MODULATING HOX GENE FUNCTIONS DURING ANIMAL BODY PATTERNING

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Abstract | With their power to shape animal morphology, few genes have captured the imagination of biologists as the evolutionarily conserved members of the Hox clusters have done. Recent research has provided new insight into how Hox proteins cause morphological diversity at the organismal and evolutionary levels. Furthermore, an expanding collection of sequences that are directly regulated by Hox proteins provides information on the specificity of target-gene activation, which might allow the successful prediction of novel Hox-response genes. Finally, the recent discovery of microRNA genes within the Hox gene clusters indicates yet another level of control by Hox genes in development and evolution.

HOMEOTIC TRANSFORMATION
The transformation of one body region into the likeness of another.

BILATERIA
A phylogenetic subdivision of animals that is characterized by left–right symmetry along the primary body axis at some stage of the life cycle.

ORAL–ABORAL AXIS
The body axis from the mouth to the body surface opposite the mouth, commonly used in animals that have no obvious bilateral symmetry.

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How has the evolution of animal genomes led to the amazing diversity of body forms that we observe in the natural world? Some of the most informative clues to this fundamental problem have come from the study of mutations in homeobox (Hox) genes. These mutations have powerful and interpretable effects on morphology, the most conspicuous being the HOMEOTIC TRANSFORMATIONS in *Drosophila melanogaster*[1,2]. Additionally, Hox genes are present and expressed in similar patterns in nearly every BILATERAL animal that has been analysed, so their roles in morphological diversification probably evolved before the appearance of the first bilateral animal. Indeed, the initial glimpses into the conservation of metazoan developmental control genes came during the study of *D. melanogaster* Hox gene clusters[3], which were originally (and more informatively) called homeotic selector genes.

In a wide variety of animals, ranging from nematodes to mice, mutations in Hox genes result in morphological defects that are restricted to discrete segmental zones along the anterior–posterior (A–P) axis, and sometimes include homeotic transformations similar to those that are seen in *D. melanogaster*[4,5]. Therefore, one conserved function of different members of the Hox gene family is to select one A–P axial identity over another. Hox genes are also interesting because their control of axial morphology has an abstract quality, exerting its influence in various organs, tissues and cell types within different A–P regions. Although emphasizing the role of Hox genes in controlling A–P or ORAL–ABORAL axial identities is a simplification of Hox gene functions, which have diversified during their 600 million years of evolution in millions of animal lineages[6–10], it is likely to be their ancestral role in developmental patterning[11].

The Hox genes map in chromosomal clusters, and the different PARALOGUES in the cluster are usually arranged in a collinear manner relative to their distinct, often overlapping, expression domains (FIG. 1a,b). In animal embryos in which mid-head and posterior abdomen can be distinguished, 'head' Hox genes have their initial anterior boundaries of expression in epidermal, neural and mesodermal cells of the mid-head region, and 'tail' Hox genes have their initial anterior boundaries of expression in the corresponding cell types of the posterior abdomen[3]. After the initial boundaries are set, Hox gene expression patterns can be labile within the larger confines of their initial domains[12,13].

The homeodomain transcription factors that are encoded by the Hox genes activate and repress batteries of downstream genes by directly binding to DNA sequences in Hox-response enhancers. *In vitro,* Hox

