Shaping segments: *Hox* gene function in the genomic age

Stefanie D. Hueber[1] and Ingrid Lohmann[1,2]*

Summary

Despite decades of research, morphogenesis along the various body axes remains one of the major mysteries in developmental biology. A milestone in the field was the realisation that a set of closely related regulators, called *Hox* genes, specifies the identity of body segments along the anterior–posterior (AP) axis in most animals. *Hox* genes have been highly conserved throughout metazoan evolution and code for homeodomain-containing transcription factors. Thus, they exert their function mainly through activation or repression of downstream genes. However, while much is known about *Hox* gene structure and molecular function, only a few target genes have been identified and studied in detail. Our knowledge of *Hox* downstream genes is therefore far from complete and consequently Hox-controlled morphogenesis is still poorly understood. Genome-wide approaches have facilitated the identification of large numbers of *Hox* downstream genes both in *Drosophila* and vertebrates, and represent a crucial step towards a comprehensive understanding of how *Hox* proteins drive morphological diversification. In this review, we focus on the role of *Hox* genes in shaping segmental morphologies along the AP axis in *Drosophila*, discuss some of the conclusions drawn from analyses of large target gene sets and highlight methods that could be used to gain a more thorough understanding of *Hox* molecular function. In addition, the mechanisms of *Hox* target gene regulation are considered with special emphasis on recent findings and their implications for *Hox* protein specificity in the context of the whole organism. *BioEssays* 30:965–979, 2008. © 2008 Wiley Periodicals, Inc.

Introduction

All bilateral animals possess a common genetic mechanism regulating development along the AP axis,[1,2] and *Hox* proteins are among the key regulators in specifying morphological diversity along this axis[3–6] (Fig. 1). In all animals studied, *Hox* genes are expressed in defined and often overlapping domains along the AP axis, and it is their activity that assigns distinct morphologies to the various body segments.[3,5] This becomes most evident when *Hox* gene function is disrupted, which frequently results in "homeotic transformations".[6,7] The term "homeotic transformation", defined by Bateson in 1894,[8] is used to describe the transformation of one structure to resemble, in form and shape, a homologous structure present in the body. For example, in *Drosophila* mutations in the *Hox* gene, *Ultrabithorax* (*Ubx*) result in the development of an additional pair of wings instead of halteres, two small balancing organs, giving rise to the famous four-winged fly, discovered by Ed Lewis.[6] Although first observed in *Drosophila*, homeotic transformations are found in many other organisms,[9,10] which led to the assumption that *Hox* proteins act as master regulators of morphogenesis. However, mutations in *Hox* genes do not always result in such dramatic phenotypes—they can also cause very subtle defects, as frequently observed in organisms with multiple *Hox* clusters (e.g. vertebrates) due to the overlapping expression and functional redundancy of paralogous *Hox* genes of different clusters.[11] In these cases, major morphological changes are only observed when paralogous *Hox* genes are simultaneously mutated. But even in organisms with a single *Hox* cluster, as in *Drosophila*, homeotic transformations are primarily observed after mutations in those *Hox* genes that either have overlapping expression domains or are engaged in a negative cross-regulation with other *Hox* genes.[5,12] Loss of function of one *Hox* gene allows the overlapping or ectopically expressed *Hox* gene to exert its function, which results in the transformation of one segment identity towards the identity of neighbouring segments.[5,13] This implies that homeotic transformations are actually not very informative with regards to the function of the mutant *Hox* gene, but rather provide insights about the function of nearby or overlapping *Hox* genes.[5] Since it is mostly the more posterior *Hox* protein repressing the expression of a more anterior one, this phenomenon was termed posterior suppression.[14,15] When no "backup" *Hox* gene is present, the functional elimination of a *Hox* gene does not result in homeotic transformation, but in structural deficiencies,[5,16] as observed for many other mutations.

On the molecular level, *Hox* genes encode proteins with a highly conserved 60-amino-acid DNA-binding motif, the homeodomain,[17–19] and function as transcription factors by directly binding to DNA sequences in *Hox* response elements (HREs)[20,21] (Fig. 2). Thus, it seems obvious that *Hox* proteins

[1]Department of Molecular Biology, AG I. Lohmann, MPI for Developmental Biology, Tübingen, Germany.
[2]BIOQUANT Center, NWG Developmental Biology, University of Heidelberg, Germany.
*Correspondence to: Ingrid Lohmann, BIOQUANT Zentrum, BQ 0032 NWG Developmental Biology, Im Neuenheimer Feld 267, 69120 Heidelberg. E-mail: ilohmann@flydev.org
DOI 10.1002/bies.20823
Published online in Wiley InterScience (www.interscience.wiley.com).

