] and hedgehog (hh) signaling pathways (Friedrich, 2006). The result of this patterning is the allocation of the dorsal head, which lies in between the eyes, into three territories (from lateral to medial): orbital cuticle, frons and ocellar complex (OCx) (Fig. 1A). The OCx comprises three small and structurally simple eyes termed the ocelli that are located at the vertices of a triangular patch of cuticle, the so-called interocellar cuticle, which also harbors a set of stereotypical bristles (Fig. 1). Ocelli are widespread in insects, where they play a number of roles, including flight stabilization and as movement detectors triggering the escape response (Goodman, 1981; Mizunami, 1995).

Work in past years has aimed at defining the functional relationships between wg, hh and otd during the process of dorsal head patterning in the disc. This work has expanded our understanding of the general mechanisms by which the conserved Wnt and Hh signaling pathways interact (Royet and Finkelstein, 1997) and of the development and evolution of the eyes and dorsal head of arthropods (Posnien et al., 2010, 2011; Samadi et al., 2015; Schomburg et al., 2015), and has helped in establishing parallels between head patterning across phyla. For instance, members of both the Wnt and Otx gene families are involved in anterior head/neural tube patterning in both invertebrates and vertebrates (Lichtneckert and Reichert, 2005; Rhinn et al., 2005; Schincko et al., 2008; Steinmetz et al., 2011; Fu et al., 2012).

The development of the OCx is a typical example of regional specification, as defined by Davidson (2001), in which the OCx progenitor field is further subdivided to give rise to the three ocelli and the interocellar cuticle. One of the earliest steps during the development of the Drosophila head is the initiation of otd expression by wg (Royet and Finkelstein, 1996; Blanco et al., 2009). In late embryos, all cells of the eye-antennal disc primordium, which can be marked by the expression of eyeless (ey) (Quiring et al., 1994; Czemy et al., 1999), express Otd (supplementary material Fig. S1). During larval development Otd expression progressively disappears from the disc, and is only maintained in its dorsal anterior region, where wg is expressed (Royet and Finkelstein, 1997). Otd in turn is required to activate hh transcription (Royet and Finkelstein, 1996). This results, in the early third instar (L3) disc, in the coexpression of wg, hh and otd in the prospective OCx region (Royet and Finkelstein, 1996) (Fig. 1A,D). However, wg transcription is next repressed in the prospective OCx to allow the development of the ocelli and the interocellar cuticle and bristles; otherwise, these structures fail to develop and are replaced by frons, a more lateral type of cuticle (Royet and Finkelstein, 1996).

During mid and late L3, the OCx region becomes further subdivided into three domains: the central domain transcribes hh and becomes the interocellar cuticle (IOC) region, while two adjacent domains express eyes absent (eya) and sine oculis (so), which encodes a Six1/2 transcription factor, and will become the anterior (a) and posterior (p) ocelli (OC) (Blanco et al., 2010; Brockmann et al., 2011) (Fig. 1B,D). The mechanism by which this aOC-IOC-pOC pattern is controlled by hh has recently been investigated (Aguilar-Hidalgo et al., 2013) and relies on the differential activation by the Hh signaling pathway of two Hh target genes: eya and the homeobox transcription factor engrailed (en). Hh first activates eya throughout the OCx region; then, en is turned on in a more restricted domain, which results in the attenuation of the Hh signaling pathway and the concomitant loss of eya from these cells.
Thus, the central region, expressing en and devoid of eya, becomes the IOC, whereas the remaining flanking eya-expressing domains become the retina-producing OC (Aguilar-Hidalgo et al., 2013).

However, a central question that remains to be answered for a comprehensive understanding of ocellar specification is how the changes in hh signaling domains are regulated during development, as this signaling morphogen plays a major role in controlling the specification and patterning of the OCx structures. Here, we have followed the regulatory steps that lead from the onset of hh expression to the establishment of its final expression domain, and define how these steps are interconnected in a gene regulatory network. We find that two transcriptional repressors, encoded by en and the Drosophila Six3/6 gene Optix, are key players in this network.