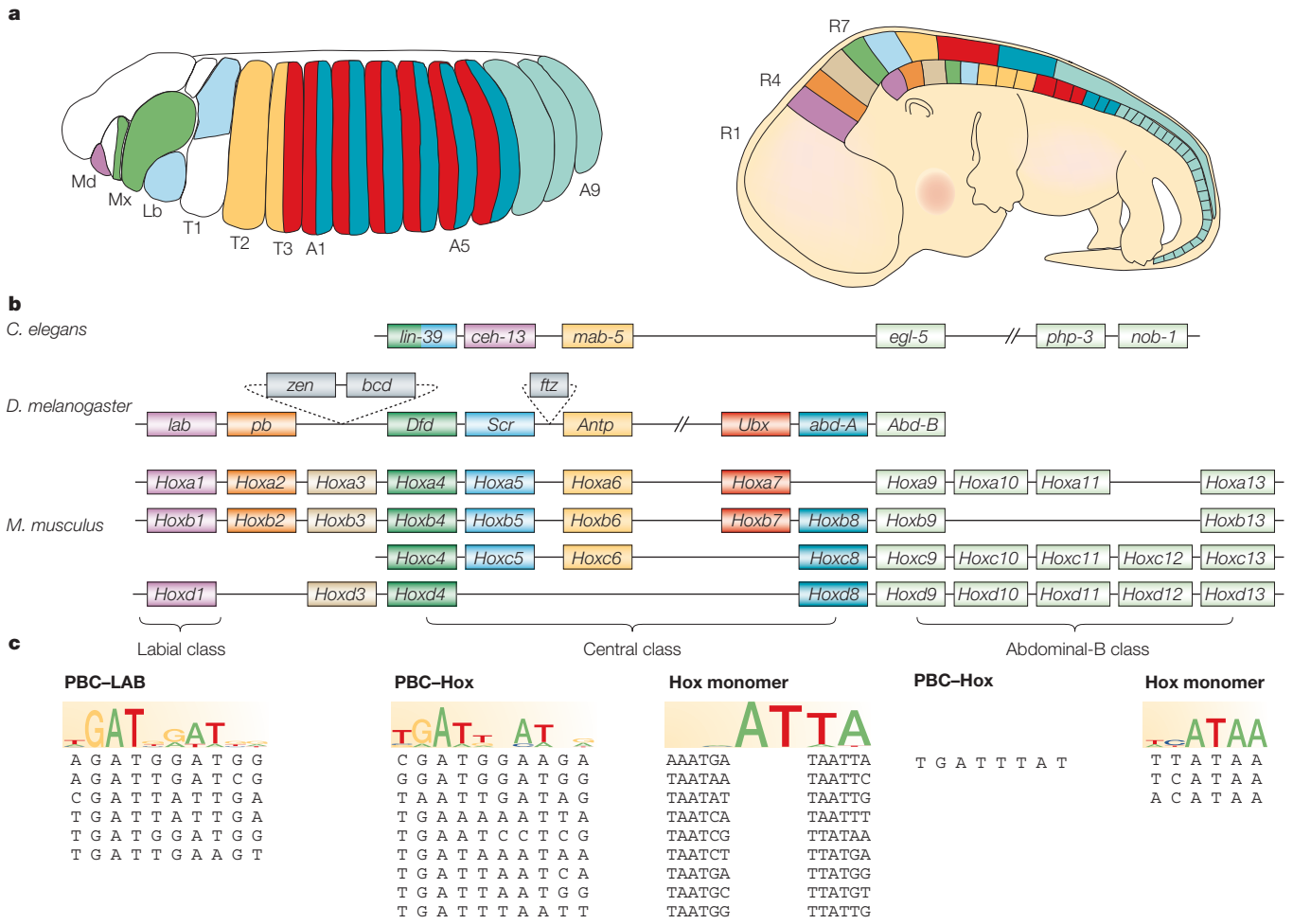


Figure 1 | **Hox expression, genomic organization, and Hox binding sequences. a** | The panel on the left shows a stage 13 *Drosophila melanogaster* embryo that has been coloured in the schematic to indicate the approximate domains of transcription expression for all Hox genes except *proboscipedia* (*pb*)[85]. The segments are labelled (Md, mandibular; Mx, maxillary; Lb, labial; T1–T3, thoracic segments; A1–A9, abdominal segments). The panel on the right shows a mouse (*Mus musculus*) embryo, at embryonic day 12.5, with approximate Hox expression domains depicted on the head–tail axis of the embryo. The positions of hindbrain RHOMBOMERES R1, R4 and R7 are labelled. In both diagrams the colours that denote the expression patterns of the Hox transcripts are colour-coded to the genes in the Hox cluster diagrams shown in **b**. Anterior is to the left, dorsal is at the top. **b** | A schematic of the Hox gene clusters (not to scale) in the genomes of *Caenorhabditis elegans*, *D. melanogaster* and *M. musculus*. Genes are coloured to differentiate between Hox family members, and genes that are orthologous between clusters and species are labelled in the same colour. In some cases, orthologous relationships are not clear (for example, *lin-39* in *C. elegans)*. Genes are shown in the order in which they are found on the chromosomes but, for clarity, some non-Hox genes that are located within the clusters of nematode and fly genomes have been excluded. The positions of three non-Hox homeodomain genes, *zen, bcd* and *ftz,* are shown in the fly Hox cluster (grey boxes). Gene abbreviations: *ceh-13*, *C. elegans homeobox 13*; *lin-39*, *abnormal cell lineage*; *mab-5*, *male abnormal 5*; *egl-5*, *egg-laying defective 5*; *php-3*, *posterior Hox gene paralogue 3*; *nob-1*, *knob-like posterior*; *lab*, *labial*; *pb*, *proboscipedia*; *zen*, *zerknullt*; *bcd*, *bicoid*; *Dfd*, *Deformed*; *Scr*, *Sex combs reduced*; *ftz*, *fushi tarazu*; *Antp*, *Antennapedia*; *Ubx*, *Ultrabithorax*; *abd-A, abdominal-A*; *Abd-B, Abdominal-B*. **c** | A compilation of *in vivo* DNA binding sequences arranged by the structural type of homeodomain that is encoded by the Hox genes. The three classes are Labial, Central, and Abdominal-B. The listed DNA binding sequences that are bound by Hox monomers and Pre-B-cell homeobox/CEH-20 (PBC)–Hox heterodimers are those that are required for the function of one or more Hox-response elements in developing mouse[36,92,101–106], fly[28,36,44–46,51–54,95,100,107–111] or nematode[29,112]. As no known HOX1-monomer-binding (mouse) or LAB-monomer-binding (fly) sites have been found to be functional *in vivo,* only PBC–LAB-heterodimer-binding sites are shown. Consensus logos were generated using all verified Hox-binding sites with WEBLOGO[113].

PARALOGUES
Genes in the same organism that have evolved from a gene duplication, usually with a subsequent, sometimes subtle, divergence of function.

RHOMBOMERE
Each of seven neuroepithelial segments found in the embryonic hindbrain that adopt distinct molecular and cellular properties, restrictions in cell mixing, and ordered domains of gene expression.

WEBLOGO
A web based application for generating sequence logos — graphical representations of an amino-acid or nucleic-acid motif weight matrix.

proteins can bind with high affinity as both monomers and multimers to specific DNA binding sites[3] (excluding Labial/Homeobox 1 (LAB/HOX1) class proteins, which bind almost exclusively as heterodimers with Pre-B-cell homeobox/CEH-20 (PBC) class proteins[14,15]). *In vivo,* however, Hox proteins bind to and regulate transcription through a broad collection of binding sites (FIG. 1c). On many target enhancers, Hox proteins cooperatively bind to canonical heterodimer-binding sites[14,15] with members of the PBC family of homeodomain proteins (called PBX in mammals, EXD in *D. melanogaster*, CEH-20 and CEH-40 in *Caenorhabditis elegans*)[16] and binding sites for the HTH/MEIS super-family of homeodomain proteins (which include MEIS

Table 1 | **Direct Hox-regulated genes: *Caenorhabditis elegans* and *Drosophila melanogaster***

Regulated gene	Expression domain that is controlled by Hox*	Regulating Hox protein(s)	Strongest evidence for direct Hox regulation	References
Caenorhabditis elegans				
hlh-8	Larval M lineage cells	LIN-39, MAB-5	Enhancers with mutated Hox sites that were tested in larvae	29
egl-17	Primary vulval cells	LIN-39	Enhancer with mutated Hox site that was tested in larvae	112
ceh-13	Embryonic dorsal body-wall muscle and ventral nerve cord	CEH-13	Enhancers with mutated Hox site that were tested in larvae	114
egl-18, elt-6	Larval vulval cells	LIN-39	Enhancers with mutated Hox sites that were tested in larvae	115
Drosophila melanogaster				
forkhead	Embryonic salivary gland	SCR	Enhancer with mutated Hox site that was tested in embryos	61,109
Distal-less	Embryonic ectoderm	UBX, ABD-A	Enhancers with mutated Hox sites that were tested in embryos	28
Antp	Embryonic tracheal and neural ectoderm	ANTP, UBX, ABD-A	Enhancers with clusters of mutated Hox sites that were tested in embryos	52
Hoxa4	Embryonic epidermis	UBX	Bicoid site swap (K50) using UBX	45
Deformed	Embryonic maxillary epidermis	DFD	Enhancer with mutated Hox site that was tested in embryos	49
1.28	Embryonic maxillary epidermis	DFD	Enhancer with mutated Hox sites that was tested in embryos	98
teashirt	Embryonic epidermis and somatic mesoderm	ANTP, UBX	Enhancers with deleted Hox sites that were tested in embryos	116
scabrous	Embryonic ectoderm	UBX, ABD-A, ABD-B	ChIP using UBX	117
Transcript 48	Embryonic epidermis, and somatic and visceral mesoderm	ABD-A, UBX	ChIP using UBX	118
La-related protein	Embryonic ectoderm, and somatic and visceral mesoderm	SCR, UBX	ChIP using UBX	119
centrosomin	Embryonic visceral mesoderm and CNS	ANTP, UBX, ABD-A	ChIP using ANTP	110
decapentaplegic	Embyonic midgut visceral mesoderm	ANTP, UBX, ABD-A	Bicoid site swap (K50) using UBX and ABD-A	27,44, 95,120
apterous	Embryonic muscle mesoderm	ANTP	Bicoid site swap (K50) using ANTP	46
connectin	Embryonic mesoderm	ABD-A, UBX	ChIP using UBX	121
serpent	Embryonic lateral mesoderm	UBX	One-hybrid assay using UBX	122
wingless	Embryonic visceral mesoderm	ABD-A	Enhancers with mutated or deleted Hox sites that were tested in embryos	123
Wnt4	Embryonic visceral mesoderm	ANTP, UBX, ABD-A	ChIP using UBX	107
β-tubulin at 60D	Embryonic visceral mesoderm	UBX	Enhancers with deleted Hox sites that were tested in embryos	108
labial	Embryonic midgut endoderm	LAB	Enhancer with mutated Hox site that was tested in embryos	53
CG11339	Embryonic midgut endoderm	LAB	Enhancers with mutated Hox sites that were tested in embryos	54
spalt major	Wing imaginal discs	UBX	Enhancers with mutated Hox sites that were tested in larvae	51
knot	Wing imaginal discs	UBX	Enhancers with mutated or deleted Hox sites that were tested in embryos	100

