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Function and specificity of *Hox* genes

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ABSTRACT The *Hox* genes specify different structures along the anteroposterior axis of bilaterians. They code for transcription factors including a conserved domain, the homeodomain, that binds DNA. The specificity of Hox function is determined by each gene controlling the expression of different groups of downstream genes. These can be other transcription factors, elements in signaling pathways or realizator genes that carry out basic cellular functions. In regulating specific targets, the Hox genes interact with members of signaling pathways and with other proteins, thus forming part of gene networks that contribute to the modification of homologous structures or to the creation of new organs.

KEY WORDS: *Hox, downstream gene, signaling, networks*

The wide variety of forms present in the animal kingdom is due to the restricted activity of different genes in particular groups of cells. The Hox genes stand out as pivotal elements in explaining this diversity. These genes are differentially expressed and required along the anteroposterior (A/P) axis of bilaterians, and mutations in Hox genes can change one structure into another one (homeotic mutations). The spectacular transformations associated with some of these mutations have fostered ideas about the role of Hox genes in development since the initial studies of homeosis in *Drosophila*. It was the discovery of Hox genes homologous to those of *Drosophila* in vertebrates, as well as the similar properties of Hox complexes in different species (Lewis, 1978; Graham *et al.,* 1989; Duboule and Dollé, 1989), what led to the proposal of Hox genes as essential players in the development and evolution of bilaterians.

Hox genes are frequently clustered, although different types of Hox clusters, or even lack of them, are found in the animal kingdom (Duboule, 2007) (Fig. 1). These genes encode proteins that act as transcription factors and include a DNA-binding domain, the homeodomain, that is conserved in different species. By controlling the expression of realizator genes, they provide unique identities to cells at different positions of the animal. That is, they select a certain pathway of development, and for that reason they are also called "selector" genes (García-Bellido, 1975). This name, however, also includes genes that may specify, say, a wing or an eye (Mann and Morata, 2000), but that are not Hox genes since they not share with them properties such as the restricted domain along the A/P axis, homeodomain class or clustering.

In this review we will deal with some aspects related to Hox gene function in development. We will describe first examples where one Hox protein can replace another one in development and refer to ideas about how Hox proteins can provide specificity in regulating different downstream genes. The description of how Hox genes integrate in networks to form different organs will be the last part of the review**.** We will discuss examples mainly from *Drosophila melanogaster* since this is the organism where Hox function in development has been more thoroughly studied.

Can a Hox protein substitute for another one?

Each Hox protein is thought to provide specific information, within a particular developmental context, to elicit a unique morphology along the A/P axis. Many examples in which a Hox gene replaces another one corroborate this assumption. In *C. elegans*, for instance, the lin-39 Hox gene, which makes the vulva, cannot substitute for the mab-5 Hox gene, which is expressed more posteriorly and makes different structures; reciprocally, mab-5 cannot substitute for lin-39 in vulva formation, either (Maloof and Kenyon, 1998; Gutierrez *et al.,* 2003). The egl-5 Hox gene also shows poor rescue of rays, copulatory sensillae determined by mab-5, in a mab-5 mutant background (Gutierrez *et al.,* 2003).

In other cases, however, one homeotic protein can substitute, to a variable extent, for another one. In the mouse, the HoxA3 and HoxD3 proteins can replace one another with no apparent mutant

Abbreviations used in this paper: abd-A, abdominal-A; Abd-B, abdominal-B; A/P, anteroposterior; Dfd, deformed; dpp, decapentaplegic; EGFR, epidermal growth factor receptor; exd, extradenticle; fkh, forkhead; hth, homothorax; HRE, homeotic response element; hkb, huckebein; lab, labial; Scr, sex combs reduced; Ubx, ultrabithorax; wg, wingless.

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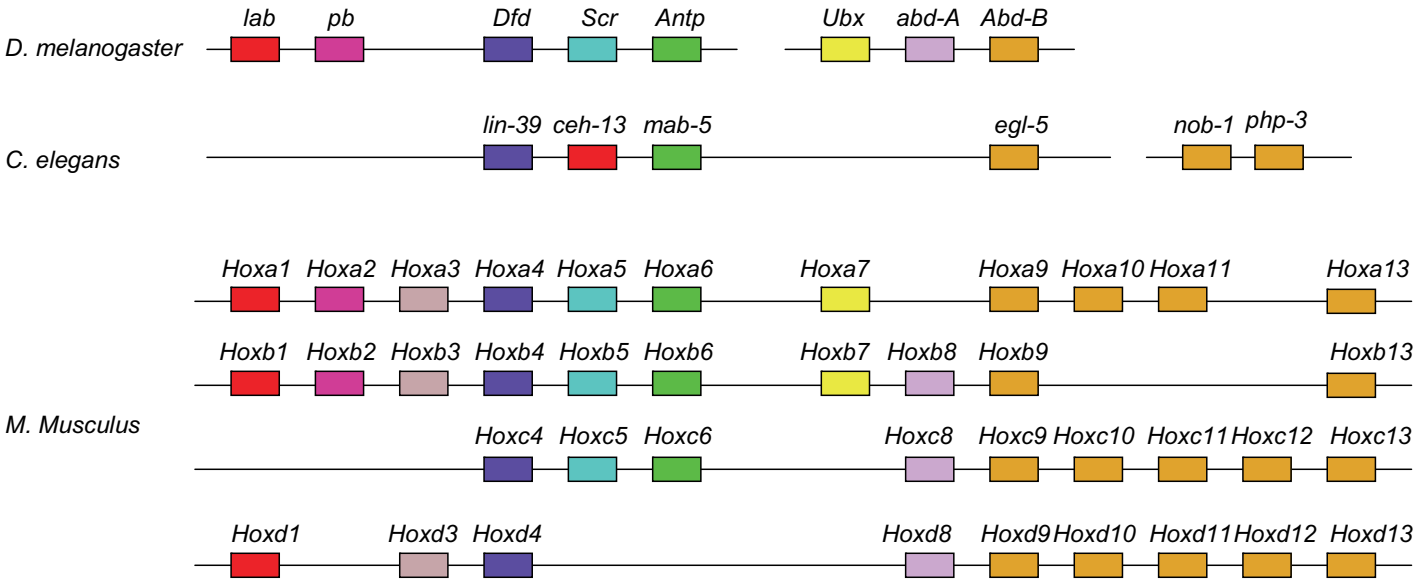