RESULTS

wg does not repress hh transcription but that of its targets eya and en

Work from the Finkelstein and Gehring labs identified many of the regulatory events between wg, hh and otd that result in the clearing of wg transcription from the dorsal-medial eye disc region to become the prospective OCx (Fig. 1D) (Royet and Finkelstein, 1995; Blanco et al., 2009). The regulatory interactions inferred from both studies highlight the same principle: wg is required for the specification of the dorsal head through the stepwise activation of otd and hh; the latter then increases Otd levels only in the medial region. Here, in a negative-feedback loop, high Otd represses wg and the OCx region is specified. However, there are also important differences between the two gene regulatory networks. Whereas Blanco and co-workers proposed that the segregation of the wg and hh expression domains is indirect, mediated through otd, Royet and Finkelstein suggested a direct mutual repression between these two signaling pathways. In particular, it was shown that removing wg function during the second half of L3 resulted in an expansion of the hh domain, pointing to wg as a hh repressor.

To test this point, we carried out the converse experiment: we overactivated the wg pathway throughout the prospective OCx and analyzed the effects on hh transcription, Hh pathway activity and the Hh pathway targets en and eya. To do so, we drove expression of a constitutively active form of Armadillo (Arm*), the Drosophila β-catenin and nuclear transducer of the canonical wg pathway (Peifer et al., 1991), using the ocellar driver oc2-GAL4 (Blanco et al., 2009) in an hh-Z background (Fig. 2). Adult oc2>Arm* flies lack all OCx structures, which are replaced by frons (Fig. 2A), a more lateral type of tissue that depends on wg (Royet and Finkelstein, 1996). In oc2>Arm* discs, transcription of hh, as monitored by the hh-Z reporter, is not repressed (Fig. 2B,D), nor is the accumulation of the activator form of Cubitus interruptus (CiA), which is an indicator of an active Hh pathway (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998) (Fig. 2C,E). On the contrary, we detect that the hh-Z domain expands in oc2>Arm* OCx (compare Fig. 2B,D). However, in this same genotype the two major hh targets, eya and en, are repressed (Fig. 2F,G). Therefore, wg signaling represses the outcome of the hh signaling pathway by blocking the expression of its targets, not by affecting hh transcription or its primary transduction. In addition, in this experiment wg signaling overactivation expands the hh expression domain. These results are thus in line with the Blanco et al. (2009) model of indirect regulatory interactions between hh and wg during OCx patterning.

hh is necessary for activity of the otd autoregulatory enhancer

Blanco and co-workers found that otd activates its own transcription (a property of the network that had not been noticed by Royet and Finkelstein) and described the oc7 DNA fragment as the otd autoregulatory enhancer (Blanco et al., 2009). Since high Otd levels build up in the prospective OCx (Royet and Finkelstein, 1995) and otd is regulated by Hh, we wondered whether these high levels could be the result of the activation of oc7 by Hh. Our experiments addressing this question reveal that oc7-Z is not active in early L3 discs, when Otd expression is homogeneous (Fig. 3A), but becomes active in later discs in the region showing highest Otd levels (Fig. 3B,C). When the Hh pathway is knocked down in the OCx region of the disc by expressing a dominant-negative form of the Hh co-receptor patched (ptc) (oc2>ptcΔloop2, or HhKD), the
expression of oc7-Z is lost, coinciding with the loss of the highest Otd levels (Fig. 3D). To test if Hh is sufficient to activate oc7, we expressed Hh in more lateral head regions using the 86c11-GAL4 driver (supplementary material Fig. S2A). In 86c11>hh discs new patches of oc7-Z expression are detected, and these are associated with new Eya-positive domains (Fig. 3E). In fact, adult 86c11>hh heads show supernumerary OC in lateral head regions associated with new Eya-positive domains (Fig. 3E).

These results confirm the functionality of the oc7 autoregulatory enhancer defined by Blanco et al. (2009) and establish a mechanism by which Hh is responsible for increasing the levels of Otd, by showing that Hh is required to turn on the otd autoregulatory enhancer oc7.