*Sorted by tissue type. ABD-A, Abdominal A; ANTP, Antennapedia; *ceh-13*, *C. elegans homeobox gene 13*; ChIP, chromatin immunoprecipitation; DFD, Deformed; *egl-17/18, egg-laying defective 17/18*; *elt-6, erythroid-like transcription factor family 6*; *hlh-8, helix-loop-helix 8;* LAB, Labial; LIN-39, abnormal cell lineage 39; MAB-5, male abnormal 5; SCR, Sex combs reduced; UBX, Ultrabithorax.

or PREP in mammals, HTH in *D. melanogaster* and UNC-62 in *C. elegans*[16]) are frequently also found nearby. The functional regulatory complex that acts on some Hox-response elements therefore often involves HOX–PBC–MEIS heterotrimers[17]. In part through the different binding preferences of distinct Hox proteins in these heterotrimer complexes, and in part through PBC/MEIS-independent mechanisms, distinct but overlapping combinations of downstream genes are activated and repressed, with the result being morphological diversity in axial domains.

In this article we review four areas in which Hox research has advanced in the past few years. First, we describe several mechanisms by which different Hox proteins can diversify A–P axial morphology at the cell biological level. Second, we look at examples of Hox-regulated enhancers and try to derive general rules about their structure. Third, we review recent studies that have revealed a surprising lability and evolvability in Hox expression patterns and protein functions in animals. Fourth, we review how microRNA functions have recently been found to be integrated within the Hox axial patterning system in many animals.

Hox targets and morphological diversification

In part, Hox proteins act as high-level executives, regulating other executive genes (including themselves, *extradenticle* (*exd*) and *homothorax* (*hth*)[18–22]) that encode transcription factors or morphogen signals. However, there is accumulating evidence that they act directly at many other levels[23], even on the 'blue collar' genes that mediate adhesion, cell division rates, cell death and cell movement. It is often lamented in print that few Hox target genes are known, but this is not true. There are at least 35 target genes, in a variety of organisms, for which there is good evidence for direct regulation by one or more Hox proteins (TABLES 1,2). In addition to these well-characterized direct targets, many other genes are influenced by Hox expression but they have not been shown to be regulated directly by Hox genes. Recent microarray experiments have identified an even larger pool of potential target genes[24–26].

Hox regulation: executive level. There are many examples of direct Hox regulation of genes that encode cell–cell signalling molecules or other transcription factors. Many of these target genes were suggested as potential targets because their mutant phenotypes showed similarities to Hox mutant phenotypes. Others were suggested because their A–P expression patterns either mimicked or complemented the patterns of one or more Hox proteins, consistent with positive or negative regulation, respectively.

One executive target gene is *decapentaplegic* (*dpp*), which is expressed in an A–P domain of VISCERAL MESODERM in *D. melanogaster*. This *dpp* expression pattern is provided, in part, by the Hox proteins Ultrabithorax (UBX) and Abdominal-A (ABD-A), which activate and repress *dpp* transcription, respectively[27]. The localized production of DPP, a secreted morphogen of the bone morphogenetic protein (BMP) class, then triggers cell shape changes in the gut that are required for normal visceral morphology[6]. UBX and ABD-A also directly repress the *Distal-less* (*Dll*) gene in the *D. melanogaster* abdominal epidermis[28] (note that Hox proteins can operate either as transcriptional activators, as UBX does on *dpp* in the visceral mesoderm, or as repressors, as UBX does on *Dll* in the epidermis). The *Dll* gene encodes a homeodomain transcription factor that promotes appendage development, so its repression by UBX results in an absence of limbs from the abdomen. In *C. elegans*, the gene that encodes the Twist transcription factor homologue, *helix-loop-helix 8* (*hlh-8*), is directly activated in mid-body mesodermal cells by the Hox proteins abnormal cell lineage 39 (LIN-39) and male abnormal 5 (MAB-5)[29] (FIG. 1a,b). The *hlh-8* gene is required for normal mesoderm development, and its absence contributes to the localized muscle defects that are observed in *lin-39* and *mab-5* mutants.

Hox regulation: cell adhesion. It has been long realized that Hox proteins must regulate cell adhesion, division, death, migration and shape in order to mould morphology[30]. However, we have only recently learned the identities of some of the Hox target genes, the realizator genes[30], that directly mediate such properties at the cellular level in developing animals. Some of the first evidence for Hox control of cell adhesion came from Yokouchi *et al.*[31]. Mouse *Hoxa13* is normally expressed in developing AUTOPODS. Ectopic activation of *Hoxa13* throughout the entire developing limb resulted in a marked reduction of the cartilage primordia for the proximal limb, cartilage that would normally develop into the radius and ulna[31]. This phenotype was associated with a *Hoxa13*-dependent increase in homophilic cell adhesion in proximal cartilage primordia.

Conversely, in mouse *Hoxa13* mutants, the mesenchymal condensations that normally form in the autopod are loosely and poorly organized, resulting in loss or abnormalities of the digit, carpal and tarsal bones that derive from the distal limb[32]. Normally the gene that encodes the ephrin receptor EPHA7 is expressed in distal limb domains in a way that closely matches *Hoxa13* expression. However, in *Hoxa13* mutants *EphA7* expression is severely reduced. Reducing EPHA7 protein function with blocking antibodies in a $Hoxa13^{+/-}$ background results in a failure to form the normal chondrogenic condensations in distal limb primordia, similar to the phenotype that is seen in $Hoxa13^{-/-}$ mutants. Since in many contexts, direct interactions between transmembrane ephrin receptors and their membrane-bound ligands are required for normal cell adhesion (as well as for many other cellular responses)[33], it seems likely that HOXA13-mediated mesenchymal condensations in the distal limb are achieved in part by the activation of *EphA7* gene expression.

The regulation of ephrin receptor and/or ephrin ligand genes by Hox proteins seems to be common. In

MicroRNAs
Small, non-coding RNAs that are components of a large protein complex (RISC) and are involved in repression of protein production from mRNAs that contain sequences with significant complementarity to the microRNA.

VISCERAL MESODERM
A subset of mesodermal cells that surround endodermal tissues such as the gut, also known as splanchnic mesoderm.