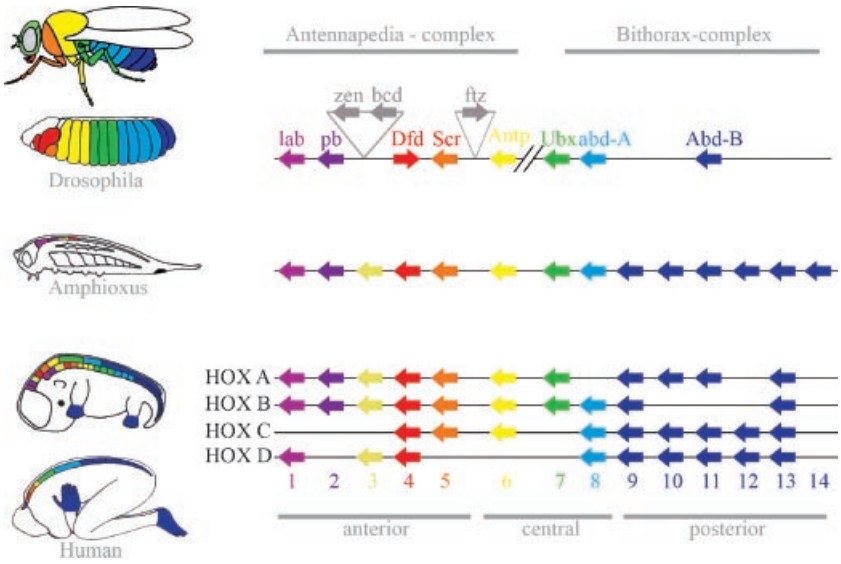

Figure 1. *Hox* expression and genomic organization in different organisms. **Left panel:** Schematic representations of different organisms, the fruit fly (*Drosophila melanogaster*, adult fly and a stage 13 embryo), amphioxus (*Branchiostoma belcheri*), mouse (*Mus musculus*) and human (*Homo sapiens*; adult and embryonic stage) are shown, with approximate RNA expression domains of most *Hox* genes. Colour-code as in the *Hox* cluster diagrams. **Right panel:** A scheme of the *Hox* gene clusters (not to scale) in the genomes of the species shown on the left panel. Genes are coloured to differentiate between *Hox* family members, orthologous genes are labelled in the same colour. Genes are shown in the order in which they are found on the chromosomes, the positions of three non-*Hox* homeodomain genes, *zen, bcd* and *ftz*, within the cluster of the fly genome are shown in grey. Gene abbreviations are: *lab, labial; pb, proboscipedia; Dfd, Deformed; Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; abd-A, abdominal-A; Abd-B, Abdominal-B; zen, zerknüllt; bcd, bicoid; ftz, fushi-tarazu*.

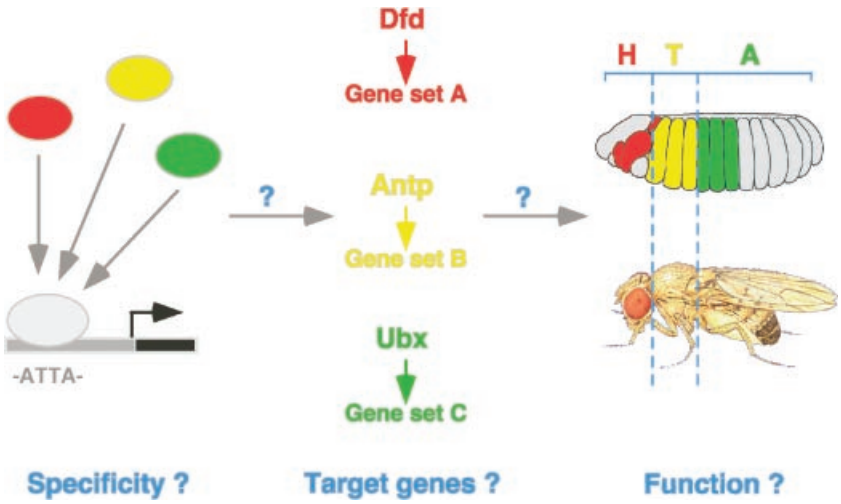

Figure 2. Schematic illustration of the "*Hox* paradox". Since *Hox* genes encode transcription factors, it seems obvious that they control different morphological outputs along the AP axis by controlling distinct sets of downstream genes. Thus, the key to understanding *Hox* function is to identify these genes and analyze their role during development. However, there are many problems: in vitro *Hox* proteins have rather unspecific DNA-binding specificities, thus they all bind to very similar and frequently occurring DNA core sequences. Therefore, it has been unclear for a long time which specific sets of downstream genes they regulate and how they can do so. The unspecific DNA-binding preference is also one of the major reasons why so few *Hox* downstream genes have been identified and functionally analyzed in the pre-genomic age. Consequently, many aspects of the morphogenetic function of *Hox* proteins are still not understood.

drive the morphological diversification of body segments by differentially controlling the expression of downstream genes (Fig. 2). While this is a straightforward assumption, there is one major problem: all *Hox* proteins display a poor in vitro DNA-binding specificity and recognize highly similar nucleotide sequences containing an -ATTA- core[22–26] (Fig. 2). In contrast, *Hox* proteins have very specific effects in vivo, and different *Hox* transcription factors target diverse sets of downstream genes.[27] Furthermore, even a single *Hox* protein is able to regulate different sets of downstream genes depending on tissue context or developmental stage, and some downstream genes are activated in one context and repressed in another.[27–29] The ambiguous nature of DNA binding by *Hox* proteins, along with the complexity of the biological processes controlled by them has hampered the identification of *Hox* target genes, despite the use of a wide range of strategies.[30–32] Only recently it has become feasible to quantitatively identify novel *Hox* downstream by genome-wide approaches.[27,33–35]

In this article, we will first summarize what was known about *Hox* downstream genes and mechanisms of *Hox* target gene regulation in the pre-genomic age, next describe recent progress in these two fields using genome-wide approaches and, finally, discuss how these recent findings have influenced our views of how *Hox* proteins exert their fundamental role in the morphological diversification of segments along the AP axis in vivo. Given the enormous amount of published data on these topics, this review cannot be exhaustive. Therefore, we focus on what is known in *Drosophila* and only include selected data from other organisms.

Hox genes—the pre-genomic era

Although *Hox* proteins have highly complex roles in specifying segment identities along the AP axis in animals, one can simplify their activities by focussing on their molecular nature. As transcription factors, *Hox* proteins control morphogenesis by regulation of distinct sets of target genes (Fig. 2). Thus, the key to understanding *Hox* function is to identify these genes and analyze their role during development.