The hh expression domain contracts during patterning of the OCx region

Even with the modifications introduced in the experiments above, the existing OCx patterning model could not explain the expression changes that we observed for hh. Most conspicuously, the mutual positive regulation between hh and otd should maintain hh transcription in the whole prospective OCx region, where Otd levels are highest, which, however, is not the case (see Figs 1 and 4).

One possibility that could explain the hh expression dynamics is that hh-expressing cells, which initially span the whole dorsal region extending anteriorly up to the antennal domain (Fig. 4A), reorganize to form a central hh-expressing patch (Fig. 4B,C), thus explaining the apparent expression loss in the anterior and posterior sides of the domain. Alternatively, the restriction of the hh domain could be the result of an attenuation of hh transcription in the prospective ocellar domains.

To clarify this, we followed the lineage of hh-expressing cells in the dorsal eye disc and compared it with hh expression in late L3 discs using a hh-GAL4 reporter and the G-TRACE method (Evans et al., 2009). In this experiment, the RFP marker reports current hh transcription, while GFP is permanently expressed in all cells that have expressed hh and their descendants (i.e. in the hh ‘lineage’). If the final hh pattern were derived from a rearrangement of cells, we would not expect a major discrepancy between RFP (current) and GFP (lineage) signals. However, if some cells had lost hh transcription during development, some GFP cells would no longer express GFP. The latter is what we observed (Fig. 4D; n=8). Therefore, we conclude that the refinement of the hh domain during L3 requires the loss or repression of hh transcription in the anterior and posterior regions of the OCx. It is in these regions that eya remains active to specify the aOC and pOC.

en is required for hh maintenance in the IOC

During L3, hh transcription is lost from the anterior and posterior regions of the OCx (Fig. 4) to become restricted to the prospective

Fig. 3. hh regulates the expression of the otd autoregulatory enhancer oc7. OCx regions of oc7-Z imaginal discs. (A-G) Spatiotemporal dynamics of the otd and oc7-Z expression patterns in early (eL3), mid (mL3) and late (lL3) L3 discs. L3 discs, stained for Otd and β-galactosidase (oc7-Z). Hh is not active in eL3 (A). Its expression starts in mL3 (B) and becomes stronger in lL3 (C, white arrow). In parallel, the medially almost Otd expression also increases as development proceeds (B,C). (D) Knockdown of the Hh signal in oc2>ptc;loop2 (HhKD) in mL3 discs results in loss of oc7-Z activity (open arrow). The peak levels of Otd are not reached. (E) Overexpression of Hh in regions lateral to the OCx using the 86c11-GAL4 driver (86c11>Hh6.1) and stained for β-galactosidase (oc7-Z) and Eya. Ectopic patches of Eya and oc7-Z expression are induced in these lateral regions (arrows). Merged and separate channels are shown for all experiments. Low, medium and high Otd levels are marked by +, ++ and +++, respectively. Scale bars mark relative magnification of panels A to D.
These observations suggested a role for *en* not in the initiation, but in the maintenance, of *hh* expression.

To test this, we first carried out two loss-of-function experiments. In the first, we drove an *en* RNAi construct in a *hh-GAL4; UAS-GFP (hh>GFP)* background. We reasoned that if *hh* transcription required *en*, then knocking down *en* in this background should result in the reduction of the *hh*-driven GFP signal. Consistent with this, we found that in *hh>GFP+enRNAi* discs the GFP signal was weaker than in *hh>GFP+UAS-lacZ* discs (in this genotype an additional UAS transgene, *UAS-lacZ*, was introduced to equalize the number of UAS transgenes in both genotypes; Fig. 5A,B). We also noted a reduction in the size of the *eya*-expressing patches, which is further compatible with weaker Hh signaling, as *eya* is a *hh* target (Blanco et al., 2009; Aguilar-Hidalgo et al., 2013). Moreover, *hh>GFP+enRNAi* adult flies showed an almost complete replacement of the IOC by OC (Fig. 5C), a phenotype similar to that previously described for *en* null clones (Aguilar-Hidalgo et al., 2013). This confirmed the efficacy of the RNAi-mediated *en* knockdown.