AUTOPODS
Distal limb primordia that give rise to structures such as the mouse paw.

Table 2 | **Direct Hox-regulated genes: *Xenopus laevis*, mouse and human**

Regulated gene	Expression domain that is controlled by Hox*	Regulating Hox protein(s)	Strongest evidence for direct Hox regulation	References
Xenopus laevis				
Hoxb4, Hoxb5	Unspecified	HOXB4	Induced nuclear importation of HOXB4 after translation inhibition	124
RAS-related protein-1a	Embryonic dorsal ectoderm?	HOXB4	Induced nuclear importation of HOXB4 after translation inhibition	125
iroquois 5	Embryonic neural ectoderm?	HOXB4	Induced nuclear importation of HOXB4 after translation inhibition	126
caspase-8-associated protein 2/FLASH	Embryonic notochord	HOXB4	Induced nuclear importation of HOXB4 after translation inhibition	127
Mouse				
Hoxb1	R4	HOXB1	Enhancers with mutated Hox sites that were tested in embryos	92
Hoxb2	R4	HOXA1, HOXB1	Enhancer with mutated Hox site that was tested in embryos	102
Hoxb3, Hoxb4	Hindbrain	HOXB4, HOXD4	Enhancers with mutated Hox sites that were tested in embryos	20
retinoic acid receptor-β	Embryonic hindbrain	HOXB4, HOXD4	Enhancers with mutated or deleted Hox sites that were tested in embryos	103
serine protease inhibitor 3	CNS	HOXB5	ChIP using HOXB5	101
Human				
ephrin B4	Human umbilical venous endothelial-cell culture	HOXA9	ChIP using HOXA9, which was screened for Ephrin B4 by PCR	35

*Sorted by tissue type. ChIP, chromatin immunoprecipitation; R4, rhombomere 4.

combination with PBX1, the HOXA1 and HOXB1 proteins can bind to and activate a mouse rhombomere-specific enhancer from the *Epha2* gene in COS7 CELLS[34], and mouse HOXA9 protein can bind and activate the *Ephb4* gene in cultured endothelial cells[35]. In addition, a recent genomic screen for Hox target genes has revealed that the mouse *Epha3* gene is repressed in a *Hoxd13*-dependent and *Hoxa13*-dependent manner in the posterior regions of developing autopods[36].

Hox regulation: cell cycle. There is ample evidence for Hox involvement in blood cell development in mammals[37], including the activation of *Hoxa10* gene expression during the differentiation of cultured MYELOMONOCYTIC CELLS into MONOCYTES. The role of *Hoxa10* in myeloid and erythroid development in bone marrow cells is complex, and it is not clear how well its function in cultured myelomonocytes recapitulates its function in animals[38]. With that caveat, forced expression of HOXA10 protein in cultured myelomonocytic cells results in premature differentiation into monocytes, accompanied by growth arrest[36]. This growth-arrest phenotype seems to be controlled by *Hoxa10*-dependent activation of the *Cdkn1a* gene, which encodes a cyclin dependent kinase inhibitor, p21. The HOXA10 protein, together with the PBX1 and MEIS1 proteins, can bind *Cdkn1a* promoter sequences *in vitro*, which are presumably part of the *cis*-regulatory DNA that mediates the effects of HOXA10 on the cell cycle *in vivo*.

Hox regulation: cell death. Another way in which Hox proteins might regulate morphology would be simply to ablate cells that are not part of the desired tissue shape. There is indeed evidence for Hox genes acting as sculptors by regulating cell death. In *D. melanogaster* embryos, maintenance of the segmental boundary between the maxillary and mandibular segments of the head (FIG. 1a) requires localized cell death at the boundary that is controlled by the apoptosis-promoting gene *reaper* (*rpr*). Mutants in the Hox gene *Deformed* (*Dfd*) have a similar head segmental defect to mutants with a deletion for several cell death genes, and this is mainly due to the absence of *rpr* expression at the maxillary–mandibular border in *Dfd* mutants[39]. When a stripe of *rpr* expression is provided at the border in *Dfd* mutants, the segmental boundary is maintained. Additionally, a small *rpr* enhancer was defined that requires four DFD-binding sites for transcriptional activation at the maxillary–mandibular border in embryos[39] (BOX 1).

Similarly, the morphology of the abdominal region of the *D. melanogaster* adult CNS is sculpted in a Hox dependent manner. In adults, the abdominal CNS is much smaller than the thoracic CNS, owing to fewer cells. Bello *et al.* found that a brief pulse of ABD-A protein expression in a large subset of the abdominal postembryonic neuroblasts triggers apoptosis in a manner that is dependent on the pro-apoptotic genes *rpr*, *head involution defective 1* (*hid1*) and *grim*, with a consequent size reduction of the adult abdominal NEUROMERES[40].

COS7 CELLS
A commonly used fibroblast-like cell line that is derived from African green monkey kidneys.

MYELOMONOCYTIC CELLS
Pluripotent cells that differentiate into immune cells such as granulocytes, dendritic cells and monocytes.

MONOCYTES
Immune cells that circulate in the blood stream and engulf foreign invaders. They can migrate into tissues, where they differentiate into macrophages.

NEUROMERES
Segmentally repeated subunits of the developing CNS.

Box 1 | Structures of representative Hox-response enhancers

The figure shows five Hox-regulated enhancers from *Mus musculus* or *Drosophila melanogaster*. Enhancers are represented by white rectangles. Linear sequence conservation from *D. melanogaster* to *Drosophila virilis* (panels **b**–**e**) or from *M. musculus* to the puffer fish *Takifugu rubripes* (panel **a**) is represented by blue bars. Hox and Pre-B-cell homeobox/CEH-20 (PBC) sites that were identified by FOOTPRINTING and/or mutation analysis are noted by H or P, respectively. Below the schematic of each enhancer are confirmed Hox or Hox–PBC binding sequences; Hox and PBC binding sites are coloured in red or green text, respectively, and conserved sequences are capitalized. The wild-type expression pattern of each enhancer is shown on the right, where one example of evidence that confirms Hox dependence is described for each enhancer.

A canonical Hox–PBC enhancer

An enhancer that responds to both HOX and PBC proteins maps upstream of mouse *Hoxb1* (panel **a**). This enhancer contains a repeat of evolutionarily conserved HOXB1–PBX (Pre-B-cell homeobox)-heterodimer-binding sites that are required for autoactivation of *Hoxb1* in rhombomere 4 (R4)[92]. Other Hox-dependent enhancers with required canonical HOX–PBC-binding sites include those in *D. melanogaster* *labial*[53] and *forkhead*[61] and in *Caenorhabditis elegans* helix-loop-helix (*hlh8*)/*twist*[29].

