Common functions of central and posterior Hox genes for the repression of head in the trunk of *Drosophila*

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Hox genes are localised in complexes, encode conserved homeodomain transcription factors and have mostly been studied for their specialised functions: the formation of distinct structures along the anteroposterior axis. They probably derived via duplication followed by divergence, from a unique gene, suggesting that Hox genes may have retained a common function. The comparison of their homeodomain sequences groups Hox proteins into Anterior, Central and Posterior classes, reflecting their expression patterns in the head, trunk and tail, respectively. However, functional data supporting this classification are rare. Here, we re-examine a common activity of Hox genes in *Drosophila*: the repression of head in the trunk. First, we show that central and posterior Hox genes prevent the expression of the head specific gene *optix* in the trunk, providing a functional basis for the classification. Loss-of-function mutations of *optix* affect embryonic head development, whereas ectopic Optix expression strongly perturbs trunk development. Second, we demonstrate that the non-Hox genes *teashirt*, *extradenticle* and *homothorax* are required for the repression of *optix* and that Wingless signalling and Engrailed contribute to this repression. We propose that an evolutionary early function of Hox genes was to modify primitive head morphology with novel functions specialising the trunk appearing later on.

**KEY WORDS:** *Drosophila*, Hox, *optix/Six3*, Head, Trunk

**INTRODUCTION**

The body plan of all bilaterian animals is composed of three morphologically distinct regions along the anteroposterior axis: the head, trunk and tail. In insects, the true head comprises three segments (antennal, labrum and intercalary) plus the anterior acron (Jürgens et al., 1986). The cephalopharyngeal cuticle of the larva is unique in that it is often pigmented and has a wrinkled morphology. Three gnathal segments (mandibular, maxillary and labium) are thought to have derived from the trunk (Snodgrass, 1938) but have acquired specific head-like characteristics. The trunk is formed by three thoracic (prothorax, mesothorax and metathorax) and eight abdominal segments (A1 to A8), each characterised in the larva by alternating anterior denticles and posterior naked cuticle. The tail comprises three segments plus the terminal telson.

The morphological diversity along the anteroposterior axis of vertebrates and invertebrates relies in part on the activity of Hox genes (Kaufman et al., 1990; Lewis, 1978). Hox proteins are evolutionarily conserved transcription factors that control the expression of downstream target genes (Graba et al., 1997; Pearson et al., 2005) through a DNA-binding domain called the homeodomain (McGinnis et al., 1984; Scott and Weiner, 1984). Hox genes in most species reside in clusters. In *Drosophila melanogaster*, they are localised in two complexes (Kaufman et al., 1990; Lewis, 1978): the Antennapedia and Bithorax complexes. Hox genes are expressed according to the spatial colinearity rule, with 3’ genes acting in the head, median genes acting in the trunk and 5’ genes in the tail parts of the embryo. Loss of Hox genes results in the transformation of one segment into a neighbouring, usually anterior one, whereas ectopic Hox activity usually involves posterior homeotic transformations (Gonzalez-Reyes and Morata, 1991; Lewis, 1978).

On the basis of expression patterns and the sequence similarity of their homeodomains, Hox proteins from a wide variety of species have been grouped into three classes (Schubert et al., 1993; Duboule, 1994; de Rosa et al., 1999): Anterior (A), Central (C) and Posterior (P). In *Drosophila*, Labial (Lab) is an A class protein functional in the head, Proboscipedia (Pb) is an A class protein with no known function during embryogenesis, Deformed (Dfd) and Sex combs reduced (Scr) are divergent C class proteins active mostly in gnathal segments, whereas Antennapedia (Antp), Ultrabithorax (Ubx) and Abdominal A (AbdA) are C class proteins active in thoracic and/or abdominal segments. Finally, Abdominal B (AbdB) is the sole P class protein active in the tail as well as in the posterior trunk (Sanchez-Herrero et al., 1985). To date, the only functional data relevant to this classification is the ability of A and C class genes to rescue the tritocerebrum mutant phenotype of the *lab* gene (Hirth et al., 2001).