In the second experiment, we induced *en* null mutant clones in a *hh-Z* background. In these clones the expression of *hh-Z* was severely reduced (Fig. 5D). We then carried out the converse experiment: to induce the expression of *en* in GFP-marked cell clones in a *hh-Z* background. In this experiment, *en*-expressing clones, located in regions adjacent to the normal ocellar *hh* expression domain, activated the *hh-Z* reporter cell-autonomously (Fig. 5E,E'). Thus, the results from both loss- and gain-of-function manipulations of *en* expression supported the hypothesis that *en* feeds back onto *hh* to maintain the characteristic high *hh* expression levels in the IOC.

**Optix** is asymmetrically expressed within the OCx and required for the development of the aOC

Having defined *en* as a positive regulator of *hh* in the OCx, we next asked which mechanisms in the OCx prevent *en* from being more widely expressed. Wider *en* expression would be expected since *en* is a *hh* target and *hh* expression extends, initially, throughout the whole prospective OCx. However, as *en* is a *hh* pathway repressor, wider expression of *en* would result in the abrogation of OC formation. This reasoning led us to hypothesize the existence of additional genes with asymmetrical expression within the OCx region, which would restrict the region within the OCx where *en* could be induced.

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**Fig. 4.** Alterations in the *hh* expression domain are the result of changes in *hh* transcription. (A-C) early (A) and late (B) *hh-Z* eye antennal discs, stained for β-galactosidase (*hh-Z*). The disc in B has also been stained for Eya. The double-headed arrow indicates the approximate extent of the OCx region. The prospective OCx region (boxed in B) is enlarged in C. The *hh-Z* domain is flanked by the OC Eya patches. (D) G-TRACE pattern of the *hh-GAL4* line. The current (red) and lineage (green) expression patterns are shown (merged and individual channels). The arrows point to regions within the *hh-GAL4* lineage where *hh-GAL4* transcription has been lost.

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IOC. In this region, the expression domain of *hh* is almost coincident with that of *en* (Royet and Finkelstein, 1997; Aguilar-Hidalgo et al., 2013). Previous work has shown that *en* acts as a *hh* activator during embryo segmentation and in wing discs (Lee et al., 1992; Mohler and Vani, 1992; Simmonds et al., 1995; Tabata et al., 1995). However, during the development of the OCx, the initiation of *en* expression is triggered by *hh*, placing *en* downstream of *hh* during early ocellar development (Aguilar-Hidalgo et al., 2013).