A non-canonical Hox–PBC enhancer

An example of a Hox target that apparently requires Hox and Extradenticle (EXD) inputs through a non-canonical site is a thoracic-limb enhancer (*Dll*-304) from the *Distal-less (Dll)* gene (panel **b**)[28], which is repressed in the abdomen by Ultrabithorax (UBX) and Abdominal-A (ABD-A) through a repression element called DMX-R[93,94]. DMX-R (panel **b**) has two Hox-binding sites (one in a non-canonical Hox–EXD-heterodimer site), as well as sites that bind a large multiprotein repression complex[94]. Curiously, when the non-canonical Hox–EXD site is changed to a canonical site with higher *in vitro* affinity, UBX and ABD-A no longer repress this *Dll* limb enhancer *in vivo*[93].

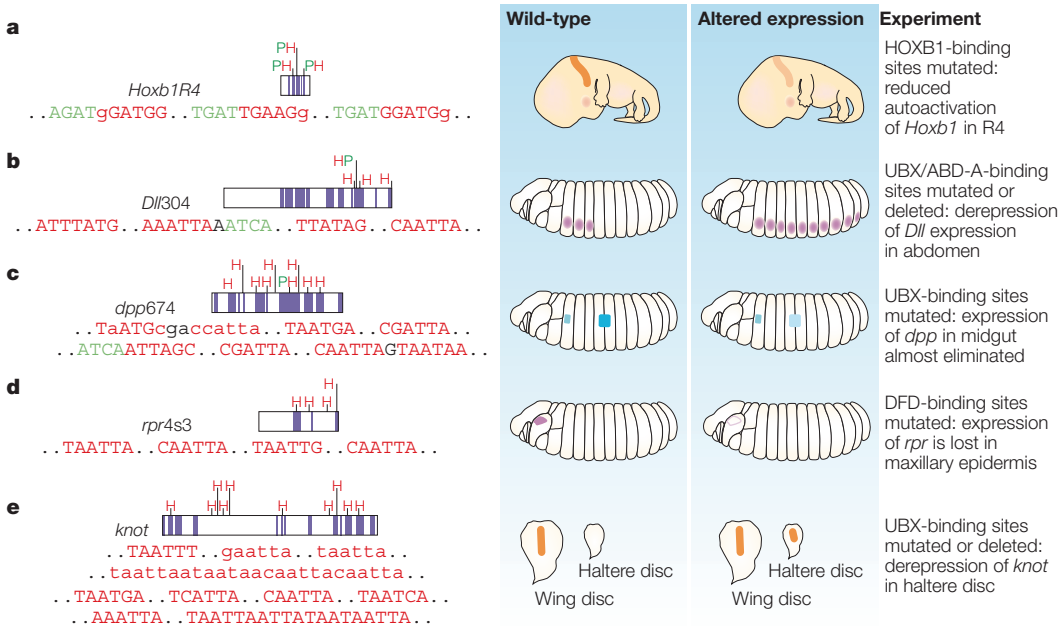
An enhancer that is activated and repressed by different abdominal Hox proteins

The *dpp*-674 enhancer of *decapentaplegic* controls expression in midgut primordia and is activated by UBX but repressed by ABD-A more posteriorly[95]. Eliminating the sites that ABD-A normally binds to repress transcription allows more posterior expression, whereas eliminating the sites that UBX binds almost eliminates expression in the midgut (panel **c**). Interestingly, a sub-element that lacks the repression Hox sites, but contains the activation sites, can be activated by either UBX or ABD-A[27].

PBC-independent enhancers

Some Hox targets appear to be regulated independently of EXD. The ambiguity exists because it is often impossible to rigorously test Hox-response elements for dependence on *PBC/exd* due to the early developmental functions of zygotic or maternally contributed EXD[96,97]. Evidence of *exd* independence/dependence is often limited to the absence/presence of EXD or EXD–HOX sites that can be identified by *in vitro* binding assays. By these criteria, two Hox-response elements that are activated by Deformed (DFD) in the maxillary epidermis are EXD-independent — a *1.28* enhancer and a *reaper* enhancer (panel **d**)[39,98,99].

Other Hox-response elements are expressed in body regions where EXD is not expressed. These include two wing-IMAGINAL-DISC enhancers that are directly repressed by UBX, one from the *knot*[100] gene (panel **e**) and one from the *spalt major* gene[51]. Both are derepressed in the HALTERE imaginal disc in *Ubx* mutants, and both possess multiple UBX-binding sites that are required for the repression of the enhancers in haltere primordia.



FOOTPRINTING
An *in vitro* assay that identifies regions of DNA that are protected from digestion by DNaseI, thereby indicating the presence of bound transcription factors.

IMAGINAL DISC
Sacs of cells in larval stages of holometabolous insects that divide and differentiate to form most adult tissues.

HALTERE
A balancing organ that is located on the third thoracic segment in Diptera and is an evolutionary modification of a wing.

Hox regulation: cell migration. Hox genes have long been known to modulate cell migration, and one of the best examples of this activity is the control of Q NEUROBLAST migration during *C. elegans* development by the Hox genes *mab-5* and *lin-39*. The function of *mab-5* is required CELL AUTONOMOUSLY for the posterior migration of the descendants of the QL neuroblast[41], and *lin-39* is required for the anterior migration of the descendants of the QR neuroblast[42,43]. The cell biological mediators that are regulated by the LIN-39 and MAB-5 proteins are not known.

The above examples barely scratch the surface of Hox-regulated morphological effector genes, yet they indicate that the cell biological effectors that are regulated by Hox proteins to sculpt morphology on the A–P axis are highly diverse. Hox proteins activate and repress multiple effector genes, in diverse cell types and tissues, throughout embryonic development. Because of the immense complexity of these interactions, it is unlikely that we will ever completely understand, at the molecular level, how Hox genes define an entire segment to have thoracic as opposed to abdominal identity. We will have to settle with understanding how cellular adhesion and other properties are controlled by the Hox system at a smaller scale in the diversification of axial morphologies.

Hox-regulated enhancers

Although we might never have a complete picture of the Hox-dependent cell-biological changes that differentiate one segment from another, it is plausible that one day we will understand the principles on which Hox target enhancers are built, at least well enough to predict their locations in the genome at a reasonable frequency. Our definition of Hox target enhancers in this review includes only those with strong evidence for direct regulation by a Hox protein in developing animals. The most rigorous test for validating a direct target element, the 'gold standard', is to subtly mutate the Hox-binding sites of an enhancer so that they prefer to bind Bicoid, a non-Hox homeodomain protein. This change results in the enhancer having reduced binding affinity and therefore a reduced response to the putative Hox *trans*-regulator. Compensatory mutations are then introduced into the DNA-binding domain of the putative Hox *trans*-regulator that allow it to bind with high affinity to the mutant sites in the enhancer. If the altered protein regains the ability to regulate the altered enhancer, it is strong evidence that a specific Hox protein is binding to a specific enhancer in embryonic cells. Only a few Hox-regulated enhancers have been validated using this rigorous test[27,44–47], which has so far only been attempted in *Drosophila* embryos. However, as is typical for most *in vivo* enhancer studies in animals, it has been more common to test whether a mutant enhancer in which all Hox-binding sites were eliminated mimics the activity of the wild-type enhancer in mutant embryos that lack the predicted Hox *trans*-regulator (BOX 1).