Identification of Hox downstream genes

Before the advent of large-scale techniques, a diverse repertoire of approaches was used to identify *Hox* downstream genes[30–32] (Table 1). Initial attempts focused on screens in heterologous systems, like yeast one-hybrid assays performed to identify regulatory elements mediating *Hox* responses.[36] However, these approaches showed limited success, since only very few *Hox* target genes could be identified. We now know that the most likely reason for this limitation lies in the fact that *Hox* proteins acquire DNA-binding specificity and thus specificity in target gene selection through interactions with additional DNA-binding proteins in vivo.[37–40] Thus, it is not surprising that most *Hox* downstream genes were initially identified by candidate gene approaches based on homeotic responses of transcript or enhancer trap patterns[3]. These findings clearly highlight the power of in vivo strategies for the identification of *Hox* target genes. For example, two well-characterized *Hox* targets identified in enhancer trap screens are *decapentaplegic* (*dpp*),[43] a gene member of the TGF-β family of signaling proteins, and the homeobox transcription factor gene *Distal-less* (*Dll*).[44] Although very powerful in identifying *Hox* downstream genes, these approaches did not allow a discrimination of direct and indirect targets without additional and tedious experimentation. This knowledge, however, was regarded as essential, since direct targets could be used not only to explore *Hox* function, but also to elucidate the mechanisms of *Hox* target gene selection and regulation. In this context, chromatin immunoprecipitation (ChIP) was developed, which has become one of the most powerful tools for the study of protein–DNA interactions in vivo. Before genomic arrays or massively parallel sequencing technologies became available, immunoprecipitation of genomic DNA fragments associated with *Hox* proteins in vivo was used to clone transcription units in their vicinity. In addition to isolating targets, this procedure had the added advantage that HREs in the regulatory regions of these target genes were identified. Some of the direct *Hox* targets identified using this technique are *scabrous* (*sca*), *Transcript 48* (*T48*) and *centrosomin* (*cnn*), which are under direct control of Ubx.[45–47] Taken together, all these methods resulted in the identification of 24 direct *Hox* downstream genes in *Drosophila* (Table 1), which then served as models to study *Hox* gene output.

Nature and function of Hox downstream genes: transcription factors—realisators—regulatory networks

In 1975, Antonio Garcia-Bellido established a concept in which he proposed a hierarchy of three classes of genes, activators, selectors and realisators to be responsible for cell differentiation in development. One key element of his proposal was that, once activated in their appropriate territories by the activator genes, the homeotic (*Hox*) selector genes would select a large number of subordinate targets, the realisator genes, that would directly influence the morphology of segments by regulating cytodifferentiation processes.[48] Based on this idea, it had been expected that realisator genes would constitute a large fraction of *Hox* target genes. However, examination of the few *Hox* downstream genes known at that time showed that many of them encoded regulatory molecules, mostly transcription factors, like Distal-less (Dll), Forkhead (Fkh) and Teashirt (Tsh), and some signalling proteins, like Decapentaplegic (Dpp), Wingless (Wg) and Scabrous (Sca)[3] (Table 1). These molecules act as high-level executives and very often function themselves to select the activity of a large number of downstream genes.

Table 1. Direct Hox target genes identified in the pre-genomic age in *Drosophila* (adapted from Pearson et al., 2005)

Target gene	Regulated by	Function	Target class	Validation	References
1.28	Dfd	unknown	unknown	reporter lines	Pederson et al., 2000[118]
Antennapedia	Antp, Ubx, Abd-A	homeobox TF; thorax development	transcription factor	reporter lines	Appel and Sakonju,1993[51]
apterous	Antp	homeobox TF; muscle identity	transcription factor	reporter lines	Capovilla et al., 2001[111]
CG11339	Lab	actin binding protein	realisator	reporter lines	Ebner et al., 2005[41]
CG13222	Ubx	cuticle protein	realisator	EMSA	Hersh et al., 2007[93]
centrosomin	Antp	centrosomal protein; PNS and CNS development	realisator	ChIP	Heuer et al., 1995[47]
connectin	Ubx, Abd-A	GPI linked cell surface protein; neuromuscular connection	realisator	ChIP	Gould and White, 1992[54]
decapentaplegic	Ubx, Abd-A	Tgf-ß protein; D/V polarity, midgut morphogenesis	signaling molecule	reporter lines	Capovilla et al., 1994[86]
Deformed	Dfd	homeobox TF; head development	transcription factor	reporter lines	Zeng et. al 1994[50]
Distal-less	Ubx, Abd-A	homeobox TF; limb development	transcription factor	reporter lines	Vachon et el., 1992[40]
empty spiracles	Abd-B	homeobox TF; head development, filzkörper specification	transcription factor	reporter lines	Jones and McGinnis, 1993[115]
forkhead	Scr	forkhead domain TF; specification of the terminal region	transcription factor	reporter lines	Ryoo and Mann, 1999[78]; Zhou et al., 2001[121]
knot	Ubx	EBF/Olf1 TF; development of wing imaginal disc	transcription factor	reporter lines	Hersh and Carroll, 2005[122]
labial	Lab	homeobox TF; head development	transcription factor	reporter lines	Grieder et al., 1997[114]
La-related protein	Scr, Ubx	autophagic cell death	realisator	ChIP	Chauvet et al., 2000[112]
reaper	Dfd	apoptosis activator	realisator	reporter lines	Lohmann et al., 2002[52]
serpent	Ubx	Zn finger TF	transcription factor	One-hybrid assay	Mastick, 1995[36]
scabrous	Ubx	secreted signal transducer; eye morphogenesis; CNS and PNS development	signaling molecule	ChIP	Graba et al., 1992[45]
spalt major	Ubx	Zn finger TF; development of wing disc	transcription factor	reporter lines	Galant et al., 2002[83]
Transcript 48	Ubx	transmembrane protein	unknown	ChIP	Strutt and White, 1994[46]
teashirt	Antp, Ubx	Zn finger TF; specification of trunk identity	transcription factor	reporter lines	McCormick et al., 1995[90]
ß-3-tubulin	Ubx	cytoskeletal protein; visceral mesoderm differentiation	realisator	reporter lines	Hinz et al., 1992[53]; Kremser et al., 1999[116]
wingless	Abd-A	Wnt signal transducer; midgut morphogenesis	signaling molecule	reporter lines	Grienenberger et al., 2003[28]
Wnt-4	Ubx	Wnt protein	signaling molecule	ChIP	Graba et al., 1995[113]