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**Fig. 5.** *en* is necessary and sufficient to maintain high *hh* transcription levels. (A,B) OCx regions of *UAS-lacZ*: *hh-GAL4; UAS-GFP (hh>GFP)* (A) and *hh-GAL4, UAS-GFP/UAS-enRNAi (hh>GFP+enRNAi)* (B), stained with anti-Eya. GFP signal is reduced in *hh>GFP+enRNAi* (B) relative to *hh>GFP* (A). In addition, in the presence of *enRNAi* the Eya signal weakens and the gap between the two Eya patches is partially filled with Eya-expressing cells. (C) *hh>enRNAi* adult head. In these flies, the IOC region is almost completely absent (one IOC bristle remains) and only one large ocellus forms. (D) *en* mutant clones (Df *en*) in the ocellar region, marked by the absence of GFP, induced in a *hh-Z* background. When these clones fall in the IOC region (arrow), they lose *hh-Z* signal. (E) Flip-out *en*-expressing clones (>>En, marked with GFP) in the dorsal anterior region of the eye-antennal disc, corresponding to the boxed region of the *hh-Z* disc shown in E’. The confocal plane shown in E’ shows the endogenous *hh-Z* expression domain (within the boxed region). The confocal plane in E is more medial. GFP-marked *en*-expressing clones (E, arrows) activate *hh-Z* expression.
In the eye-antennal disc, the Six3/6 homolog Optix has been shown to be expressed in the undifferentiated cell population of the developing eye (Seimiya and Gehring, 2000; Kenyon et al., 2005). In this context, Optix is required to promote retinal differentiation (Li et al., 2013). Interestingly, Optix is also expressed in the prospective dorsal head of the eye-antennal disc in a single domain (Seimiya and Gehring, 2000; Kenyon et al., 2005). We examined expression using an Optix-specific antibody, and found it to be expressed in a single anterior domain that overlaps with the aOC Eya domain but not with the IOC region (Fig. 6A) and that falls entirely within the broader otd domain (supplementary material Fig. S3). To investigate the function of Optix in this region, we used UAS-dsRNAi specific to Optix driven by oc2-GAL4 (oc2>OptixRNAi or OptixKD), which strongly reduced the Optix protein signal. Concomitantly, the anterior domain of Eya was variably reduced or absent (Fig. 6A,B). In oc2>OptixRNAi adult flies, the anterior ocellus was frequently unfused, small or absent, again with variable expressivity, whereas other ocellar structures, including the posterior ocellus, remained unaffected (Fig. 6C,D). By contrast, when we overexpressed Optix in the prospective IOC using a hh-GAL4 driver (Hh>Optix), the IOC was reduced, as expected from en repression (Fig. 6C,E).

To map more precisely the region of the adult head that derives from this Optix-expressing domain, we drove expression of UAS-lacZ with the OptixNP2631-GAL4 line, which is a GAL4 insertion in the vicinity of the Optix gene (see Materials and Methods) that recapitulates Optix expression in the dorsal eye disc (not shown). X-Gal-stained adult heads showed expression in a medial stripe running from the anterior edge of the dorsal head capsule to the aOC, but not beyond (Fig. 6C), in agreement with the requirement for Optix in the aOC only. Therefore, the anterior ocellus primordium, but not the posterior ocellus primordium, falls within the medial-anterior dorsal head domain marked by Optix, and requires Optix for its development.

**Optix acts as an en repressor in the aOC primordium**

The fact that the pOC develops without the need of Optix made us think that, rather than acting as a positive factor, Optix could be working to alleviate an ocellus fate repressor. Two pieces of evidence suggested that en could be such a repressor. First, the expression patterns of Optix and en were almost mutually exclusive (Fig. 7A). Second, the gain of Optix function phenocopied en loss (Fig. 6D) (Aguilar-Hidalgo et al., 2013).

To test whether Optix is required to set the anterior en expression limit, we knocked down Optix expression by driving OptixRNAi in the whole OCx region (using oc2-GAL4) or specifically in the anterior ocellus (with NP2631-GAL4) and assayed their effects on the expression of the en reporter en-Z. In discs of either genotype (oc2>OptixRNAi or NP2631>OptixRNAi) we still detected low levels of Eya expression in the aOC (see above and Fig. 7A-C), which was likely to be due to residual Optix function. Accordingly, the aOC was often reduced in oc2>OptixRNAi (Fig. 7B’; see also Fig. 6B) or incorrectly fused along the dorsal midline in NP2631>OptixRNAi (Fig. 7C’). When we checked the expression of en-Z in oc2>OptixRNAi discs we observed an anterior expansion of en-Z along the lateral regions of the OCx (Fig. 7B), which corresponded in the adult head to a lateral-anterior extension of the en-Z domain, surrounding the vestigial aOC (Fig. 7B’). When OptixRNAi was driven by NP2631-GAL4, en-Z also expanded anteriorly, but this time preferentially along the medial region of the OCx. Accordingly, in the resulting adults, en-Z signal spread anteriorly in the cuticle that splits the unfused aOC (Fig. 7C’).