Common principles of Hox target enhancers. The five enhancers that are shown in BOX 1 represent a sample of diverse Hox-responsive DNA elements. Although they differ in many ways, including organism of origin, they also share several properties.

One common property is tissue specificity. For example, the UBX-dependent enhancer from *Drosophila dpp* is active only in the visceral mesoderm (BOX 1), and is inactive in the epidermal, CNS and somatic mesoderm cells that also contain UBX protein. This specificity is due to the *dpp* enhancer also being regulated by Biniou/FOXF, a visceral-mesoderm-specific forkhead-type transcription factor[48]. Two autoactivation enhancers from the *Dfd* gene also exemplify this 'tissue-specificity rule'. One, which maps 5 kb upstream of the *Dfd* transcriptional start site, is active only in the epidermal cells that express DFD protein at the maxillary–mandibular border[49]. Although DFD protein also autoactivates *Dfd* transcription in the CNS, this process is mediated through another enhancer that maps to the large intron of the *Dfd* gene[50].

A second common property of Hox-response elements is the requirement for multiple Hox-monomer-binding sites (FIG. 1c) (BOX 1), most of which possess an ATTA (or TAAT) core sequence. Many Hox-response elements also require Hox–PBC-heterodimer-binding sites (BOX 1), and often contain MEIS-binding sites as well, at variably spaced distances from the Hox–PBC sites. The range of both Hox-monomer-binding and Hox–PBC-binding sequences is broad (FIG. 1c). This is consistent with the evidence indicating that there is no systematic relationship between the affinities for monomer or heterodimer sites *in vitro* and their functional importance *in vivo*[27,51–54]. How the functional specificity of Hox-regulated enhancers is strengthened without the help of PBC or MEIS sites is unknown, but it is not surprising that natural selective pressures will 'use' any available mechanism to generate meaningful Hox-enhancer expression patterns. On the basis of genetic evidence in *D. melanogaster*, there are at least two other evolutionarily conserved transcription factors, Teashirt and Disco, that probably operate as Hox cofactors in specifying A–P axial identity[55–58]. Whether these two proteins mechanistically interact with Hox proteins to activate or repress target enhancers, and how they do so, is unknown.

In silico *searches for Hox targets.* The best hope for identifying at least a subset of Hox-response elements by bioinformatic means is to search for genomic regions that are enriched for Hox, PBC and MEIS consensus sites. To test the utility of this strategy, Ebner *et al.* searched the *D. melanogaster* genome for canonical LAB–EXD-heterodimer-binding sequences within 40 base pairs of an HTH-consensus-binding sequence, and identified 30 genomic regions that met these requirements[54]. The expression patterns of genes near to 16 of these loci were tested for overlap with the LAB expression pattern. Besides the *lab* autoregulatory enhancer (the source of the sequence motifs), only one other potential LAB-response element was identified. It

Q NEUROBLAST
A *C. elegans* neural lineage that divides to make QL and QR neuroblasts, which in turn generate identical neural cells on left and right sides of the body.

CELL AUTONOMOUS
If the activity of a gene has effects only in the cells that express it, its function is said to be cell autonomous; if it causes effects in cells other than (or in addition to) those expressing it, its function is cell non-autonomous.

mapped to the first intron of the *CG11339* gene, which encodes an actin-binding protein that is activated in a LAB-like expression pattern in the endoderm[54].

Tests of a 2 kb genomic fragment that contains the LAB–EXD–HTH consensus indicated that it did not function as a Hox-response element. However, the authors tested other DNA fragments around the *CG11339* transcription unit and identified an upstream fragment that acted as a LAB-dependent enhancer when fused to a reporter gene. When tested with *in vitro* binding assays, this enhancer was found to possess an HTH-binding site as well as a LAB–EXD site. Interestingly, the latter was highly divergent from the canonical site that was used in the bioinformatic search, but is still required for enhancer activation *in vivo*. This LAB–EXD site also bound LAB protein as a monomer, contesting the prevailing belief that LAB had little or no DNA-binding affinity in the absence of EXD[59]. It is possible that *CG11339* was identified as a LAB-responsive gene by accident, albeit an accidental find that led to interesting new insight concerning *in vivo* LAB–EXD regulation[54]. In any case, the results of this study do not bode well for bioinformatic predictions of naturally evolved Hox–PBC response elements that use the current version of the 'DNA-binding-selectivity model'[60,61].

On the basis of the current body of knowledge, it is clear that Hox target elements do not observe simple rules. Even individual enhancers seem to be regulated by both PBC-dependent and PBC-independent mechanisms[20]. Given the great diversity in Hox-response enhancer structures, it seems that even modest success in predicting Hox-response elements will require more knowledge about the range of Hox protein interactions with cofactors and target DNA sites.

Hox genes and morphological evolution

In the past 10 years, many studies have supported the idea that Hox gene mutations contribute to morphological evolution[1], as there is evidence that animal body plans have been altered by mutations occurring at many levels of Hox regulatory hierarchies[62]. Apparently, some of this mutational variation is in *cis*-regulatory sequences of the Hox genes. For example, variations in the expression pattern of UBX and ABD-A orthologues in different crustacean groups correlate well with the evolution of their anterior thoracic limbs into specialized feeding appendages (maxillipeds)[63]. At the micro-evolutionary level, subtle variations in the expression pattern of UBX protein correlate with differences in hair patterns on the second thoracic femur between closely related species of *Drosophila*[64]. These are only a few of the many studies that are dedicated to revealing connections between Hox-expression-pattern changes and changes in animal body patterning. Many other examples can be found in Carroll *et al.*[62]

There is also ample evidence that mutations in Hox protein sequences have contributed to morphological diversification during evolution[65]. For example, two groups found correlative and experimental evidence indicating that between distantly related arthropods, differences in carboxy-terminal regions of UBX proteins, which changed them into better transcriptional repressors, might have contributed to the reductions in limb number during the evolution of insects from multilimbed arthropods[66,67]. In both cases, the changes in UBX protein functions were accompanied by changes in their expression patterns in the trunk region of developing embryos.

The most striking findings on the evolution of Hox-expression-pattern change and Hox-protein-function change come from studies of the evolutionary origins of the *D. melanogaster* homeodomain genes *bicoid* (*bcd*), *zerknullt* (*zen*), *fushi tarazu* (*ftz*) and *even skipped* (*eve*)[65]. All of these genes derive from Hox ancestors, but their functions in present day *D. melanogaster* range from the control of early embryonic polarity (*bcd*) to segment number (*ftz* and *eve*). Three of these Hox derivatives still map to the *D. melanogaster* Hox cluster (FIG. 1b).