Fig. 1. The *Hox* gene complexes of *Drosophila melanogaster, Caenorhabditis elegans* and *Mus musculus*. *The scheme shows homologous genes with the same colour. Four complexes exist in most vertebrates, with a variable loss of* Hox *genes. There is an expansion of* Abd-B-like *genes in* C. elegans *and vertebrates. In* Drosophila, *there is a single* Abd-B *gene, but it codes for two different proteins with different expression patterns.*

phenotype (Greer *et al.,* 2000), but these are paralogous genes that present similar axial expression domains. Examples that may indicate partial redundancy between Hox genes with different expression domains have been described in mouse limb development, where digit formation seems to depend more on the amount of Hox proteins than in their nature (Zákány *et al.,* 1997). In *C. elegans*, mutations in the mab-5 or lin-39 Hox genes have mild effects in the development of the mesodermal M lineage and the double mutant has much stronger effects; moreover, the expression of either the MAB-5 or LIN-39 Hox proteins in the double mutant background can partially rescue the mutant phenotype (Liu and Fire, 2000). In *Drosophila*, the Ultrabithorax (Ubx) and Abdominal-A (Abd-A) proteins can similarly develop gonadal mesoderm (Greig and Akam, 1995) or halteres (Casares *et al.,* 1996; de Navas *et al.,* 2006a) and all Hox proteins except Abdominal-B (Abd-B) can substitute for Labial (Lab) in the specification of the tritocerebral neuromere (Hirth *et al.,* 2001). The need to mutate both the Ubx and abd-A genes to obtain certain mutant phenotypes in the embryonic *Drosophila* abdomen, for example the formation of ectopic Keilin´s organs (Lewis, 1978), and the fact that different Hox proteins can repress the same targets in the *Drosophila* embryo (reviewed in Graba *et al.,* 1997), also suggest there is partial redundancy between different Hox products. These results indicate there are some functions particular to a Hox protein and others that are common to several ones.

The need for cofactors

Most Hox proteins bind *in vitro* to simple sequences with similar affinity (Hoey and Levine, 1988; Ekker *et al.,* 1994). The specificity of Hox protein activity *in vivo* is most likely due to the activity of cofactors. The best characterized cofactor is Extradenticle (Exd) in *Drosophila* and its related proteins PBX in vertebrates and CEH-20 and CEH-40 in *C. elegans* (Mann and Chan, 1996; Van Auken *et al.,* 2002; Moens and Selleri, 2006). Exd, a protein that also contains a homeodomain, was characterized as a possible cofactor of Hox products because exd mutations produced homeotic phenotypes without affecting Hox expression (Peifer and Wieschaus, 1993). Two models have been proposed to account for the activity of cofactors (particularly Exd) *in vivo:* 1) the selective binding model and 2) the activity regulation model.

1) The selective binding model. This model argues that different Exd-Hox combinations discriminate, among similar sequences (about 10 bp of length), which of these are bound by each heterodimer. Different Exd/Hox heterodimers show different binding specificities *in vitro* (Chang *et al.,* 1996; reviewed in Mann and Chan, 1996) and the presence of Exd increases the selectivity of a Hox protein in binding to composite sites (reviewed in Mann and Chan, 1996, Mann and Affolter, 1998; Akin and Nazarali, 2005). Experiments that underpin this model have been carried out in some enhancer elements in mouse and *Drosophila*. An autoregulatory enhancer of the Hoxb1 gene in mouse includes a 20 bp sequence (repeat 3) that is activated specifically by the HoxB1/PBC heterodimer *in vivo* (Pöpperl *et al.,* 1995). A similar sequence is present in lab550, an autoregulatory enhancer of labial (lab), the *Drosophila* homologue of Hoxb1, and also reproduces lab expression in the *Drosophila* embryo. If two base pairs from the central region of a composite Hox/Exd binding site included within this sequence are changed, so that the sequence is now bound preferentially *in vitro* by the Deformed/Exd dimer, there is a change of specificity from Lab to Deformed (Dfd) *in vivo:* a reporter gene regulated by this altered sequence is expressed in a Dfd pattern instead of a lab one, and responds to Dfd and not to lab in *Drosophila* embryos (Chan *et al.,* 1997) (Fig. 2).

Some experiments, however, argue against the selective binding model. Although, as we have seen, a small change in a sequence present in the lab550 enhancer of the lab gene of *Drosophila* changes specificity of Lab to Dfd *in vivo*, a similar

alteration made in the context of the entire lab550 enhancer does not produce a similar change (Marty *et al.,* 2001). There are also homeotic response elements (HREs) that do not seem to contain Exd/Hox composite consensus binding sites (Pederson *et al.,* 2000), and Hox targets that do not require the presence of Exd. In the regions of the thoracic imaginal discs of *Drosophila* which give rise to the distal part of appendages such as legs or halteres, the Exd protein is inactive (González-Crespo and Morata, 1995; Rauskolb *et al.,* 1995). Therefore, a certain Hox protein should not be specifically required to make these organs if specificity is conferred just by Exd activity. In fact, several Hox proteins can transform wings into halteres in a similar way (Casares *et al.,* 1996), suggesting some lack of Hox specificity in making a haltere. However, distal legs, even though they not need Exd, are differently determined by Hox genes, the first leg by Sex combs reduced (Scr) and the third one by Ubx (Struhl, 1982; Pattatucci *et al.,* 1991). This example, nevertheless, do not rule out that cofactors different from Exd may provide specificity to Hox proteins (see below).