Next, we overexpressed Optix in the prospective IOC domain using a hh-GAL4 driver in an en-Z background. In these experiments, en-Z expression was attenuated in the prospective IOC, although not completely absent (Fig. 7D). This level of reduction, however, seemed sufficient to block en action, as now the expression ofeya extended in between the aOC and pOC patches, forming a continuous domain (Fig. 7D). In the adult, this resulted in the reduction of the IOC fate (Fig. 6E), which depends on en (Aguilar-Hidalgo et al., 2013). However, the size of the OC was not increased, suggesting that the overexpression of Optix in the IOC does not allow for expansion of OC fate, even when it now expresseseya (Fig. 7D). Therefore, the finding that reduction of Optix results in the anterior expansion of en, which impedes the activation ofeya by Hh signaling, would explain the loss or reduction of the aOC in Optix knockdowns. In addition, the capacity of en to repress Optix suggests that the border between the Optix and en domains might be refined by mutual repression.

**DISCUSSION**

The relative simplicity of the OCx makes it an ideal system with which to study in detail the mechanisms involved in the
specification and patterning of a visual structure. Previous work had described the functional relationships between wg, hh and otd during the specification of the dorsal head, the region where the OCx forms. The outcome of these interactions, a wg-cleared OCx region, allows the subsequent specification of the ocellar structures, namely the three ocelli (an eya/so-dependent structure) and the intervening interocellar cuticle (an en-dependent structure), by the Hh signaling pathway. However, it is not clear how this aOC-IOC-pOC pattern is generated. More specifically, since this pattern depends on hh, the question is to understand how hh controls alternative fate decisions in this region. In this work, we have shown that the hh signaling domain changes during this process and that this change is essential for proper ocellar development. This dynamics depends on the establishment of a feedback loop with the hh signaling pathway target en, which, in turn, is restricted in its expression domain by the action of the Drosophila Six3/6 homolog Optix.

During the first half of L3 two Hh-related events occur. First, wg transcription clears from the prospective ocellar region. This is mediated by high Otd levels (Royet and Finkelstein, 1995), which are achieved through the activation of an otd autoregulatory enhancer [oc7 (Blanco et al., 2009)] by Hh signaling (Fig. 3). Second, and parallel to the wg clearing, low levels of eya expression are induced throughout the whole OCx (Aguilar-Hidalgo et al., 2013). During the second half of L3, though, the initially uniform expression domain of hh fades away to become restricted to its central region, associated with the activation of en. This central domain becomes the non-retinal interocellar cuticle (IOC), where en represses the transduction of the hh signal. This change in hh expression pattern, which defines the aOC-IOC-pOC domain organization in the disc and the structure of the adult OCx, occurs through transcriptional changes (Fig. 4). In particular, the maintenance of hh in the central domain depends on en. Therefore, after en expression is turned on by hh signaling (Aguilar-Hidalgo et al., 2013), en feeds back positively on hh transcription to maintain high hh expression levels (Fig. 5). In the prospective IOC, the expression of en represses hh signaling transduction and the initial expression of eya is lost. In the adjacent regions, though, eya expression is maintained at high levels through an autoregulatory loop that involves so (Brockmann et al., 2011). In addition, a potential non-autonomous contribution of Hh, produced at the IOC region, cannot be excluded. The en-to-hh maintenance function that we describe in the OCx resembles the well-established role of en as a hh transcriptional activator in other contexts, such as the embryo segmental stripes and the posterior compartment of the wing disc (Mohler and Vaní, 1992; Tabata et al., 1992, 1995; Zecca et al., 1995), and could constitute a regulatory module that is deployed in several developmental contexts, such as the developing head.