The evolution of *ftz* from a *Hox5/6*-like gene is particularly interesting. In many arthropod embryos, including some insects, ancestral *ftz* genes are expressed in Hox-like patterns, but in beetles and flies the expression patterns have evolved multiply striped patterns, characteristic of genes that specify segment number[68–72] as opposed to segment identity. In parallel with this change in embryonic expression pattern, the coding region of insect *ftz* has undergone mutations that eliminated the YWPM amino-acid motif (a signature of the Hox class of homeodomain protein) and added an LXXLL motif. The current evidence indicates that these changes altered the ancestral FTZ protein so that it lost its axial identity specification functions, and gained the ability to regulate segment number in early embryos[73,74].

All the above studies indicate that Hox expression patterns and Hox protein functions are amazingly labile for high-level developmental regulators. It will be fascinating to discover the mechanisms that allow such large changes in Hox function during evolution without interfering with animal viability and reproductive fitness. It might be possible in the near future to test homologous gene replacements between closely and distantly related organisms, involving Hox regulatory or coding sequences (or both). This might shed light on how their evolution can so profoundly contribute to morphological change.

Hox genes as microRNA targets

MicroRNAs (miRNAs) are a recently discovered class of regulatory molecule that has dramatically enhanced the standard view of gene regulation. These ~22-nucleotide RNAs are made from transcripts that are generated by RNA polymerase II in a two-step cleavage process that is catalysed by the nuclear RNaseIII Drosha and the cytoplasmic RNaseIII Dicer[75,76]. After processing, the mature miRNA is packaged into a functional RNA-INDUCED SILENCING COMPLEX (RISC). The RISC can catalyse precise endonucleolytic cleavage of target RNAs if they have perfect or near perfect complementarity to RISC-bound miRNAs, or reduce protein production by unknown post-transcriptional mechanisms if there is imperfect

RNA-INDUCED SILENCING COMPLEX
A large protein complex that packages microRNAs or siRNAs, silencing expression of proteins from target mRNAs by endonucleolytic cleavage or other unknown mechanisms, depending on the complementarity of mRNA sequences to the packaged small RNAs.

GNATHAL APPENDAGES
Outgrowths, usually from head segments, that are used to aid feeding.

RACE CLONES
Partial cDNA sequences that are generated from transcripts by the rapid amplification of cDNA ends (RACE) to determine the start and end points of gene transcription.

complementarity. Considering the large number of identified miRNAs, few validated *in vivo* targets are known at present. However, there are indications that Hox genes might be an important class of miRNA targets.

Potential regulation of Hox by miRNAs. For some time, there has been evidence for post-transcriptional regulation of Hox gene expression. Mouse HOXB4 protein and *Hoxb4* transcript patterns of accumulation were similar in somites 7–13 and in the posterior hindbrain of developing embryos, but transcripts were also detected in the posterior neural tube, whereas protein was not[77]. In addition, there is evidence for post-transcriptional regulation of *HOXC6* in the chick embryonic hindlimb[78]. There is also evidence that post-transcriptional regulation of the *Sex combs reduced* (*Scr*) orthologue in the crustacean *Porcellio scaber* might be involved in modification of the first thoracic segment to produce GNATHAL APPENDAGES as opposed to walking legs[79]. It is unknown whether these discrepancies between transcript and protein expression patterns are due to miRNA regulation or other mechanisms such as localized protein instability.

Although a few Hox genes are post-transcriptionally regulated by unknown mechanisms, many Hox genes have been predicted *in silico* to be direct targets of miRNAs in both vertebrates[80] and invertebrates[81]. On the basis of partial complementarity between miRNA sequence and 3′UTR sequences, Enright *et al.* proposed that *D. melanogaster Scr*, *Antennapedia* (*Antp*), *Ubx*, *abd-A* and *Abd-B* transcripts are targets of miRNA regulation[81]. If true, this indicates that miRNA regulation of Hox protein expression, at least in flies, is the rule rather than the exception. However, the validity of these proposed interactions have yet to be tested *in vitro* or *in vivo*.

The sole validated Hox miRNA target is the protein-encoding Hox transcript from mouse *Hoxb8*. The *Hoxb8* transcript contains a near perfect miR-196 target site in its 3′UTR, and analysis of mouse embryonic RACE CLONES showed that some of the *Hoxb8* transcripts are cleaved endonucleolytically at the tenth nucleotide of the miRNA target site[82,83], which is typical for sites with perfect complementarity to miRNA regulators. This target site is conserved in vertebrate *Hoxb8* genes, but it is not known what influence this miRNA-mediated regulation exerts on vertebrate axial morphology.

In addition to miR-196, other miRNAs that are encoded within Hox gene clusters might also be regulators of Hox protein expression. The microRNA-10 gene (*mir-10* in *D. melanogaster*) is phylogenetically conserved and is present in a conserved position in both insect and vertebrate Hox clusters, between the *Dfd/Hox4* and *Scr/Hox5* genes (FIG. 2a). In mammals, the *mir-10a* gene maps adjacent to *Hoxb4*, and the *mir-10b* gene is adjacent to *Hoxd4*. The *mir-57* gene of *C. elegans* is likely to be a diverged orthologue of the *mir-10* family (FIG. 2b), although it maps to a different chromosome than the *C. elegans* Hox genes. Additionally, at least one other miRNA gene maps among the posterior Hox genes in both insect and vertebrate genomes, but the *D. melanogaster mir-iab-4* and vertebrate *mir-196* miRNA genes do not seem to be structural orthologues, as they have only fragmentary sequence identity (FIG. 2b).

Expression patterns of Hox-complex miRNAs. The spatial expression patterns of Hox-cluster miRNAs also indicate that they have roles in A–P axial patterning (FIG. 2c). One way to detect the expression domains of miRNAs is to use ubiquitously transcribed reporter transgenes with 3′ UTRs that contain sequences perfectly complementary to putative miRNA *trans*-regulators (miRNA sensors). The body region in which reporter protein expression is abolished is inferred to represent the expression pattern of the miRNA that is