If each Hox/Exd composite binding site could determine specificity of binding by Hox proteins, downstream genes of a particular Hox gene could be pinpointed by interrogating the genome for binding sites of a particular Hox/Exd heterodimer. These sequences will be located in the regulatory regions of each Hox target gene. This approach was recently carried out for the downstream genes of the *Drosophila* lab gene and the authors could identify one putative target. However, although the gene seems to be a bona fide target of lab, the HRE is not that predicted by the consensus sequence but another one, in a different location within the gene, and that does not match the consensus Lab/Exd binding sequence (Ebner *et al.,* 2005).

2) The activity regulation model. This model proposes that Hox proteins have promiscuous binding, and that differences in binding to a composite Hox/Exd binding site are not crucial in conferring specificity. Different Hox-Exd heterodimers could bind to the same composite site but specificity would be provided by the different activity of the dimer (by recruiting co-activators or co-repressors). In some conditions, the role of Exd could be to change the activity of the heterodimers from being repressors to being activators (Biggin and McGinnis, 1997; Pinsonneault *et al.,* 1997); in this way, Exd could make a Hox protein active without increasing its DNA binding affinity. The model is based on experiments that show extensive, non-selective, binding of homeoproteins on target genes in the *Drosophila* embryo (reviewed in Biggin and McGinnis, 1997; Mann and Morata, 2000) and in the change of activity of Dfd when bound to a certain sequence in the absence or presence of Exd (Pinsonneault *et al.,* 1997). Support for the model also stems from the observation that Dfd does not activate a target on simple Dfd binding sites but does it when the protein is fused with a strong activation domain (Dfd-VP16), suggesting that Dfd can bind, but not activate, alone: Exd will be the protein required to make the Dfd protein active (Li *et al.,* 1999a). Also supporting the model, when a 21-bp sequence required for activity of a Dfd autoregulatory element, but that does not include any Hox or Hox/Exd binding site, is juxtaposed to a Lab/Exd composite site (which directs Lab expression), the reporter linked to the compound construct is expressed in a Dfd-dependent (and not Lab-dependent) pattern of expression in *Drosophila* embryos (Li *et al.,* 1999b) (Fig. 2).

This second model does not explain some results obtained with different HREs (see Mann and Morata, 2000), apart from those supporting the first model. It is likely, therefore, that the specificity of Hox proteins is provided by different mechanisms in different HREs. This argues for a relatively high flexibility in the response to Hox activity in downstream genes, probably due to the fact that sequences adjacent to Hox-binding sites can help to provide selective activity. There are, nevertheless, some HREs that are clearly regulated by one Hox protein with preference to others: a sequence from the forkhead gene of *Drosophila*, required to form salivary glands, is activated specifically by the Scr Hox protein, and only the Scr–Exd dimer binds this sequence with high affinity (Ryoo and Mann, 1999). Recent results (Joshi *et al.,* 2007) have provided evidence to explain the specific recognition of this sequence by the Scr-Exd dimer. The specificity lies in the presence of two key aminoacids (Arg and His), in the linker region and in the N-terminal arm of the Scr homeodomain, which recognize a particular DNA structure in its minor groove. These results provide a basis to explain specific recognition by Hox proteins that have similar homeodomains but differ in the N-terminal arms.

Plenty of cofactors?

The Exd protein belongs to a particular class of homeodomain proteins, the TALE group, characterized by the presence of three aminoacids between helix 1 and 2 of the homeodomain. Another protein belonging to this class is Homothorax (Hth) in flies and their related Meis and Prep proteins in vertebrates (Bürglin, 1997; Berthelsen *et al.,* 1998). These proteins regulate Hox activity in

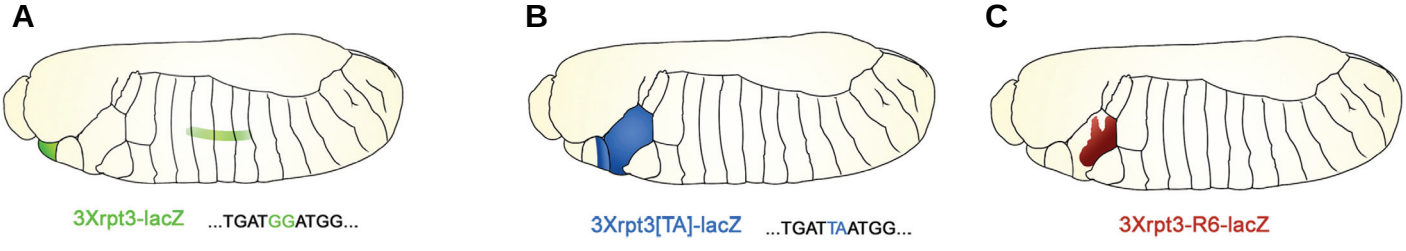

Fig. 2. Two models to explain Hox specific activity. (A) *The repeat3 sequence (3xrpt3), recognized by a Lab/Exd heterodimer, can direct the expression of a reporter gene in the* Drosophila *embryo at the positions where lab is expressed. The sequence bound by the heterodimer is also shown.* **(B)** *If two base pairs from the central region of this sequence are changed (from GG to TA), the expression of the reporter corresponds now to that of the* Dfd *gene, and the sequence is bound* in vitro *preferentially by a Dfd/Exd heterodimer (Chan* et al., *1997).* **(C)** *If the repeat3 sequence is juxtaposed to a sequence from a Dfd autoregulatory enhancer not bound by Dfd (R6), the expression of the reporter gene is similar to that of the* Dfd *gene, and not to that of* lab *(Li* et al., *1999b).*

several ways: first, they are required for the nuclear localization of Exd/PBC proteins (Rieckhof *et al.,* 1997; Kurant *et al.,* 1998; Berthelsen *et al.,* 1999); second, Meis proteins can interact with Hox proteins (Shen *et al.,* 1997; Williams *et al.,* 2005a), and third, they can form part of tripartite Hox - Exd/Pbc - Hth/Meis/Prep complexes that bind to specific DNA sequences (reviewed in Mann and Affolter, 1998; Mann and Morata, 2000; Akin and Nazarali, 2005; Moens and Selleri, 2006). In this way, these protein complexes can increase Hox specificity.