As Hox genes are physically clustered on the chromosome, they probably arose by tandem duplication and acquired novel functions during evolution (Lewis, 1951). Many targets of Hox genes have been described (Graba et al., 1997; Pearson et al., 2005; Svingen and Tonissen, 2006) that are controlled directly by individual, or at most three, Hox proteins. Established targets therefore represent novel, acquired functions, as none of the targets are regulated by all or the majority of Hox genes. There is evidence however that most Hox genes have a common function for the repression of head in the trunk, as their loss results in the differentiation of head structures in each trunk segment in the fly and the beetle Tribolium (Lewis, 1978; Stuart et al., 1991; Struhl, 1983). This common activity may therefore represent an ancient function. If so, Hox genes might be expected to regulate common target genes or activities involved in head development. Although some putative, common targets have been reported recently in a microarray approach following ectopic expression of most, but not all, Hox genes in the embryo (Hueber et al., 2007) no candidate for a common target of Hox proteins has yet been identified.

Hox genes do not act alone for segment diversity of the embryo. In the trunk, the zinc-finger protein Teashirt (Tsh) is co-expressed and acts with Scr, Antp, Ubx, AbdA and AbdB (hereafter referred to...
as HoxCP) to promote trunk. Importantly, the loss of all of these proteins causes a complete transformation of the ventral trunk into head identity (Roder et al., 1992). Consistent with the notion that Hox genes and tsh suppress head identity, ectopic activity of Antp, Ubx, AbdA or Tsh replace certain head identities with trunk structures (Gonzalez-Reyes and Morata, 1990; de Zulueta et al., 1994). Hox and tsh genes thus share a common activity to repress head formation in the trunk. Additionally, to achieve their function, Hox proteins usually need the presence of their co-factors, Extradenticle (Exd) and Homothorax (Hth) (Mann, 1995; Mann and Affolter, 1998), which are also homeodomain proteins (Ryoo and Mann, 1999) and are highly conserved in bilaterians (Moens and Selleri, 2006). Together, they form complexes that increase Hox DNA-binding specificity to regulate their target genes.

In vertebrates and invertebrates Wnt genes have been implicated in a variety of processes during embryogenesis (Klingensmith and Kaufman, 1981), showing that Wnt signalling promotes trunk at the expense of head. Head formation in the trunk. Additionally, to achieve their function, Hox proteins usually need the presence of their co-factors, Extradenticle (Exd) and Homothorax (Hth) (Mann, 1995; Mann and Affolter, 1998), which are also homeodomain proteins (Ryoo and Mann, 1999) and are highly conserved in bilaterians (Moens and Selleri, 2006). Together, they form complexes that increase Hox DNA-binding specificity to regulate their target genes.

Here, we show that the Six-gene family member optix, encoding a conserved homeodomain protein normally limited to the head (Kawakami et al., 2000; Seimiya and Gehring, 2000), is repressed by all C and P class Hox genes in the trunk or tail. Additionally, we demonstrate that the Hox co-factors Extradenticle, Homothorax and Teashirt are necessary for the repression of optix in the trunk, and that Wg signalling and Engrailed contributes to this repression. Therefore, *Drosophila* has developed multiple inputs to repress head development in the trunk. From an evolutionary standpoint, we propose that one of the most ancient functions of Hox genes is to repress head, a function shared with other non-Hox genes.

**MATERIALS AND METHODS**

*Drosophila* strains

The following strains were used: *Scr^C1* Antp^{Nvs+Rc3} Df(3R)P9, *Scr^C1* Antp^{Nvs+Rc3} Ubx^{MX12} abdAb^{AM1} AbdB^{AM6}, Antp^{Nvs+Rc3} Ubx^{MX12} abdAb^{AM1} AbdB^{AM6}, *Scr^C1* Antp^{Nvs+Rc3} Ubx^{MX12} abdAb^{AM1} AbdB^{AM6}, *Scr^C1* Antp^{Nvs+Rc3} Ubx^{AM1} AbdB^{AM6} Ubx^{AM1} AbdB^{AM6}, Antp^{Nvs+Rc3} Df(3R)P109, *Dfd^{RX1}* lab^{abf8}, *pb^{5}*, and *Df(2R)en* (Bloomington) (Seimiya and Gehring, 2000) and *Df(3R)Ubx^{109}* (Bloomington).