We have further demonstrated that the peripheral reduction of the hh domain is due to transcriptional regulation rather than cellular rearrangements. This raises the question of how the reduction of hh transcription outside the IOC region occurs. The most likely possibility is that a hh activator is lost as the development of the OCx region progresses through L3. We noted that hyperactivation of the wg canonical pathway in the OCx region results in an expansion of the hh-Z domain (Fig. 2). If wg were required to activate hh, the indirect negative-feedback loop that results in the wg clearing from the OCx region would also result in the loss of its activating action on hh and the loss of hh transcription. This would be prevented only in places where en was expressed. This hypothesis (wg being required for hh expression in the OCx) is supported by the fact that, in the embryonic head, wg activates hh expression (Bejsovec and Martinez Arias, 1991; Lee et al., 1992). Nevertheless, it seems contradictory to previous reports in which, using a temperature-sensitive wg allele, the reduction of wg signaling activity resulted in an enlargement of the OC and the IOC (Royet and Finkelstein, 1996). However, this result could be reconciled with wg acting as a hh activator. Early during L3, wg would activate hh transcription (Fig. 2) while simultaneously preventing the expression of en and eya, two targets of hh (Blanco et al., 2009) (Fig. 2). Later in L3, high levels of Otd, produced after the activation of the oc7 enhancer, result in the transcriptional repression of wg in the prospective OCx region. This repressive step leaves the wg expression domain restricted to more lateral regions, where the frons and the orbital cuticle will be specified. Removing wg function during this late period, as in the Royet and Finkelstein (1996) experiment, would allow the activation of Hh targets eya and en in a broader domain due to the non-autonomous action of secreted Hh. Since en maintains hh transcription at high levels, late removal of wg should result in an enlarged hh expression domain and an increase in the overall size of
the OCx, as observed. It is also important to mention that previous work has shown that the Iroquois Complex (Iro-C) genes araucan and caupolican participate in the restriction of the OCx to the medial region (Yorimitsu et al., 2011) (Fig. 8).

Key to the establishment of OCx patterning is where en becomes expressed. In contrast to hh transcription, which changes over time, that of en is stable once initiated in the prospective IOC. We found that Optix is expressed in a dorsal anterior strip in the eye disc, contained within the otd domain (supplementary material Fig. S3), that abuts posteriorly the en domain (Fig. 7). Our results suggest that Optix is partially responsible for setting up this anterior border of en. Since en, by acting as a Hh pathway repressor, prevents eya transcription, such an expansion is the most likely cause of the effects on the aOC. The definition of a clear-cut Optix/en border might be further refined by mutually repressive interactions, as indicated by two results: overexpressing Optix in the IOC results in the downregulation of en (Fig. 7) and, reciprocally, the overexpression of en in the prospective aOC represses Optix (supplementary material Fig. S4). Therefore, as en is initially activated by hh, the anterior limit of the en domain may result from the integration of activator and repressor inputs provided by hh signaling and Optix, respectively, a limit that might be further refined by reciprocal repression of Optix by en. We hypothesize that a similar mechanism sets the posterior border of the en expression domain to allow for an en-free, hh-receiving domain that becomes specified as the pOC. Potential candidates for the posterior anti-repressor are the Sp genes buttonhead (btd) and Sp1, and hunchback (hb). These genes are expressed in the preoptic region of the embryonic head of all arthropods (see Schaeper et al., 2010; Birkan et al., 2011; Janssen et al., 2011; and references therein). However, neither Sp1 nor btd is expressed in the prospective OCx region of the eye disc (Estella et al., 2003; C. Estella, personal communication). We have checked for hh expression using an anti-Hb antibody and found no expression in L3 eye-antennal discs (not shown). Therefore, the nature of the posterior regulator(s) involved remains to be determined. Fig. 8 summarizes these results into a schematic gene network (for an extended network representation, see supplementary material Fig. S5).

Our results indicate that, despite their structural similarity and shared requirement of otd, hh signaling and eya activation, the patterning of the aOC and pOC are under different genetic control. This might be expected, as only the anterior ocellar patches fuse during metamorphosis to form the adult aOC. In fact, evidence for this difference in genetic control had previously been obtained by Maynard-Smith and Sondhi in population selection experiments in Drosophila subobscura (Maynard-Smith and Sondhi, 1960). In this study, the authors used an ocelliless (oc) mutant population that showed loss of OCx structures, including the aOC and pOC, with variable penetrance. Through breeding, they were able to establish independent sublines in which the aOC, but not the pOC (or vice versa), were preferentially lost, even when these flies were still carrying the otd mutation. In light of these results, the authors proposed that, on top of a common precursor for OC and bristle (i.e. cuticle) determined by otd, an additional ‘system’ would control the amount of ocellar or cuticle precursors, and this system would differ along the anterior-posterior axis. In this context, Optix would not instruct an ocellar fate but rather control the amount of anterior ocellus precursor cells within the OCx primordium, thus acting as a pre-patterning gene.