Consequently, mutations in these genes resulted in major morphological and patterning defects, and sometimes even in homeotic transformations similar to the ones observed in *Hox* mutants. This is surely one of the reasons why initially transcription factors were preferentially identified as *Hox* targets (in genetic screens). One well-studied example of *Hox* target gene coding for a transcription factor is *Dll*, which is required for appendage formation in ventral regions of *Drosophila* embryos.[44] The *Hox* proteins Ubx, Abdominal-A (Abd-A) and Abdominal-B (Abd-B) repress *Dll* expression, resulting in the absence of limbs in the abdomen[44] (Fig. 3A), whereas *Hox* transcription factors expressed in the head and thorax preferentially do not repress *Dll* transcription (Fig. 3A). This allows the formation of appendages, while the precise spatial context dictates which kind of appendage will develop[49] (Fig. 3A). When *Dll* is co-expressed with *homothorax* (*hth*), another homeodomain containing transcription factor gene under the control of *Hox* proteins,[49] antennae are formed, whereas cell-specific expression of *Dll* (in the absence of *hth* expression) results in the formation of legs[49] (Fig. 3A). Other interesting examples of *Hox* target genes coding for transcription factors are the *Hox* genes themselves. Although there are many examples, we would like to focus on two that have been understood at the molecular level. First, Deformed (Dfd), a head-specific *Hox* protein, is known to maintain its own expression in the maxillary and mandibular segments by interacting with specific binding sites in Dfd autoregulatory enhancer elements.[50] Second, *Antennapedia* (*Antp*), a *Hox* gene primarily expressed in thoracic segments, has been shown to be directly regulated by three different *Hox* proteins, Antp, Ubx and Abd-A:[51] Antp positively autoregulates its own expression in neuronal cells of the thorax by binding to specific DNA sites in a P2-specific enhancer, whereas Ubx and Abd-A prevent this autoregulation in abdominal neuronal cells by competitively interacting with the same sites. If this cross-regulation of *Hox* genes fails in *Hox* mutants, *Hox* proteins are

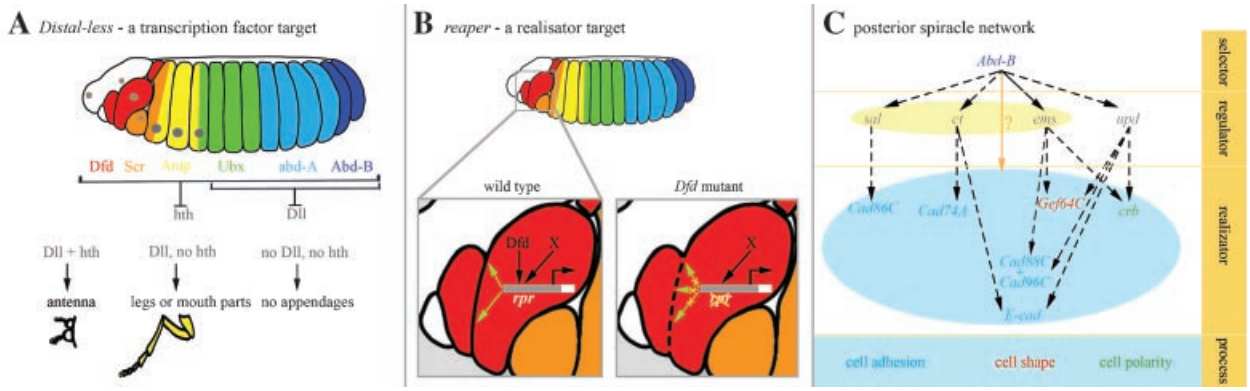

Figure 3. Examples of *Hox* target gene regulation on the executive, realisator and network level. **A:** The *Hox* target gene *Distal-less* (*Dll*), required for appendage formation, encodes a homeobox transcription factor and is one of the high-level executives regulated by *Hox* proteins. The area of *Dll* expression in the embryo (shown as grey patches) corresponds to sites where in the course of development imaginal discs and, subsequently, appendages are formed. This is due to the inability of *Hox* transcription factors expressed in the head and thorax to repress *Dll* expression. In the abdomen, where no appendages are formed, the *Hox* transcription factors Ubx, Abd-A and Abd-B repress *Dll* transcription. **B:** The apoptosis gene *reaper* (*rpr*) is an example of a *Hox* realisator gene target. In wild-type embryos, Deformed (Dfd) directly activates the apoptotic gene *rpr*, leading to localized cell death (marked by green arrows), which sculpts a 'groove' between two head structures, the mandibular and maxillary segments (marked in red). In *Dfd* mutants, *rpr* is not activated, thus localized cell death is not induced, resulting in the fusion of the mandibular and maxillary head segments (indicated by dashed line). **C:** The selector protein Abd-B, supposedly through the interaction with cofactors, activates the spatially and temporarily restricted expression of four "intermediate" regulator genes, *spalt* (*sal*), *cut* (*ct*), *empty spiracles* (*ems*) and *unpaired* (*upd*). Subsequently, their gene products (potentially in combination with Abd-B) control (directly or indirectly) the local expression of a battery of realisator genes, which confer unique properties to cells by influencing morphogenetic processes like cell adhesion, cell polarity or organization of the cytoskeleton. This ultimately leads to the proper morphogenesis of the posterior spiracle. Figure adapted from Lovegrove B, Somoes S, Rivas ML, Sotillos S, Johnson K et al. 2006 Curr Biol 16:2206–2216.

expressed outside their normal expression domains, which in turn results in homeotic transformations.[5]

Only very few *Hox* target genes initially identified coded for realisator genes, which was rather unexpected and suggested that *Hox* proteins exert their function primarily through the regulation of other high-executive genes. However, analysis of the few known realisator genes has been instrumental for understanding the morphogenetic function of *Hox* proteins.[52–56] For example, one of the best-studied *Hox* realisator genes in *Drosophila* is the apoptosis inducing gene *reaper* (*rpr*). *rpr* is expressed in a small number of cells in the anterior part of the maxillary segment in *Drosophila* embryos, and is directly controlled by the *Hox* protein Dfd[52] (Fig. 3B). In *Dfd* mutant embryos, *rpr* expression in the maxillary segment is abolished, which results in a loss of the boundary between the maxillary and mandibular segments (Fig. 3B). When *rpr* expression is restored in *Dfd* mutants, the segment boundary is maintained, showing that the Dfd-dependent activation of *rpr* and, consequently, the local activation of apoptosis is necessary and sufficient for the maintenance of the maxillary–mandibular segment boundary.[52] Looking at this example, it becomes clearer why the identification of realisator genes among the *Hox* targets has been so difficult. Realisator proteins are required for general functions (cell adhesion, cell proliferation, cell death etc.) in many cells at many different developmental stages. Consequently, mutations in realisator genes either result in early embryonic lethality or in pleiotropic effects. Alternatively, realisators very often act redundantly or have very subtle and context-dependent effects, like in the case of *rpr*. These complications make it difficult to correlate their mutant phenotypes to those found in *Hox* mutants. And here lies another problem: although mutations in *Hox* genes have been analyzed for decades, their phenotypic analysis is far from complete and many of the subtle morphological changes in *Hox* mutants may have gone unnoticed. Knowledge of these phenotypes, however, is a prerequisite to correlate *Hox*-dependent morphological output with the activity of downstream gene(s). Taken together, several lessons can be learned. First, although it was easier to identify and study transcription factors and signalling molecules as *Hox* target genes, per se these two classes of *Hox* target genes are not so informative in elucidating the role of *Hox* proteins in the specification of morphological properties on a cellular level (but more so in understanding the patterning properties of *Hox* proteins). Second, in order to gain an in-depth understanding of Hox-dependent morphogenesis, it is essential to study the function of *Hox* realisator genes irrespective of whether they are direct or indirect *Hox* targets. Third, since many realisator genes will be indirectly regulated by several executive *Hox* target genes, we need to elucidate