Standard techniques were used for cuticle preparations. Genotypes were maintained by the transgenic *UAS-lab* (Southern Biotechnology Associates) and analysed with an LSM 510 Zeiss confocal microscope.

**RESULTS**

Central and posterior Hox and tsh genes repress head development in the trunk

In order to study the potential common function of Hox genes to repress head in the trunk of the *Drosophila* embryo, we have re-examined the morphology of larval cuticles lacking the HoxCP genes. As reported earlier (Lewis, 1978; Roder et al., 1992; Sanchez-Herrero et al., 1985; Struhl, 1983), prothoracic-like structures (denticles and beard) develop in the anterior part of each trunk segment, owing to Tsh activity, and head-like cuticle forms in the posterior (Fig. 1A,B), because Tsh expression is not maintained (Roder et al., 1992). Moreover, the loss of tsh and HoxCP genes replaces all ventral trunk structures of the larva (denticles and smooth cuticle) with wrinkled cuticle (Roder et al., 1992) (inset Fig. 1C) found normally only in the head of larvae (Fig. 1A,C). Therefore, like Hox genes, tsh contributes to the suppression of head in the trunk (Roder et al., 1992).

To analyse the individual capacities of HoxCP genes to ensure this function, we examined cuticles from embryos carrying individual Hox gene activities in tsh HoxCP larvae. Cuticle preparations show that Scr function represses head in the extreme anterior trunk but is incapable of making denticles without Tsh (Fig. 1D) (see Roder et al., 1992). Antp, Ubx and AbdA similarly repress head (Fig. 1E-G), but each acts in a specific way: Antp induces denticles similar to those found in the thorax (Fig. 1E, see legend), Ubx to those found in the first abdominal segment (Fig. 1F) and AbdA induces denticles with a second abdominal identity (Fig. 1G). Finally, AbdB represses head identity producing naked cuticle but does not induce denticles (Fig. 1H).

At the molecular level, we have examined the expression of *shaven baby* (*svb*), which is expressed in the cells destined to form denticles in the larva (Payre et al., 1999). For each Hox gene capable of inducing denticles (Antp, Ubx or AbdA), *svb* is detected ventrally in each trunk segment where these genes are active (see Fig. S1 in the supplementary material). The activities of Scr and AbdB, in the absence of Tsh, are incapable of inducing *svb* expression or denticles ventrally (see Fig. S1 in the supplementary material).
In conclusion, though only Tsh, Antp, Ubx and AbdA are capable of inducing denticles, Tsh, Scr, Antp, Ubx, AbdA and AbdB all contribute independently to the shared role of repressing head development.

The head gene optix is repressed by central and posterior Hox and tsh genes in the trunk epidermis

In order to identify genes involved in the head to trunk homeosis, we compared the expression of eight candidate genes (spalt, cap-n-collar, optix, empty-spiracles, orthodenticle, buttonhead, forkhead, sine oculis) with prominent head expression. Of these, only optix expression is strictly localised to the wild-type head and gnathal regions (Fig. 2A-C, not shown), and altered in HoxCP and tsh HoxCP embryos (Fig. 2D-F and data not shown).

In wild type, optix is expressed from the blastoderm stage in a ring of cells at the anterior end of the embryo that is destined to form the clypeolabrum, the pharynx and the acron, according to the projections on the fate map (Fig. 2A) (Seimiya and Gehring, 2000). At the end of germ band elongation (Fig. 2B), the anterior cephalic domain extends dorsally then is separated in two parts, while new small patches of optix expression appear in cells located in the lateral parts of the maxillary and labial segments. By stage 14, optix is detected in the ectoderm covering the supra oesophageal ganglion, which gives rise to certain parts of the head skeleton. Inset in A shows the pigmented crinkled cuticle typical of the head skeleton. Inset in C highlights the crinkled cuticle. In D, the distance from the anterior border of the crinkled cuticle to the anterior trunk (line) is larger than that in C. The inset in E shows the small thoracic denticles (white arrow) invisible in the larger photograph. Head-like cuticle develops in A5-8 (arrowheads). Anterior is on the left for all panels.