Other cofactors of Hox proteins have been described: the disconnected (disco) and disco-related (disco-r) genes, which code for zinc finger proteins, are required for the activity, but not the expression, of Dfd and Scr proteins in the *Drosophila* embryo (reviewed in Mahaffey, 2005). tea-shirt (tsh), a gene encoding a zinc finger protein as well, is also needed for Hox proteins to confer a specific development to the thorax and abdomen of the *Drosophila* embryo (Röder *et al.,* 1992). Proteins like Tsh and Disco/Disco-r are unequally distributed along the A/P axis of the *Drosophila* embryo and it has been suggested that they act as cofactors of Hox proteins, establishing "fields" in the embryo and serving as ancillary products to give specificity to Hox proteins (Mahaffey, 2005). Also in the *Drosophila* embryo, the proteins encoded by the sloppy paired and engrailed genes, required for embryonic segmentation, act as cofactors of Ubx and Abd-A to repress the expression of Distal-less, an essential gene to make appendages (Gebelein *et al.,* 2002), thus providing a link between the segmentation process and Hox activity. As more and more Hox target sequences are functionally dissected, more cofactors of Hox proteins are likely to be characterized; these cofactors will be different in distinct developmental stages and may link Hox proteins with processes like segmentation and tissue and organ specification.

The structure of enhancers with Hox binding sites

When a Hox gene is uniformly expressed in the *Drosophila* embryo it activates ectopically a HRE in just a few cells (see for example Chan *et al.,* 1997). Moreover, the activity of an isolated HRE is different when integrated in a whole enhancer. This suggests that some factors may limit Hox activity on this responding element. Binding sites for Hox proteins are frequently juxtaposed to binding sites for other transcription factors, like effectors of signaling pathways, or proteins that determine tissue-specific expression (Manak *et al.,* 1994; McCormick *et al.,* 1995; Guss *et al.,* 2001; Marty *et al.,* 2001; Zhou *et al.,* 2001; Di Rocco *et al.,* 2001). For example, Lab and Decapentaplegic (Dpp) signalling cooperate to establish the proper expression of the lab550 enhancer in the endoderm of *Drosophila* (Grieder *et al.,* 1997) and abdA and Dpp signalling cooperatively regulate a wingless (wg) enhancer in the visceral mesoderm of the fly (Grienenberger *et al.,* 2003). Similarly, the binding of the SOX/OCT heterodimer close to the sequences of the autoregulatory domain bound by HoxB1 is required for the full transcriptional activity dictated by this sequence (Di Rocco *et al.,* 2001). These examples show that the enhancer where the HRE is located integrate different signals and can modify the output of Hox activity bound to its HRE.

Searching for downstream genes

A crucial issue in unravelling Hox function and specificity is to identify the target genes of Hox proteins. Different methods have been used to characterize Hox downstream genes, such as the study of genes based in their restricted expression pattern, immunopurification, immunoprecipitation of DNA fragments bound by Hox proteins, binding to polytene chromosomes, differential screening and subtractive hybridization of cDNAs, or transcriptional activation of a reporter gene in yeast. The genes under Hox control include transcription factors and genes belonging to signaling pathways but also, as was originally predicted (García-Bellido, 1975), genes directly regulating cellular functions such as cell proliferation, cell polarity or cell growth (reviewed in Graba *et al.,* 1997; Hombría and Lovegrove, 2003; Akin and Nazarali, 2005; Pearson *et al.,* 2005). Recently, more thorough analyses to identify downstream genes have been carried out by the use of microarrays in *Drosophila*, mouse and cell culture (Leemans *et al.,* 2001; Zhao and Potter, 2001; Valerius *et al.,* 2002; Hedlund *et al.,* 2004; Barmina *et al.,* 2005; Cobb and Duboule, 2005; Klebes *et al.,* 2005; Lei *et al.,* 2005; Williams *et al.,* 2005b; Mohit *et al.,* 2006; Hersh *et al.,* 2007; Hueber *et al.,* 2007; Rohrschneider *et al.,* 2007). The general outcome of these studies is similar to those obtained previously, although a higher number of genes implementing cellular functions were obtained. As a summary, Hox downstream genes include different classes of genes, many of which directly carrying out basic cellular functions.

One microarray study made in the *Drosophila* embryo (Hueber *et al.,* 2007) catalogued targets of most Hox genes. This analysis led to several significant conclusions: one is that genes with a related function (say, cell death) are similarly repressed or activated by a certain Hox gene, implicating a coordinated function (see also Leemans *et al.,* 2001); another one is that different groups of genes are activated or repressed by different Hox genes, suggesting specificity of Hox genes for particular targets; the authors of this work claim that 63-69% of the targets are unique for a particular Hox protein. This seems to be at odds with the genetic analysis that predicts a high number of targets common to, for example, Ubx and abd-A in the *Drosophila* embryo, since these genes are required to make similar structures (although the existence of particular cell types in the Ubx and abd-A domain has to be taken into account). A third conclusion is that Hox genes do not preferentially activate regulatory genes; rather, realizator genes are more represented among Hox targets. However, detailed analysis of how two different Hox genes specify salivary glands or posterior spiracles in *Drosophila* suggests that Hox genes control morphogenesis mainly by regulating other transcription factors (see below).

Hox downstream genes are different according to the tissue and stage of development analyzed. The first and second leg of *Drosophila*, coming from the first and second leg discs, respectively, are different owing to the expression of Scr in the former but not in the latter (Struhl, 1982; Pattatucci *et al.,* 1991). As pointed out by Barmina *et al.* (2005), one study identified 17 genes differently expressed in the two discs (Klebes *et al.,* 2005) and another one 14 genes (Barmina *et al.,* 2005), but the two gene sets do not overlap; the reason may be that the two microarray studies were done at different times of development (third instar larvae in the former and prepupal stages in the latter) apart from the fact that different methods and analyses were used.