the nature of these Hox-modulated regulatory networks to understand how *Hox* genes control morphogenesis.

One such regulatory network, which is fairly well understood in *Drosophila*, is the posterior spiracle network (Fig. 3C). This network is activated in the abdominal segment 8 (A8) by *Abdominal-B* (*Abd-B*), the *Hox* gene specifying the morphology of the posterior region in *Drosophila* embryos.[57] Activation of the posterior spiracle network results in the formation of an ectodermal structure composed of the spiracular chamber, a tube connecting the trachea to the exterior, and the stigmatophore representing the external protrusion, in which the spiracular chamber is located.[57] The formation of these structures is dependent on the activation of four primary Abd-B target genes, the transcription factors *cut* (*ct*), *empty spiracles* (*ems*) and *spalt* (*sal*), and the signal transduction ligand of the JAK/STAT pathway *unpaired* (*upd*). The partially overlapping activity of the four primary Abd-B targets subsequently activates (potentially in combination with Abd-B) different sets of realisator genes in particular subsets of spiracle cells[58] (Fig. 3C). The targets include cell adhesion molecules, like E-cadherin or non-classical cadherins, like Cad86C or Cad74A, the cell-polarity protein Crumbs (Crb), and two regulators of the actin-cytoskeleton organization, the Rho GTPases RhoGAP88C and Gef64C[58] (Fig. 3C). Although not yet understood at the cellular level, it is thought that the region-specific expression of these and probably numerous other realisators confers unique morphogenetic properties to the cells, which ultimately lead to the formation of a segment-specific organ, the posterior spiracle.

The development of the posterior spiracle is one example for the complexity of Hox-modulated regulatory networks and illustrates that *Hox* proteins are able to regulate, directly and indirectly, many levels of such a network. Thus, in order to fully understand all functions of *Hox* proteins, it is necessary to elucidate these regulatory networks, which includes a complete knowledge of all direct and indirect *Hox* downstream genes.

Specificity in Hox target gene regulation: the Hox paradox

The functional analysis of direct *Hox* target genes, which inevitably includes the identification and characterization of regulatory sequences directly mediating the homeotic response, the so-called *Hox* response elements (HREs), has been extremely difficult. The limited success in identifying in vivo relevant HREs can be primarily attributed to an important intrinsic property of *Hox* proteins: a poor specificity in sequence recognition and binding exhibited by *Hox* proteins in vitro. Why is that? Why do *Hox* proteins (at least when present as monomers) recognize very similar and rather unspecific DNA sequences? The answer lies in the DNA-binding domain of *Hox* proteins, the homeodomain, which is, including its functionality, greatly conserved over large evolutionary distances.[59–61] This high conservation is reflected in an almost identical three-dimensional structure of the homeodomain in all *Hox* proteins studied so far.[60] As a consequence of this almost invariant molecular structure, the majority of Hox proteins, including the paralogues within one species, preferentially recognize a conserved, but fairly unspecific, -ATTA- core motif.[26] This low DNA-binding specificity, however, sharply contrasts with the highly specific effects *Hox* transcription factors exert on distinct and different sets of target genes in vivo.[27] Another dimension of *Hox* transcriptional specificity is reflected in their ability to act both as transcriptional repressors and activators and to regulate their target genes in highly specific spatial and temporal patterns in the animal, despite their rather large domains of expression.[27–29] This paradox of high in vivo (functional) but low in vitro (binding) specificity has raised the fundamental question: how do *Hox* proteins achieve regulation of selected target genes and what are the molecular mechanisms allowing *Hox* proteins to achieve their high developmental specificity in vivo?

The most-likely explanation is that other factors influence the functional specificity of *Hox* proteins. Initial support for this hypothesis came from studies that tested the effects of chimeric *Hox* proteins in vivo in order to identify functional domains within the *Hox* proteins.[15,62–65] All these studies suggested that multiple domains within any given *Hox* protein are essential for in vivo specificity. Based on these findings, the idea emerged that *Hox* proteins would heterodimerize with many other factors, so-called cofactors, which would subsequently enhance their sequence selectivity and binding specificity. This "cooperative/co-selective binding model" seemed the most-plausible mechanism used by *Hox* proteins (Fig. 4), since the cooperative binding of the yeast homeodomain transcription factors a1 and α2 had only been discovered a couple of years before.[66] Both transcription factors, a1 and α2, bind poorly to DNA alone, but specificity of DNA binding is greatly enhanced when both factors form a complex via protein–protein interactions. Therefore, it is not surprising that a hunt for factors began that were able to influence the binding behaviour of *Hox* proteins in a similar manner.[4,49,40,42,67] The resolution to the *Hox* pardox seemed close, when it was demonstrated that one candidate, the homeodomain protein Extradenticle (Exd), which was shown to directly interact with *Hox* proteins via a conserved YPWM hexapeptide motif,[68] was able to cooperatively bind DNA with many *Hox* proteins, thereby selectively increasing Hox-binding affinity on specific DNA sites.[69–71] In the following years, much research focused on Exd, not only because of its important role in *Hox* target gene regulation, but also because of major difficulties (despite large efforts) in identifying additional *Hox* cofactors of the Exd-type. The results showed that *Hox* target gene regulation is amazingly diverse and complex even when only a single *Hox* cofactor is considered. For example, the activity of Exd is regulated at the level of its