![Generic Hox function](image)

Fig. 1. Hox genes repress head and promote trunk. Cuticles of (A) wild type, (B) HoxCP, (C) tsh- HoxCP, (D) tsh- Hox Scr+, (E) tsh- Hox Antp+, (F) tsh- Hox Ubx+, (G) tsh- Hox abdA+ and (H) tsh- Hox AbdB+, larvae. White arrows, denticles; black arrows, naked cuticle; arrowheads, crinkled head cuticle. Head cuticle is mostly in the posterior part of each trunk segment in B, but occasionally also differentiates in the anterior (left and right arrowheads). Inset in A shows the pigmented crinkled cuticle typical of the head skeleton. Inset in C highlights the crinkled cuticle. In D, the distance from the anterior border of the crinkled cuticle to the anterior trunk (line) is larger than that in C. The inset in E shows the small thoracic denticles (white arrow) invisible in the larger photograph. Head-like cuticle develops in A5-8 (arrowheads). Anterior is on the left for all panels.
The role of optix in head development

In light of these observations, we wondered whether Optix is required for the normal development of the embryonic head and for the transformation of trunk in tsh HoxCP embryos. We examined the cuticular phenotypes of loss-of-function larvae either using three optix mutant alleles (FlyBase), or induced by the injection of three different dsRNAi constructs into wild-type embryos (Fig. 3A, n=400 embryos for each probe). Both techniques gave similar phenotypes, suggesting that the insertion alleles are amorphs or strong hypomorphs. Indeed, homozygotes and hemizygotes are indistinguishable from each other and from optix RNAi-injected embryos. For the latter (n=210), 70%-80% gave no signal upon hybridisation with optix probes compared with control water-injected embryos (n=154), where 95% of embryos exhibited expression (not shown).

The most common phenotypes are the absence of structures deriving from the labrum (Fig. 3B-E) (Jürgens et al., 1986) and defects in mouth hook formation, which are absent (Fig. 3E), deformed or reduced (Fig. 3D). Together these abnormalities correlate with the domains of optix expression in the head and maxillary segment (Fig. 2A-C). However, we found no evident abnormality in structures deriving from the labial segment where a small group of optix-expressing cells is observed (Fig. 2B).

As optix has an embryonic function in the head, it could be responsible for the crinkled cuticle present in the trunk of tsh HoxCP embryos. However, loss of optix, tsh and HoxCP genes or inactivation of optix and tsh by dsRNAi injection in HoxCP embryos were indistinguishable from tsh HoxCP mutants (Fig. 3F). We conclude that Optix is not the only factor responsible for the head morphology present in the trunk of such embryos. However, it represents an excellent molecular marker for head.

Finally, we examined the effects of early (Fig. 3G) and late (not shown) ectopic optix expression, to determine whether optix expression in the trunk is functional. In both cases, the trunk is abnormal with penetrant (95%, n=230) defects in germ band retraction (Fig. 3G) and in dorsal closure (not shown), suggesting that the negative regulation of optix in the trunk is important.

Individual capacities of Hox genes to repress optix

Next, we examined the individual capacities of HoxCP genes to repress optix. Addition of Scr, Antp, Ubx, abdA or AbdB genes individually in tsh HoxCP context results in the loss of ectopic patches of optix in the domains where each Hox gene is expressed (Fig. 4A-F). Similar results were found on restoring individual Hox gene functions to HoxCP embryos (data not shown). We conclude that Scr, Antp, Ubx, abdA and AbdB are sufficient to repress optix in homologous anterior parts of each trunk segment, where they are active.