To characterize the regulatory networks controlled by Hox genes, it is relevant to know how many Hox downstream genes

are direct targets. Hueber *et al.,* 2007 calculate that this class comprises 20%-30% of the genes they identify as regulated by Deformed in the embryo. Some studies have made use of the polytene chromosomes of *Drosophila* to address this issue: by using antibodies against Hox products, the sites bound by the antibody (putative direct targets) could be mapped on the chromosomes. The results obtained, as expected, were different depending on the method used and tissue analyzed: in the polytene chromosomes of the salivary glands, after forced expression of Ubx (this gene is not normally expressed in this tissue), 103 loci bound by Ubx were found (Botas and Auwers, 1996); by contrast, the same experiment carried out in the fat body (a Ubx naturally-expressing organ) discovered 188 loci bound by this same protein (Marchetti *et al.,* 2003), only 53 of which were in common with the previous analysis. Another approach to determine the direct targets of a Hox gene is to dissect the genetic networks controlled by a Hox gene to make a particular structure.

Building organs with *Hox* genes

The function of Hox genes has been largely inferred from the effect of Hox mutations on development. We can roughly subdivide these effects into two classes (Hombría and Lovegrove, 2003): a) Some mutations change one structure into another one. These are the remarkable homeotic transformations observed in *Drosophila* or mouse, such as the change of halteres into wings (Lewis, 1963) or of lumbar vertebrae into thoracic ones (Wellik and Capecchi, 2003). These changes occur among structures that are homologous and rely on the modification of a basic set of patterning clues established by signaling pathways, like the Hedgehog, Wingless/Wnt, Decapentaplegic/BMP or Notch pathways (Weatherbee *et al.,* 1998). The different morphology of the homologous structures suggest that Hox genes specify them by regulating cellular processes like cell proliferation, cell death, cell differentiation, etc, without altering the underlying positional information. This common information is revealed, for instance, by the existence of common targets of Hox genes in the primordia of structures with similar ontogenetic rules, like limbs and external genitalia (Cobb and Duboule, 2005). b) Another effect of Hox mutations is to eliminate or modify an organ or structure without transforming it into a similar one. For instance, the absence of Hoxa/Hoxd genes prevent the formation of distal limbs in mice (Kmita *et al.,* 2005) and that of Abd-B eliminates posterior spiracles in the *Drosophila* embryo. This second effect implies that the normal function of the Hox gene is, in this case, morphogenetic (Hombría and Lovegrove, 2003), and that Hox genes do not simply change the outcome of the activity of signaling pathways and transcription factors that set up a common information in homologous structures. The same mutation may produce these two kinds of effects depending on the tissue, organ and time analyzed.

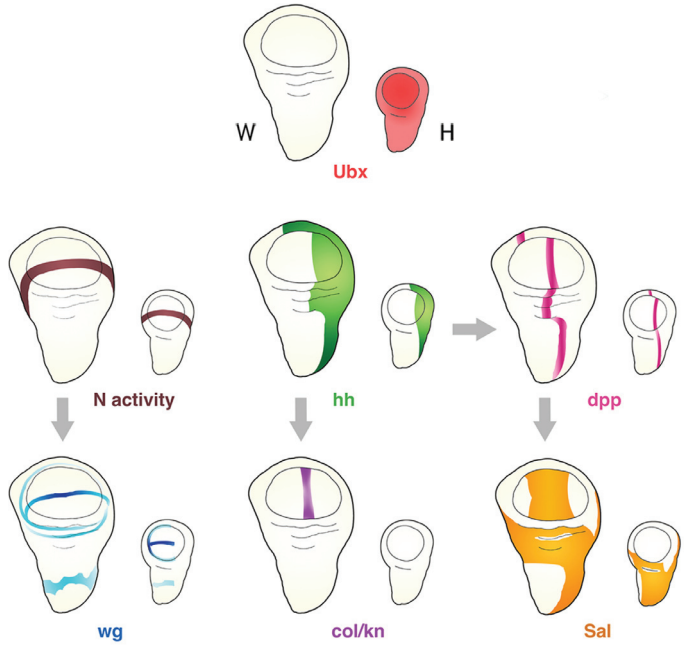

Fig. 3. Ubx modulates the activity of several signaling pathways in the haltere disc. *Ubx is expressed in haltere (H) but not in the wing (W) disc. In both discs the Notch (N), Hedhehog (Hh) and Decapentaplegic (Dpp) signaling pathways are active at similar positions. However, the expression of wingless (wg), a target of the N pathway, collier/knot (col/kn), a target of the Hh pathway, and spalt (sal), a target of the Dpp pathway, is suppressed in the pouch of the haltere disc (only in the posterior compartment for wg) due to Ubx activity. Dpp expression responds to Hh signaling and is also weaker in the haltere than in the wing disc (after Weatherbee et al., 1998, Hersh and Carroll, 2005, Crickmore and Mann, 2006, de Navas* et al., *2006; Makhijani* et al., *2007).*

The interaction with signaling pathways

Whether they modify an underlying positional information or develop new structures, the Hox genes interact with signalling pathways in different ways:

a) The Hox genes can alter the expression of ligands, receptors, intermediate elements or effectors of signaling pathways. For instance, in *Drosophila,* Ubx modifies the activity of the Notch, Hedgehog, Dpp, Wingless (Wg) and Epidermal growth factor receptor (EGFR) signaling pathways in the haltere disc (with respect to the wing one) to make a haltere instead of a wing (Weatherbee *et al.,* 1998; Galant *et al.,* 2002; Mohit *et al.,* 2003; Hersh and Carroll, 2005; Crickmore and Mann, 2006; de Navas *et al.,* 2006b; Pallavi *et al.,* 2006; Makhijani *et al.,* 2007) (Fig. 3). Similarly, also in *Drosophila*, proboscipedia determines pattern differences between the proboscis and the leg by modifying the Hedgehog pathway (Joulia *et al.,* 2005), whereas Abd-B determines pattern differences between the genitalia and the leg by changing Dpp and Wg signaling (Estrada and Sánchez-Herrero, 2001). Another example is the formation of denticles in the embryonic ventral cuticle of the *Drosophila* embryo. The abdominal segments bear more rows of denticles, which are also of bigger size, than the thoracic ones. This is due to Ubx and abd-A activating in the abdomen a ligand of the Notch pathway, Serrate, which, in turn, triggers EGFR signalling to form denticles. In the thorax, the Hox genes do not activate Ser expression, there is no EGFR signaling and the denticle bands are thinner and with more slender denticles (Willete and McGinnis, 1999). Similarly, in the mouse, HOXB13 inhibits TCF-4-mediated Wnt signaling in prostate cells by decreasing both Tcf-4 expression and expression of its targets