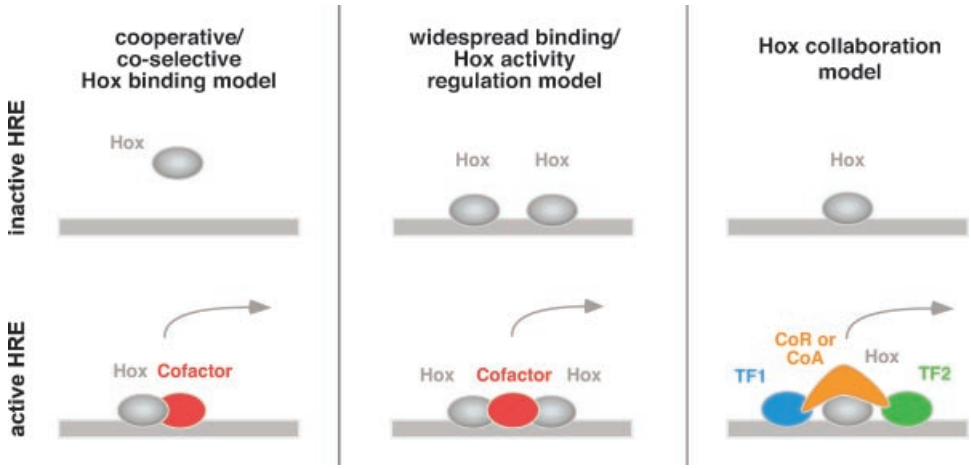

Figure 4. Three models of *Hox* target gene regulation in vivo. In the cooperative/co-selective binding model, *Hox* proteins cannot significantly occupy any HRE without the aid of cooperative interactions with cofactors (like Exd). Only in those cells co-expressing the *Hox* protein and the cofactor specific interactions occur, allowing *Hox* proteins to recognize and occupy individual HREs with increased binding specificity, and thus enabling them to activate or repress target genes accordingly. In the widespread binding/activity regulation model, *Hox* proteins bind many HREs in the absence of cofactors, but are unable to change target gene expression under these conditions. In the presence of cofactors, the activity of *Hox* proteins is changed, thus allowing them to activate or repress specific target genes. In the collaboration model, *Hox* proteins and many other context-specific transcription factors independently bind to HREs. The sign of the transcriptional output is determined by the combination of bound factors and the recruitment of specific co-repressors and/or co-activators. TF, transcription factor; CoA, co-activator; CoR, co-repressor.

subcellular localization, since Exd requires direct interaction with another homeodomain protein encoded by the gene *homothorax* (*hth*) for its nuclear translocation.[72] Once in the nucleus, Exd acts as a *Hox* cofactor by several means.

Detailed analysis of several *Hox* response elements led to the conclusion that Exd, as predicted by the "cooperative/co-selective binding model"[25] (Fig. 4), helps *Hox* proteins achieve DNA-binding specificity.[69,73–75] A couple of studies have meanwhile underlined the significance of this model in vivo.[76–78] For example, it has been shown that a 37 bp HRE from the *forkhead* (*fkh*) gene, a direct target of the *Hox* protein Scr, is not only cooperatively bound by Exd and Scr in vitro, but that activation of this element in vivo requires both genes, *Scr* and *exd*, and that other *Hox*–Exd heterodimers do not exert these specific in vitro and in vivo effects.[78] When two base pairs within this element were mutated, the element was bound by different *Hox*/Exd heterodimers with almost the same affinities in vitro and was specifically regulated by different *Hox* proteins in vivo. Structural analysis of both *Hox*–Exd–DNA ternary complexes, containing either the natural occurring *Hox*–Exd consensus sites or the mutated versions, now elegantly revealed a potential mechanism used by *Hox* proteins to select specific binding sequences in vivo: it was well established that *Hox* proteins recognize generic Hox-binding sites through major groove-recognition helix interactions,[60,61] but the structural analysis of both Scr–Exd–DNA complexes showed that selection among sites is critically dependent on minor groove interactions determined by two positively charged amino acid residues located in the N-terminal arm and linker region of the *Hox* protein Scr,[79] These residues, which are only correctly positioned through interaction with Exd, recognize the structure of the minor groove in a sequence-specific fashion.[79] Interestingly, the Scr-dependent regulation of *fkh* highlights another level of complexity in *Hox* target gene regulation: Scr and Exd are able to regulate the *fkh* HRE only during early stages of embryogenesis, since Scr negatively regulates *hth* expression and thus nuclear translocation of Exd later in development.[78] Thus, *Hox* proteins are also able to regulate their own activity (on specific enhancers) by regulating the availability of their cofactors. Finally, Hth does not only promote Exd's nuclear translocation, but it also acts as a *Hox* cofactor itself by increasing the DNA-binding specificities and/or affinities of *Hox*–Exd complexes through direct protein–protein interaction.[40,41,70,77]

Alternative to the assembly of large protein complexes on HREs, recent findings on *Hox*–Exd interactions indicate that only two protein partners, such as Exd and *Hox* proteins, are sufficient to generate specificity in target sequence recognition simply by interacting via different protein domains. Although *Hox*–Exd interactions for a long time were thought to be mediated primarily by the short hexapeptide N-terminal to the homeodomain.[68,80,81] newer evidence suggests that the physical interactions of both proteins are more complex than anticipated. For example, it has been shown for the *Hox* protein Ubx that the unrelated UbdA motif, located C-terminal

to the homeodoamin, mediates Exd recruitment, and that this interaction is essential for repression of the Ubx target gene *Dll in vivo*.[82] In contrast, it is well established that Ubx–Exd interaction via the hexapeptide motif is important for the specification of other Ubx-dependent segmental features.[14,83] Thus, specificity in *Hox* target gene recognition is (at least in some cases) achieved by at least two distinct interactions of *Hox* proteins with a single cofactor, Exd. This presumably allows the Hox–Exd complex to adopt different conformations, which can in turn recognize different target sequences. An open question in this context remains how the different Hox–Exd interactions are regulated in vivo. In addition, other *Hox* cofactors of the Exd type have been identified in recent years, including Teashirt and Disconnected.[67,84] However, the cooperative interaction of these factors with *Hox* proteins and the selective enhancement of Hox-binding specificities have remained unclear.