We then asked whether the more anterior Hox genes lab, pb and Dfd affected optix expression. Dfd is a divergent C type Hox gene (Schubert et al., 1993), expressed in the maxillary and mandibular segments in wild-type embryos (Chadwick and McGinnis, 1987). Loss of Dfd induces ectopic optix expression in the ventral part of the maxillary segment (arrow, Fig. 4G). Products of the A class gene lab are detected in the intercalary segment of wild-type embryos (Diederich et al., 1989). Loss of lab causes no detectable effect on the localisation of optix transcripts (compare Fig. 4H with Fig. 2B). To ensure that the loss of lab does not affect the expression of optix, we expressed lab ectopically in the absence of HoxCP genes using the prd-Gal4 driver, which is expressed in a pair rule pattern. No alteration in the ectopic expression of optix was detected ventrally.
Fig. 3. optix is required for the development of the labrum and mouth hooks during embryogenesis. (A) Genomic region of optix showing the insertions and the probes (red lines) used for the RNAi experiments. (B-G) Cuticle preparations of wild type (B, dorsal; C, lateral), Df(optix)(D, laterodorsal; E, ventral) head skeletons, tsh–optixC01718 HoxCP (F, ventral) and nullo-Gal4 UAS-optix (G, lateral) larva. Derivatives of the labrum (red; lr, labrum; es, epistomal sclerite) and labral sense organ are absent and the maxillary-derived, mouth hooks (MH) are missing (E) or reduced (D) in Df(optix) (compare B-E). All sense organs, apart from the labral one, are unaffected in the different optix alleles (D,E) and RNAi-treated larvae (not shown). In E, the asterisk indicates head cuticle in place of the mouth hooks, labral and adjacent structures normally found in this position (see B,C). H piece (H), hypostomal sclerite (hys) and lateralgräten (LG) derive from segments posterior to the labrum (Jürgens et al., 1986). The head-like cuticle present on the ventral side of tsh–optixC01718 HoxCP (arrowhead in F) and tsh HoxCP (Fig. 1C) embryos are indistinguishable. In G, early ectopic expression of optix results in a failure in retraction of the germ band.

Effects of ectopic expression of Hox and tsh genes upon optix expression

Next, we asked whether ectopic Hox or tsh production could repress optix expression in its normal domains in the head and gnathal segments. Ubiquitous expression in the epidermis from stage 10 (using 69B-Gal4) of Tsh, Scr, Antp, Ubx, abdA and AbdB represses optix in the late gnathal domains, but not in the initial domain in the true head (Fig. 5A-C, not shown). However, earlier production of Hox or Tsh proteins ubiquitously from the blastoderm stage, using the nullo-Gal4 driver, strongly reduced or removed optix expression in the initial head, as well as the gnathal regions (Fig. 5D,E, not shown).

We then analysed the effect of ubiquitous activity of Dfd, Lab and Pb on optix expression. Mis-expressed Dfd does not repress optix in the head or in the gnathal segments, the normal domain of Dfd expression (Fig. 5F) (Chadwick and McGinnis, 1987); however, the gnathal patches of optix are enlarged (compare Fig. 5F, Fig. 2B,C and Fig. 5A) and in the thorax ectopic optix is detected in pairs of lateral groups of cells. These patches possibly correspond to ectopic maxillary structures (mouth hooks) known to develop in these positions in larvae upon expression of Dfd (Fig. 5F) (Kuziora and McGinnis, 1988) and correlate with one role of optix for normal mouth hook development (Fig. 3B-E). Similarly, early (but not late) ectopic induction of Lab also results in ectopic optix expression in the thorax (Fig. 5G), where three pairs of lateral patches of cells are detected. In addition in the maxillary and labial segments, the patches of optix are enlarged (compare Fig. 5A with F). Neither early nor late ectopic Pb expression has any effect on the expression of optix (not shown).

These results corroborate the idea that tsh and all Hox genes active in the embryo can regulate the optix gene. However, only tsh, central and posterior Hox genes have a common function to repress the expression of optix, each acting in distinct domains of the gnathal and/or trunk domains of the ventral epidermis. The A class genes lab and pb are the only Hox genes that cannot repress optix expression. Although Dfd represses optix in the ventral maxillary epidermis (Fig. 4G), ectopic assays indicate that Dfd and Lab, but not Pb, are capable of activating optix in more lateral positions.