(Jung *et al.,* 2004).

b) Hox gene activity is also governed by signaling pathways, as in the regulation of the lin-39 Hox gene of *C. elegans* by the Ras signaling pathway to instruct vulval development (Maloof and Kenyon, 1998), or in the control of egl-5 by the EGFR pathway in the formation of the P12 neuroectoblast (Jiang and Sternberg, 1998).

c) There are cases of cooperation between signaling pathways and Hox genes in inducing the expression of target genes. This interplay may be a protein-protein interaction, like that of HoxD proteins and Gli-3, a member of the Sonic hedgehog signaling pathway, in specifying digits in vertebrate limb (Chen *et al.,* 2004), or may be effected, as we have described, through the integration of Hox genes and signalling pathways in the promoters of downstream genes. There are even more complicated relationships, as Hox genes can be both upstream and downstream of the Sonic Hedgehog pathway in the formation of digits in the mouse (Zákány *et al.,* 2004).

The multiple roles of *Hox* genes in organ formation

The control of cell death

Several examples have been described about the role of Hox genes in regulating apoptosis. In *Drosophila*, Hox genes regulate cell death required for the correct formation of the head or the larval central nervous system (Lohmann *et al.,* 2002; Bello *et al.,* 2003; Miguel-Aliaga and Thor, 2004). In vertebrates, Hox genes also control cell death in the spinal cord of the mouse (Economides *et al.,* 2003) and in the nematode *Pristionchus Pacificus* the Hox gene lin-39 prevents apoptosis in cells that will form the vulva (Eizinger and Sommer, 1997). The regulation of apoptosis by Hox genes can impinge on the morphology of the animal in several cases. Thus, in the *Drosophila* embryo, the Hox gene Deformed activates the pro-apoptotic gene reaper in cells located between the mandibular and maxillary lobes. This activation, and the subsequent cell death, is required to maintain the boundary between the two segments. The regulation is direct, and demonstrate that Hox genes can directly regulate processes like cell death to shape morphology (Lohmann *et al.,* 2002). In other situations the control is mediated by other genes: for example, the MAB-5 protein of *C. elegans* governs the expression of egl-1, a BH3-domain encoding gene which regulates, in combination with the co-factor CEH-20, the death of two cells in the posterior nerve cord (Liu *et al.,* 2006). As in other examples, the activity of a Hox gene is limited in space and time, suggesting there is coordination of different signals, including Hox activity, to prevent or stimulate apoptosis.

The control of cell proliferation

Hox genes have been implicated in the control of cell proliferation (reviewed in Del Bene and Wittbrodt, 2005; Pearson *et al.,* 2005). As examples, Ubx and abd-A regulate proliferation of neuroblasts in the *Drosophila* embryo (Prokop *et al.,* 1998) and lin-39 is necessary for cell divisions in the vulva of *C. elegans* (Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002). Genes with specific roles in cell cycle progression have been shown to be controlled by Hox genes; an example is the regulation by HOXA10 of p21, a cyclin-dependent kinase (Bromleigh and Freeman, 2000). In some cases this control is direct: for instance, Abd-B regulates directly dacapo, a gene coding for an inhibitor of CyclinE/Cdk2 complexes (Meyer *et al.,* 2002). In other cases, the effect of Hox genes is not mediated by the control of transcription: there is mutual regulation between Hox proteins and the cell cycle regulator geminin, a protein that prevents S-phase initiation (Luo *et al.,* 2004). The control of cell proliferation may explain the different size of homologous structures that differ in the expression of Hox genes. One example is found in the determination of the *Drosophila* halteres. Wings and halteres are homologous