While work on the ''cooperative/co-selective binding model'' focused on the regulation of Hox–DNA interactions, much less progress has been made to elucidate how the transcriptional activity of *Hox* proteins bound to DNA is modulated in vivo. According to the ''widespread binding/activity regulation model'' cofactors, such as Exd, function to convert *Hox* proteins, which are bound to a very large number of Hox-binding sites in vivo, from a neutral to an active state capable of transcriptional activation or repression[25] (Fig. 4). This ''widespread binding/activity regulation model'' has three major implications: first, *Hox* proteins should be able to bind many genes in the genome, which has been confirmed for homeodomain-containing transcription factors by in vivo cross-linking experiments.[85] Second, *Hox* proteins should be able to bind to DNA independently of Exd. And consistent with this assumption, some naturally occurring Hox-dependent enhancers contain functionally important high-affinity Hox-binding sites that are not closely juxtaposed to high-affinity Exd sites.[50,86,87] In addition, it has only been shown recently that, for some enhancers, even binding of a Hox–Exd complex alone is not sufficient for target gene regulation.[40] Third, the ''widespread binding/activity regulation model'' implies that Exd should be able to switch *Hox* proteins into both transcriptional activators and repressors. Although Exd is able to change a *Hox* protein from a transcriptional repressor into a transcriptional activator (at least in one case), probably by masking a repressor domain contained in some *Hox* proteins,[88] a switch from activator into repressor has, to our knowledge, never been shown. Thus it has been postulated that the sign of transcriptional effect is not primarily determined by the Hox–Exd interaction, but due to the recruitment of additional factors into the Hox–cofactor complex. Interestingly, two such factors have been identified recently: in addition to the binding of a Hox–Exd–Hth complex, which is itself not sufficient for target gene regulation, two segmentation proteins, Engrailed (En) and Sloppy paired 1 (Slp1), and their sequence-specific recruitment have been shown to be required for the repression of the *Hox* target gene *Dll*.[40] Since En and Slp1 harbour motifs for interaction with the co-repressor Groucho, it is assumed that both proteins (through the recruitment of Groucho) act as intermediate regulatory molecules determining the sign of the transcriptional *Hox* output (in this case, repression of *Dll* transcription). In this scenario, the Hox–Exd–Hth complex serves to select the correct Hox-binding site(s) without regulating target gene expression, whereas the regulatory activity of the *Hox* protein is dependent on the surrounding binding sites and the activity of the factors interacting with these sites.

The finding that transcription factors with very-well-known functions in development (in the case of En and Slp1, the generation of anterior and posterior compartments in segments) work directly with *Hox* proteins in regulating their target genes was not completely unanticipated, since it had been realised before that other transcription factors and conserved sequences surrounding Hox-binding sites are important for Hox-dependent transcriptional control.[28,50,89,90] However, it seems that, in these days, the time was not right for the idea that *Hox* protein function and activity does not only dependent on specific Hox-cofactor interactions, but also (and perhaps more so) on the combinatorial interaction with other transcription factors, allowing *Hox* proteins to mediate context-specific activation or repression of target genes (''*Hox* collaboration model'') (Fig. 4). Evidence for the general importance of this new concept was provided only recently, when it was shown that the *Hox* protein Ubx collaborates with two transcription factors downstream of the Dpp/TGF-β pathway, Mothers against Dpp (Mad) and Medea (Med), to repress the *Hox* target gene spalt major (*sal*) in the haltere.[42] In addition, this study showed that cooperative interaction of *Hox* proteins with other regulatory factors is not required to modulate *Hox* target gene selection. On the contrary, the repression of *sal*, which does not require Exd and Hth activity, is mediated by the independent binding of Ubx and the collaborating transcription factors Mad and Med to the *sal* HRE.[42] And again, as in the case of En and Slp1, Mad and Med do not themselves function as transcriptional repressors, but they determine the regulatory activity of the *Hox* protein through recruitment of the co-repressor Schnurri (Shn), which was shown to be necessary for *sal* repression.[42] Taken together, these findings have revolutionized our picture of *Hox* target gene regulation: previously, much attention has focused on cofactors of the Exd- and Hth-type and their control of *Hox* DNA-binding selectivity via cooperative interactions with *Hox* proteins. However, *Hox* proteins (positively and negatively) regulate in a context-dependent manner a large diversity of target genes that are also regulated by other transcription factors. Thus, it has been argued that it would be too great a constraint to require that *Hox* proteins physically interact with

the large and diverse repertoire of transcription factors with which they act. In the light of recent findings, it seems more plausible that, even in the absence of any direct physical interaction, *Hox* proteins work together with many other transcription factors in a combinatorial fashion through a selective recruitment of all regulatory factors to target-specific and nearby binding sites in HREs, a transcriptional control mechanism meanwhile termed collaboration.[42] Since all of the collaborating transcription factors identified so far (En, Slp1, Mad and Med) harbour motifs for interactions with co-repressors/co-activators, it seems quite attractive to assume that the different transcriptional inputs from *Hox* proteins and collaborators are integrated and mediated to the transcription machinery via the recruitment and assembly of different co-repressor and/or co-activator complexes. In addition, the collaboration model offers a very simple, but nonetheless elegant explanation to the mystery how *Hox* proteins can act as repressors in one context and as activators in another: it seems very likely that *Hox* proteins primarily function as placeholders in HREs, but that the sign of *Hox* action (and thus the transcriptional output) is mainly dictated by the regulatory activity of all collaborating transcription factors assembled on these HREs. Finally, we would like to take the collaboration model to the next level: we propose that, in principle, every transcription factor could act as *Hox* collaborator. Since every cell has a unique combination of transcription factors, the combinatorial interactions for the broadly expressed *Hox* proteins would be almost limitless in such a scenario. This would allow for a very precise modulation and fine-tuning of *Hox* target gene regulation, even on the level of the individual cell, eventually leading to the amazing functional diversity that *Hox* proteins achieve in development and evolution. Thus, previously identified tissue-specific transcription factors or sequence elements shown to be necessary for the regulation of Hox target genes[28,67,84,89–91] could represent, in our view, additional collaborators of *Hox* proteins or their respective binding sites in HREs.