The Hox co-factors Extradenticle and Homothorax repress optix expression in the trunk

To increase their DNA-binding specificity and regulate their targets, Hox proteins require the activity of co-factors (Mann, 1995; Mann and Affolter, 1998; Mann and Chan, 1996) including Exd and Hth. We examined optix expression in exd and hth embryos. For both, optix is expressed ectopically in a large ventral part of twelve trunk [maxillary (Mx) to A7] segments from stage 11 onwards (Fig. 6A-D). Ectopic optix expression is more intense in the posterior two gnathal and thoracic segments compared with the abdominal domains of these embryos, indicating a thoracic transformation to a more anterior identity, which is coherent with the differentiation of head-like, crinkled cuticle in the thorax of the larvae (insets Fig. 6E,F). In addition, in A8, where AbdB is strongly detected in the
epidermis (Fig. 6A,B), and A9, optix is neither detected in exd nor hth embryos. We conclude that these Hox co-factors are required for the prevention (by Hox protein) of head development and/or optix expression in the trunk, with the exception of A8 and 9.

Wingless signalling and Engrailed contribute to the repression of optix in the trunk

The absence of tsh and HoxCP genes results in the derepression of optix only in the ventral, anterior and median, but not in the posterior part of trunk segments, corresponding to the normal domain of wg and en (Fig. 2F, Fig. 7A,B) (Wodarz and Nusse, 1998). Loss of En abrogates Hh signalling and En and Wg are required to maintain the expression of one another from stage 10 onwards (reviewed by Wodarz and Nusse, 1998). As the location of en and wg stripes is approximately complementary to the domains of ectopic optix expression in the trunk segments of HoxCP and tsh HoxCP embryos (Fig. 2F, Fig. 7A,B), we asked whether Wg and En contribute to the repression of optix in the posterior parts of trunk segments in tsh HoxCP embryos.

With this aim, we removed en or wg in tsh HoxCP embryos. In both contexts, optix expression is detected throughout the ventral part of each trunk segment with far fewer ventral cells that are devoid of optix signal (Fig. 7C,D). In embryos that lack only wg or en function, no ectopic activation of optix is seen (not shown) implying that the repressive action of Wg or En upon optix relies on the absence of Hox and Tsh proteins. Ectopic Wg or En production has no clear effect on the normal expression of optix (not shown). These results are perhaps not surprising, as these genes are normally co-expressed in specific cells in the head (Fig. 2F, Fig. 7A). These results favour the idea that Wg signalling and En contribute to the suppression of head development in the Drosophila trunk, but these activities are only revealed in the absence of Hox and Tsh (Fig. 7E).

DISCUSSION

On the basis of their homeodomain sequences and expression patterns, Hox proteins across the animal kingdom fall into A, C or P classes (de Rosa et al., 1999; Schubert et al., 1993). Here, we show that C and P class Hox genes share a function, repressing head development in the trunk, which, on the basis of morphology, has been described both in Drosophila and Tribolium (Lewis, 1978; Stuart et al., 1991). Our novel observation is the molecular manifestation of these concerted Hox activities involving the restriction of a common molecular target, optix/dSix3, and the Six-class homeodomain protein it encodes. Indeed, though optix expression is normally restricted to the head, it can be differentially extended to the trunk on removing Hox or co-factor activities there.
The common ability of C and P Hox genes to repress ‘head’ suggests that this function is an ancient one compared with novel functions, which are required for morphological novelties observed in distinct parts of the body, which evolved later on. Although we have been unable to demonstrate a function for Optix in the trunk of tsh HoxCP embryos (Fig. 3F), Optix perturbs trunk morphogenesis when ectopically expressed uniformly (Fig. 3G). The Hox co-factors Exd and Hth also repress optix in the trunk, consistent with a common role for C and P Hox genes in the repression of head and indicating that Hox/Exd/Hth complexes are ancient acquisitions, in accordance with their universality in bilaterians. Furthermore, we show that additional, conserved genes, including tsh, en and wg have a common function with C and P Hox genes to repress head identity (Fig. 7E).