The expression pattern and function of six3/Optix have been studied in Drosophila (Seimiya and Gehring, 2000; Coffier et al., 2008) and Tribolium (Posnien et al., 2009, 2011; Kittelmann et al., 2013) embryos. In both insects, six3/Optix expression is restricted to the head region, and includes the clypeolabrum and maxillary
segment in Drosophila and the larval and middle head regions in Tribolium. Accordingly, six3/Optix mutant Drosophila larvae show reduced or absent larval-derived head skeletal elements, such as the larval organ and the maxillary segment-derived mouth hooks in Drosophila (Coiffier et al., 2008) and loss of the labrum and anterior vertex bristle in Tribolium (Posnien et al., 2009, 2011). Here, we have shown that in Drosophila the expression domain of Optix in eye discs, which runs along the anteriormost medial disc region, maps to the anterior medial dorsal head, where it is required. In addition, OptixNP2631-OptixRNAi adults show defects in the clypeal skeleton (not shown), recapitulating the defects seen in larvae (Coiffier et al., 2008). Therefore, all these results point to six3/Optix as a medial head-patterning gene also during eye-antennal disc development. However, we have not been able to detect Optix expression in the embryonic primordium of the eye-antennal disc, marked with the disc primordium marker ey-Z and using either an anti-Optix antisera or the OptixNP2631-GAL4 line (not shown). We have also analyzed the expression of all available regulatory constructs associated with the Optix locus generated by the Janelia Project (Jory et al., 2012). Neither of the lines expressed in the eye disc showed overlapping expression with the embryonic eye primordium (not shown). Therefore, and provided that we have not missed a low number of Optix-expressing cells in the eye-antennal disc primordium, the expression of Optix in the head primordium is likely to be initiated during larval life.

The mechanisms that initiate Optix expression in the most anterior region of the dorsal head need to be investigated further. Our results also raise the question of whether en, otd and hh might be similarly engaged in adult head patterning in other insects.

**Immunostaining and imaging**

Immunofluorescence in eye imaginal discs and embryos was carried out according to standard protocols. Antibodies used were: guinea pig anti-Otd (gift of Tiffany Cook, Cincinnati Children’s Hospital, USA; 1/1000), rabbit anti-Optix (gift of Francesca Pignoni, Upstate Medical University, Syracuse, USA; 1/500), guinea pig anti-Hb [gift of Karl Wotton (Kosman et al., 1998); 1/2000], mouse anti-Eya (10H6; 1/1000), rat anti-CiA (2A1; 1/5) [which detects the activator form of Ci (Aza-Blanc et al., 1997)] and mouse anti-En (4D9; 1/15) from Developmental Studies Hybridoma Bank. lacZ reporters were detected using a rabbit anti-β-galactosidase antibody (Cappel, catalog number 55976; 1/1000). Appropriate Alexa Fluor-conjugated secondary antibodies were used. Image acquisition was carried out using a Leica SP2 AOBs confocal microscope. Images were processed with Photoshop CS5 (Adobe).

**Adult cuticle preparation**

Dorsal head cuticle pieces were dissected from adult or late pharate heads in PBS, and mounted in Hoyer’s solution:acetic acid (1:1) as described (Casares and Mann, 2000). lacZ expression in enhancer trap line-derived adult fly heads was monitored using the β-galactosidase substrate X-Gal as described (Hama et al., 1990). Images were obtained using a Leica DMS500B microscope with a Leica DFC490 digital camera and processed with Photoshop CS5.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

F.C. conceived the study and wrote the paper; M.A.D.-C. carried out most of the research; M.A.D.-C. and F.C. designed the experiments and analyzed results.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.125179/-/DC1

**References**