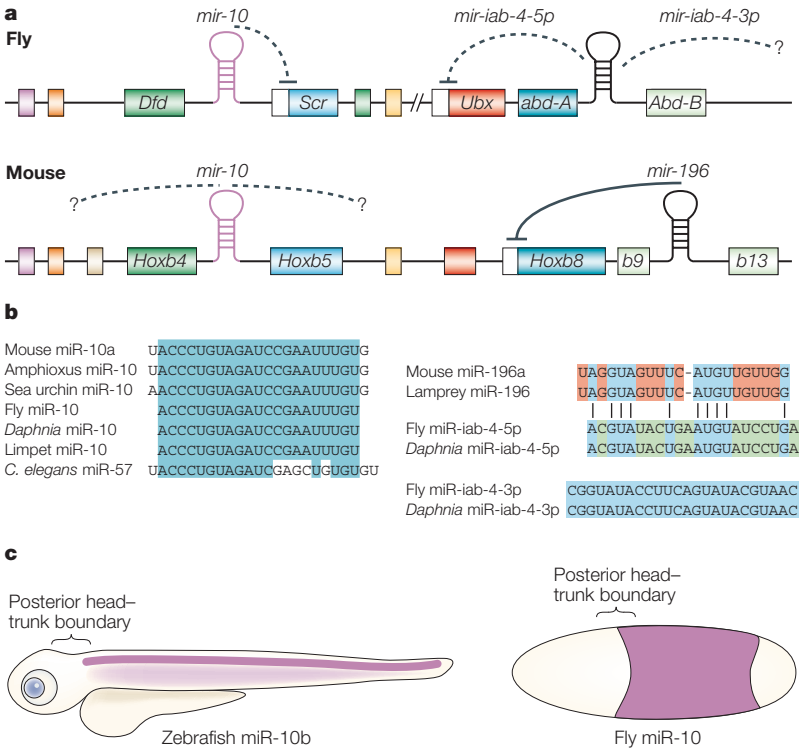


Figure 2 | **MicroRNA genes of the Hox cluster. a** | Hox microRNA (miRNA) genes of *Drosophila melanogaster* (upper panel) and mouse (lower panel), with hairpins indicating their positions within the Hox complexes. For mouse, only the Hoxb cluster is shown; additional paralogues of *microRNA-10 (mir-10)* and *mir-196* map in the other Hox clusters. Proposed interactions (dashed lines) or interactions for which there is existing evidence (solid lines) are shown for nearby Hox target genes. Both *mir-iab-4-5p* and *mir-iab-4-3p* are indicated, since both strands (5′ and 3′) of the *infraabdominal 4* (*iab-4*) Dicer-cleavage product have been cloned from developing *D. melanogaster*[99]. **b** | Left: an alignment of miR-10 orthologues from mouse (*Mus musculus*), amphioxus (*Branchiostoma floridae*), sea urchin (*Strongylocentrotus purpuratus*), fruitfly (*D. melanogaster*), a crustacean (*Daphnia pulex*), a gastropod limpet (*Lottia gigantea*) and the *microRNA-57* sequence from *Caenorhabditis elegans*, showing a high degree of sequence conservation in bilaterian genomes. Right: miR-iab-4-5p sequences from fly (*D. melanogaster*) and crustacean (*D. pulex*) are aligned with vertebrate miR-196 sequences from mouse (*M. musculus*) and lamprey (*Petromyzon marinus*), showing very little sequence identity between these two miRNAs. This indicates that they are probably not orthologues, even though they reside in similar locations in the Hox complexes. Also included below is an alignment of fly and crustacean miR-iab-4-3p. **c** | A diagrammatic comparison of embryonic expression patterns for miR-10b in a 72-hour zebrafish embryo and *pri-mir-10* in a cellular-blastoderm-stage *D. melanogaster* embryo. In both embryos, the anterior boundary of miR-10 expression lies approximately at the boundary between posterior head and trunk (brackets). Both embryos are orientated with dorsal at the top and anterior to the left.

LOCKED NUCLEIC ACID OLIGONUCLEOTIDE PROBES
Modified nucleic acids that have increased thermal stability relative to DNA or RNA when complexed with complementary DNA or RNA.

DEUTEROSTOME
A major bilaterian subdivision that includes chordates and echinoderms.

PROTOSTOME
One of two major subdivision of bilateria, to which arthropods and molluscs belong.

complementary to the target site[84]. This strategy has allowed a rough mapping of miR-10a and miR-196 functional domains in mouse embryos[83]. The miR-10 sensor indicates that miR-10 activity is present in regions of the posterior thorax and abdomen, but not in the head or tail bud. This is similar to the expression pattern of the adjacent gene, *Hoxb4*, which is consistent with the possibility that they are coordinately regulated[83]. One of the limitations of current 'sensor' studies has been the difficulty of interpreting the 'negative' patterns at cellular resolution.

The primary transcripts of miRNAs are typically much longer than the mature miRNAs[75], and *in situ* hybridization conditions have been developed that allow the detection of these nascent transcripts at their chromosomal sites of transcription. This method was used to detect the *D. melanogaster pri-mir-10* expression pattern in early embryos[85]. The *pri-mir-10* transcripts are detected in a Hox-like pattern that spans the future thoracic and abdominal segments in blastoderm-stage embryos. Another method to detect miRNA expression patterns involves the use of LOCKED-NUCLEIC-ACID OLIGONUCLEOTIDE PROBES[86]. This allowed the *in situ* localization of a large subset of zebrafish mature miRNA transcripts[87], including those of miR-10a, miR-10b and miR-196a. All three of the Hox miRNAs are detected in zebrafish embryos in Hox-like patterns in the developing nerve cord and trunk, with the miR-10a and miR-10b expression borders being anterior to that of miR-196a. A comparison of the three studies that localized miR-10 expression in mouse, zebrafish and *D. melanogaster* embryos indicates that its Hox-like expression pattern in the posterior head, thoracic and abdominal primordia is conserved in many animal embryos[83,85,87].

Why have *mir-10*, and perhaps *mir-196/iab-4*, genes been conserved in Hox clusters since the DEUTEROSTOME–PROTOSTOME divergence? It might be that, like the protein-coding Hox-cluster genes, they rely on regulatory mechanisms such as shared enhancers and long-range Polycomb–Trithorax-mediated repression and activation[88–90]. It has been suggested that miRNAs are derived from inverted duplications of the 3′ UTRs of future target genes[91]. It is possible that this is the case for the Hox miRNAs, especially considering that they might be targeting adjacent Hox-protein-coding genes.

With no mutants specific to the *mir-10*, *mir-196* or *mir-iab-4* genes, it remains to be seen what significance they might have for regulating developmental patterning during bilaterian embryogenesis. It will be exciting to discover the roles of these highly conserved miRNA genes that have been residents of animal Hox clusters for at least 550 million years. It is conceivable that the gain or loss of Hox miRNA target sites, or changes in spatio-temporal expression of Hox-targeting miRNAs could be an important mode of Hox gene regulation in animal development and evolution.

Conclusions

We have discussed recent advances in four areas of Hox regulatory biology. Although a few Hox realizator genes have been identified that illustrate how Hox genes accomplish their function of sculpting variations on a basic segmental shape, many remain to be identified. We think it entirely plausible that the number of known Hox morphological effector genes will expand until almost every gene that can mediate cell adhesion, division, migration and so forth will be found to be directly regulated by Hox proteins in some developmental context.

Recent evidence has revealed a surprising lability in Hox protein functions during evolution, and this lability makes them the best current system for understanding how transcription-factor functions evolve in animals. As we have reviewed here, this lability might be facilitated by their ability to interact with a great range of binding sites within enhancers, either with or without cofactors from the PBC and MEIS families of proteins. From this perspective, the difficulty with coming to a general understanding of how different Hox proteins achieve their functional specificity might simply be due to their basic principles of operation. As Hox proteins operate in so many different cell types and developmental stages, selective pressure might have acted on their functions so that they will observe as few 'rules' as possible, allowing them to fit into nearly all developmental genetic circuits to tweak morphology. To look at this in anthropomorphic terms, it is amazing what the Hox proteins can accomplish when they let the tissue-specific transcription factors get the credit for making muscle, bone, skin and nerve.

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