Common and divergent activities of Hox genes
Hox genes are conserved in higher animals and have well described activities concerning their specific roles in segment morphology. For example Ubx and AbdA directly repress the transcription of the Distal-less (Dll) gene in the abdomen of the embryo (Gebelein et al., 2002; Gebelein et al., 2004). Moreover, direct Hox targets specific to individual or up to three Hox genes have been widely described (reviewed by Graba et al., 1997; Pearson et al., 2005). Hox genes are thought to have derived by gene duplication (Lewis, 1951) and divergence of activity (Carroll, 1995). If Hox genes derived from a common ancestral gene, then two extreme hypotheses seem plausible: either ancient Hox functions have been retained or been lost in current-day Hox genes. Our results favour the former idea, as all C and P class Hox genes repress head formation and the expression of optix, in their domains of function, in the trunk epidermis (Fig. 4A-G). Similar functional equivalence of Hox proteins has been reported in the nervous system for A and C genes by Hirth et al. (Hirth et al., 2001).

The A class Hox genes, lab and pb, are not involved in the repression of optix during embryogenesis. As Lab, like optix, is expressed and active in the true head, this is not surprising. However, lab is able to activate optix, following early and continuous ectopic expression. pb is not active in the embryo and is not able to repress or activate optix. Thus, optix is a common target of all seven fly Hox genes that are active in the embryo.

Genes outside of the Hox complex contribute to head repression in the trunk
We further document that tsh contributes to the morphological repression of head (de Zulueta et al., 1994; Roder et al., 1992), and the molecular repression of optix in the trunk (Fig. 2E). However, loss of Tsh alone does not cause derepression of optix in the trunk; its activity is only revealed when HoxCP genes are absent, showing that Tsh and HoxCP genes have common functions. Interestingly three vertebrate orthologues of tsh have been described that are expressed in distinct caudal rostral and dorsal ventral domains of the mouse embryo (Caubit et al., 2000; Manfroid et al., 2004).

In addition, Exd and Hth repress optix in the maxillary, labial, thoracic and first seven abdominal segments (Fig. 6A-D). The highly conserved nature of these Hox co-factors and our observations suggest that the Hox/Exd/Hth interaction is an ancient one. However, there is one exception to this rule. Ectopic expression of optix is not detected in the eighth or ninth abdominal...
segments, where the AbdB, P class, Hox protein acts, in either exd (Fig. 6A) or hth mutant embryos (Fig. 6B). Exd binds to Hox proteins via the hexapeptide motif and specific C-terminal regions (Chang et al., 1995; Knoepfler and Kamps, 1995). Unique among Hox proteins, AbdB does not possess the classical hexapeptide motif and has not been reported to bind Exd in vitro (Mann and Affolter, 1998). Our results suggest that AbdB can repress optix (Fig. 4F, Fig. 6A,B) in the absence of these co-factors; its ability to act independently of these co-factors has already been shown for the formation of the filzkörpers (Peifer and Wieschaus, 1990).

In the thorax of exd and hth larvae, the patterns have been interpreted as naked in the case of exd (Peifer and Wieschaus, 1990) or a posteriorly directed transformation for hth (Rieckhof et al., 1997). As there is strong ectopic expression of optix in the thorax (E,F insets), crinkled head-like cuticle differentiates. A and B are lateral views, C-F are ventral views.

Fig. 6. Expression of optix in mutations for exd and hth.
Expression of optix (green) in exd (A,C) and hth (B,D) mutant embryos, and cuticle preparations (E,F). (A,B) Stage 12; (C,D) retracted germ band stage. Expression of optix is strongest in the maxillary (Mx), labial (Lb) and thoracic segments and weaker in A1-7. There is no significant expression of optix in the A8, where AbdB is detected (A,B), nor in A9 segments. There is a stronger segmentation defect in exd compared with hth embryos. In the thorax of exd and hth (E,F insets), crinkled head-like cuticle differentiates. A and B are lateral views, C-F are ventral views.

Fig. 7. The role of Engrailed and Wingless signalling in the repression of optix.
Expression of wg (red) and optix (green) in HoxCP (A) and tsh−HoxCP (B) embryos at the retracted germ band stage. optix and wg patterns of expression are complementary in each trunk segment and are colocalised in parts of the labrum and gnathal domains (yellow). (C,D) Expression of optix in wg− tsh− HoxC (C) and tsh− en− HoxC (D) embryos; optix expression covers a larger region of the ventral side of each trunk segment compared with B. (A,B) ventral views; (C) lateral view. (E) Summary of the repression activities of HoxCP, Tsh and Wg in a typical trunk segment.
(Fig. 6A-F) we propose another possibility that the thorax is replaced with structures normally found in a more anterior position (Fig. 6E,F): i.e. in the head.