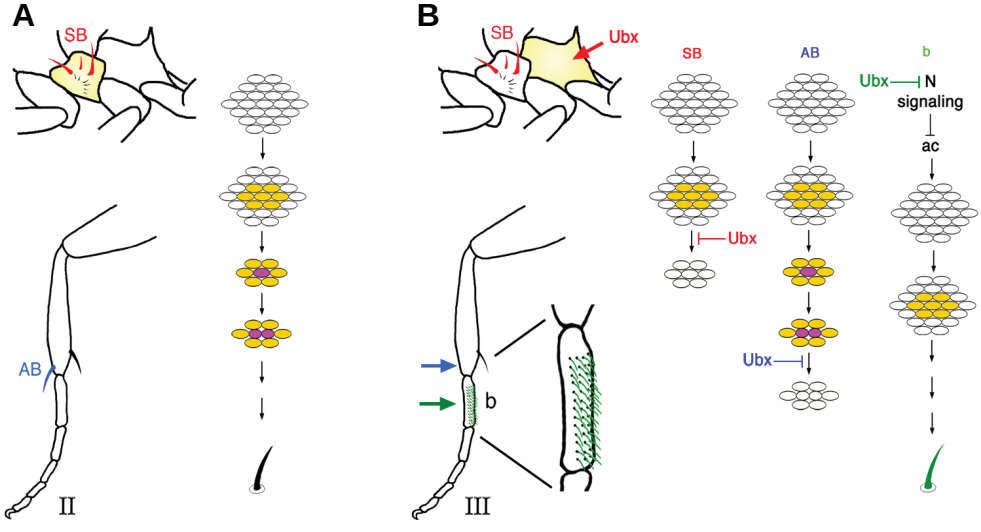

Fig. 4. Ubx regulates bristle development at different times of development. *Ubx determines the different development of bristles in the pleura of the second (mesopleura) and third (metapleura) thoracic segments, and in the second and third legs of* Drosophila, *by acting at different times of development.* **(A)** *Scheme showing the pleura (above) and the distal part of the second leg (II). The mesopleura is in pale yellow and bears bristles (in red). The apical bristle (AB), in the tibia, is in blue. To the right of this drawing, a simplified view of how the sensory mother cell (in pink), that will divide and form the bristle, is singled out from a group of cells expressing scute (in orange).* **(B)** *Scheme of the pleura (above) and third leg (III) showing that Ubx represses the formation of specific bristles by acting at different developmental times and at different steps of bristle formation. The development of the sternopleural bristles (SB; in red) is repressed in the metapleura (pale yellow) by Ubx (red arrow) acting at an early step of bristle formation (first drawing to the right). The formation of the apical bristle is repressed by Ubx (blue arrow) at a late stage in bristle formation (middle scheme of bristle formation). In both cases Ubx is active in larval stages. A group of bristles in the basitarsus (b) (in green) is activated by Ubx in the third leg (green arrow) by repressing Delta expression and the Notch pathway; this pathway, in turn, down-regulates achaete, a gene required to form bristles (scheme to the right) (after Rozowski and Akam, 2002 and Shroff* et al., *2007).*

dorsal thoracic appendages, the wings being much bigger than the halteres. The difference between them is due to the activity of the Ubx gene, present in the haltere but not in the wing disc. Much of the size difference is due to Ubx controlling cell growth during pupation, wing cells being much bigger than haltere cells (Roch and Akam, 2000). However, Ubx also reduces the size of the larval haltere disc, as compared to the wing one (while cell size in the two discs is similar), by down-regulating the transcription and spread of the Dpp morphogen. The reduction of both parameters results in a narrow domain of different values of Dpp activity. This, in turn, will likely reduce the number of divisions in the haltere disc with respect to the wing one leading, finally, to a smaller appendage (Crickmore and Mann, 2006; de Navas *et al.,* 2006b; Makhijani *et al.,* 2007).

The control of pattern and cell specification

In *Drosophila*, cell morphology or fate may depend on the activity of a Hox gene. Ubx regulates cell growth, shape and differentiation in making haltere tissue instead of wing one during pupal development (Roch and Akam, 2000) and abd-A changes the shape and physiological properties of cells of the *Drosophila* cardiac tube during metamorphosis (Monier *et al.,* 2005). As in the cases of cell death or cell proliferation, the effect of Hox genes in differentiation is sometimes brought about by direct control of realizator genes. One example is the direct regulation of the gene yellow, which gives pigmentation to the cuticle, by Abd-B in the *Drosophila* male abdomen. The two posterior segments of the male abdomen are pigmented and this pigmentation depends on the sex of the fly and on Abd-B. This Hox gene is expressed in the posterior abdomen and directly activates the yellow gene, thus explaining the darker colour of these metameres when compared to more anterior abdominal segments (Jeong *et al.,* 2006).

Cells are competent to the activity of Hox genes at a precise time in a particular developmental process (Castelli-Gair *et al.,* 1994; Castelli-Gair and Akam, 1995; Monier *et al.,* 2005; Iimura and Pourquié, 2007). Thus, in the *Drosophila* leg discs, Ubx regulates the presence or absence of bristles in the third leg, as compared to the second one, at several steps of bristle formation and at different developmental times (Rozowski and Akam, 2002; Shroff *et al.,* 2007) (Fig. 4). Hox gene activity may even be the opposite at different developmental stages: Abd-B represses abd-A in the embryo but activates it in the larval genital disc (Foronda *et al.,* 2006) and abd-A represses, in the pupal cardiac tube, the gene Ih, homologous to the vertebrate hyperpolarization-activated cyclic nucleotide-gated channels gene, whereas it activates this same gene in the embryo (Monier *et al.,* 2005).

The conclusion from all these results is that the Hox genes are involved in many cellular processes throughout development. In such role they do not behave as "master" genes but, rather, as "micromanagers", modulating developmental routes (Akam, 1998). This is more evident when they modify the outcome of signaling pathways and differentiate between homologous structures; in these circumstances, Hox mutations result in homeotic transformations. In other conditions, Hox genes coordinate signaling pathways and establish regulatory networks that contribute to organ formation (Hombría and Lovegrove, 2003).

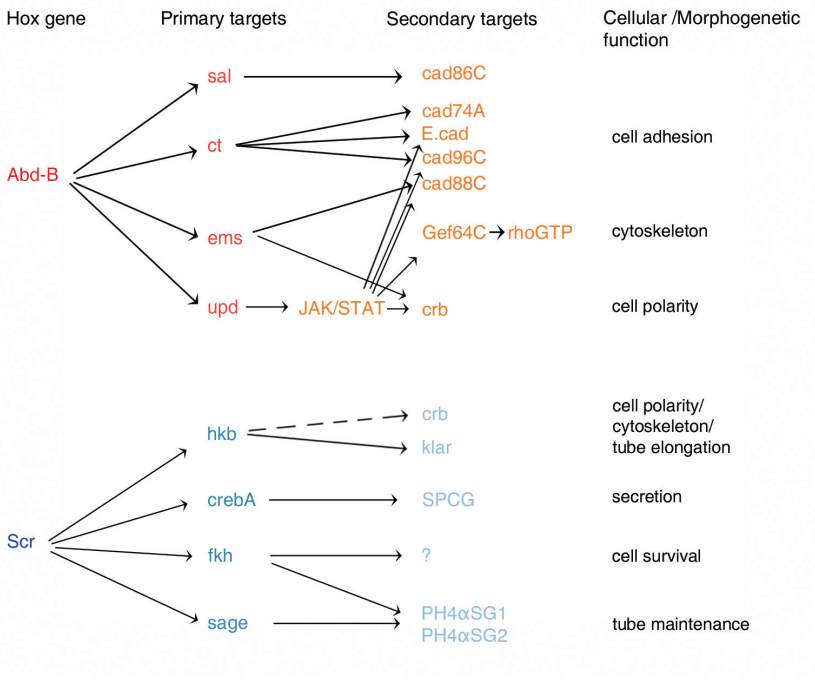

Fig. 5. Comparison of the Abd-B and Scr networks in Drosophila. *Abd-B forms posterior spiracles and Scr salivary glands, respectively. In both cases the* Hox *genes regulate a few genes, mainly transcription factors (primary targets), that control the expression of many other genes (secondary targets), implementing basic cellular functions (after Lovegrove* et al., *2006 and Kerman* et al., *2006).*

Hox networks

The way Hox genes control cell growth, proliferation, polarity or migration might be done: a) by regulating a few transcription factors which, in turn, regulate other genes in a repeated cascade of regulatory signals, or b) by controlling many genes simultaneously, which will integrate signals to form organs. We describe now the architecture of some of the best-characterized Hox-regulated networks studied in *Drosophila*.