Tsh and HoxCP proteins do not repress the expression of optix in ventral posterior regions of the trunk segments (Fig. 2E), but do so when Wg or En are also abrogated. As loss of en, wg or tsh or in double combinations does not lead to ectopic optix expression in the trunk, our results favour the idea that Wg signalling and En share a common function with tsh and HoxCP genes to repress head in the trunk. We note that Tsh has been implicated in the late function of Wg signalling for the patterning of the trunk segments (Gallet et al., 1998; Gallet et al., 1999) and that Exd is required for the maintenance of wg, en and tsh transcription (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994), which could explain the extent of optix ectopic patches in this context (Fig. 6A,D).

This is the first evidence suggesting that Wg signalling is capable of repressing head in any invertebrate species, though this is a well documented function for Wnt signalling in vertebrates (Niehrs, 1999; Popperl et al., 1997). The role of wg for the suppression of head in the fly trunk is masked by a redundant, activity shared by HoxCP and Tsh factors. In the brain of mice lacking Six3 (optix orthologue), Wnt1 expression is extended anteriorly, leading to posteriorisation of the brain. Thus, forebrain regionalisation requires the repression of Wnt1 by Six3 (Lagutin et al., 2003).

Role of Optix during embryonic head development

The phenotypic analysis of optix during embryogenesis reveals that it acts in the labrum (Fig. 3): its initial blastodermic domain of expression (Fig. 2A). Additionally, optix is involved in the normal development of the mouth hooks (Fig. 3D) and is detected in a pair of cells from stage 11 in the maxillary segment from which the hooks derive (Fig. 2B). Previously, Optix has been shown to induce ectopic eye development (Seimiya and Gehring, 2000) in the adult, following ectopic optix production. In all organisms tested, the expression of Six3 is limited to the forebrain and the optic vesicles. In vertebrates, gain of function induces an enlarged brain and eye or ectopic retina or lens development (Bovolenta et al., 1998; Liu et al., 2006; Loosli et al., 1999; Zuber et al., 1999), whereas loss of function leads to the failure of forebrain development or eye formation (Carl et al., 2002; Lagutin et al., 2003). Six3 family members, including Optix, therefore have activities restricted to parts of the head.

Evolutionary considerations

Bilaterians (especially the chordates) possess both Hox and paralogous Para Hox complexes (Garcia-Fernandez, 2005). Analysis of these clusters indicates that they arose by gene duplication and diversification. A recent study has compared the Hox and paraHox complexes from both triploblast and diploblast (cnidarians) species and suggests that the original ‘protoHox’ complex was made up of only anterior class Hox genes (Chourrout et al., 2006). This idea is consistent with the observation that, during embryogenesis of vertebrate and some invertebrate (Scholtz et al., 1994) species, the head is the first to develop morphologically, with the central parts added on during later steps of development. Furthermore, one school of thought favours the idea, from the observation of gene expression patterns in chordian species, that ancient organisms possessed a large head with no trunk and reduced tail parts (Meinhardt, 2002). Our results favour the idea that head is evolutionarily more primitive than trunk, as the C and P Hox genes repress head and the head specific gene optix in the trunk. Early ectopic expression of the sole A class Hox that is active in the embryo, Lab, can activate optix (Fig. 5G). This effect may represent a vestige of the most ancient Hox function: the modification of head identity (see Hirth et al., 2001).

In conclusion, we show that HoxCP genes share a common role to suppress head development in the trunk in addition to their well documented, novel roles for segment identity. This common function has been retained by all central and posterior Hox proteins, as well as by their co-actors Exd, Hth, Tsh, Wg and En. Clearly, our results favour the idea that complex organisms have acquired multiple factors to repress head, as well as the acquisition of novel functions to diversify the trunk. As these factors are conserved in vertebrates, we expect these roles, at least in part, to be conserved.

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Supplementary material

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References


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