One example of these networks is the formation of the posterior spiracles, small openings of the tracheal system at the back of the embryo (Hu and Castelli-Gair, 1999; Lovegrove *et al.,* 2006). The Hox gene Abd-B is needed for posterior spiracle formation (Sánchez-Herrero *et al.,* 1985) and regulates four "primary" downstream genes (three of them transcription factors) which, in turn, govern several cellular properties: cell adhesion is controlled through the genes cut and spalt, which regulate the expression of cadherins, the cytoskeleton is regulated through the control of a Rho GTPase by the empty spiracles transcription factor, and cell polarity is controlled through the Jak-Stat pathway regulating crumbs, a transmembrane protein needed for apical-basal cell polarity (this pathway also impinges on the regulation of the other cellular properties) (Hu and Castelli-Gair, 1999; Lovegrove *et al.,* 2006; Fig. 5) The data obtained from these studies allow to draw several conclusions: a) the primary targets link Hox gene activity with the activation of genes in charge of basic cellular functions, such as cell

polarity, cell shape, etc.; b) the co-expression of more than one primary target is sometimes required to activate secondary downstream genes; c) the primary targets are expressed in just some of the cells that express Abd-B and only at a certain time of development; the interaction with signaling pathways spatially limit the activity of the Hox gene and subdivide the morphogenetic field; d) secondary targets can also be transcription factors, which work as intermediates between primary targets and realizator genes; e) the ectopic expression of the four primary targets is enough to activate the realizator genes and make ectopic spiracle-like structures, suggesting that the formation of a simple structure by Hox genes does not need the control of a large amount of primary genes (Lovegrove *et al.,* 2006) (Fig. 5).

The salivary glands are secretory organs whose formation depends on the Hox gene Scr (Panzer *et al.,* 1992). This Hox gene (together with exd and hth) activates primary downstream targets like crebA, encoding a leucine zipper protein, sage, which codes for a bHLH protein, forkhead (fkh), encoding a winged-helix DNA binding protein and huckebein (hkb), encoding a Spi/egr-like transcription factor (reviewed in Kerman *et al.,* 2006). These genes, in turn, regulate genes that carry out basic cellular functions: fkh and sage govern the expression of PH4αSG1 and PH4αSG2, which encode α-subunits of enzymes that hydroxylate proline residues in collagen and help to maintain tube size (Abrams *et al.,* 2006); hkb regulates crumbs, required in the apical region of the membranes to polarize cell shape changes during salivary gland invagination, and klarsich, a gene coding for a protein that is proposed to regulate the motor protein dynein and to mediate the microtubule-dependent organelle transport; the regulation of these and other genes allows hkb to regulate the morphology of salivary cells during invagination (Myat and Andrew, 2002). Finally, crebA controls the expression of genes that are part of the secretory pathway (Abrams and Andrew, 2005). Therefore, the formation of salivary glands includes changes in tube elongation through hkb, tube maintenance through fkh and sage, tube formation through fkh and unknown downstream targets, and secretory capacity through the secretory pathway component genes (SPCG), regulated by the CrebA transcription factor (reviewed in Kerman *et al.,* 2006) (Fig. 5). Some of the general rules that we have described for the formation of posterior spiracles can also be applied to salivary gland determination. For instance, the expression of some downstream genes in salivary gland take place in either duct cells or secretory cells, showing an spatial subdivision within the Hox network. The primary targets are also few and, in general, code for transcription factors, which may regulate many genes. Thus, over 200 genes require fkh for their expression in salivary glands (Kerman *et al.,* 2006). These examples seem to favour a model whereby Hox genes regulate cellular functions through a short number of intermediate transcription factors, and seems to contradict the conclusion from microarray studies discussed above (Hueber *et al.,* 2007), which indicates that many realizators are direct targets. However, it is possible that both redundant direct and indirect control of realizator genes by Hox genes takes place in organ formation.

Conclusions

The Hox genes keep on attracting attention as key elements in development and evolution. Although in several cases they have a morphogenetic role, their activity is generally linked to those of signaling pathways, so that Hox products form new structures by modulating different signals. The specificity of Hox gene activity relies on the set of particular downstream genes they activate or repress, and to identify them, and how they are regulated, seems a priority if we want to understand Hox gene function. To this aim we probably need chromatin immunoprecipitation studies and better bioinformatic tools to select the genes under direct control of each Hox protein. Another fruitful approach to determine Hox function is to dissect Hox regulatory networks in simple organs and to analyze how they regulate different cellular properties. This method will allow a comparison between similar networks in related species, thus providing a path to evaluate the role of Hox genes in creating new forms in evolution.

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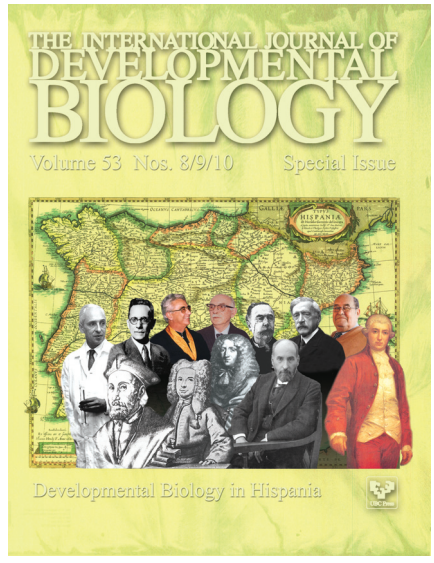

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