Taken together, many aspects of *Hox* function and *Hox* target gene regulation were understood in much detail in the pre-genomic age. However, the fact that only few *Hox* target genes were known severely limited our ability to assess the relative contribution of the various modes of *Hox* action during development of the entire organism. The focus on a few selected target genes and HREs implicates that we could be dealing with the exceptions rather than the most widely used mechanisms for *Hox* target gene regulation. In addition, the small number of known target genes made it impossible to infer how *Hox* proteins carry out their morphogenetic function in vivo. Therefore, to draw more general conclusions about *Hox* target recognition and regulation, as well as Hox target function, a genome-wide inventory of *Hox* targets and HREs is required.

Large-scale analysis of *Hox* downstream genes and regulation: the genomic era

With the advent of genome-wide approaches in the last decade, we are now in a position to overcome some of the limitations outlined above and characterize *Hox* downstream genes and HREs on a large-scale to more fully understand all aspects and mechanisms of *Hox* function. Again, we will focus our review on findings made in *Drosophila*.

Large-scale identification of Hox downstream genes: microarray expression profiling

With the introduction of DNA microarray technology, transcript profiling was used to systematically detect genes that showed differential expression in response to *Hox* proteins. Though this method is extremely powerful, additional methods are required to distinguish between direct and indirect targets. DNA microarray technology has been used quite extensively in vertebrates (summarized in Table 2), while only few groups have used it for the identification of *Hox* downstream genes in *Drosophila* (summarized in Table 2). Recent papers report genes regulated by the *Hox* genes *Dfd*, *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* in the embryo,[27] as well as downstream genes of Ubx in developing wing and haltere imaginal discs.[92,93] What can be learned from these studies? Which concepts established previously were confirmed, which ones have to be newly defined?

First of all, both embryo and imaginal studies show that *Hox* genes regulate a large number of downstream genes despite the fact that only very tightly defined developmental stages were analyzed (Table 2). Since *Hox* genes are required throughout development, it follows that they very likely regulate thousands of genes during a fly's life. Although at first glance this might be a surprising finding, it is not a new one. Already in 1998, Liang and colleagues[85] have characterized the expression of randomly selected genes at different stages of *Drosophila* embryogenesis, and their results suggested that selector homeoproteins, including *Hox* proteins, regulate the expression of most genes throughout development,[85] a view now supported by genomic data. Is this also true for other organisms? In vertebrates, various groups have used microarray expression profiling to identify downstream genes of different *Hox* proteins (Table 2). One major problem in higher organisms is the functional redundancy of *Hox* genes, thus it is almost impossible to study the effects of a single *Hox* gene in vivo. To circumvent this problem many groups have expressed individual *Hox* genes in cell cultures and assessed gene expression changes with high-density gene arrays.[35,94–97] However, as outlined above, *Hox* genes fulfil only a subset of their functions if taken out of context due to the lack of assisting transcription factors. This is also reflected in the outcome of the studies mentioned above, since they very often report low numbers of putative *Hox* downstream genes (Table 2). However, when such studies were performed in vivo, the

Table 2. Large-scale identification of *Hox* downstream genes and *Hox* response elements in the genomic age

References	*Hox* genes	Organism	Tissue	Stage	#Targets
Leemanns et al., 2001[117]	Lab	*Drosophila*	whole embryo	embryonic stage 10-17	96
Mohit et al., 2006[92]	Ubx	*Drosophila*	haltere and wing disc	3rd instar larvae	541
Hersh et al., 2007[93]	Ubx	*Drosophila*	haltere and wing disc	3rd instar larvae	447
Hueber et al., 2007[27]	Dfd, Scr, Antp, Ubx, Abd-A, Abd-B	*Drosophila*	whole embryo	embryonic stage 11+ 12	1508
Shen et al., 2000[97]	*Hox*A1	mouse	cell culture – teratocarcinoma		28
Zhao and Potter, 2001[120]	*Hox*A13	mouse	uterus and cervix tissue	4.5 weeks old	unclear
Valerius et al., 2002[119]	*Hox*A11	mouse	kidney tissue	embryonic stage 18.5	10
Hedlund et al., 2004[34]	*Hox*D10	mouse	spinal cord tissue	embryonic stage 12.5	69
Martinez-Ceballos et al., 2005[96]	*Hox*A1	mouse	cell culture – embryonic blastocysts		145
Lei et al., 2005[95]	*Hox*C8	mouse	cell culture – embryonic fribroblasts		34
Cobb et al., 2005[33]	*Hox*D cluster genes	mouse	mouse tissue of limbs and genitalia	embryonic stage 12.5	16
Williams et al., 2005[35]	*Hox*A13	mouse	cell culture – embryonic fribroblasts		68
Schwab et al., 2006[99]	*Hox*A11 + HoxD11	mouse	whole embryonic kidneys and urogenital tissue	embryonic stage 11.5, 12.5 13.5, 16.5 + adult	1518
Rohrschneider et al., 2007[98]	*Hox*B1a	zebrafish	whole embryo	19-20 hours post fertilization	471
Ferrell et al., 2005[94]	*Hox*A10	human	cell culture – umbilical cord cells		115

Large-scale identification of *Hox* response elements

References	*Hox* genes	Organism	Approach
Ebner et al., 2005[41]	Lab	*Drosophila*	*in silico* prediction
Hueber et al., 2007[27]	Dfd	*Drosophila*	*in silico* prediction
McCabe et al., 2005[101]	*Hox*A13 + HoxD13	mouse	ChIP

results were often similar to those obtained in *Drosophila*,[98,99] again highlighting the importance of in vivo studies.

Another important observation of the large-scale analyses in *Drosophila* is that *Hox* downstream genes can be found across diverse functional classes, ranging from regulatory molecules, like transcription factors and signalling components, to realisators. This notion is also mirrored by studies performed in vertebrates, which resulted in the identification of similar classes of *Hox* downstream genes. These findings contrast with the view, prevalent in the pre-genomic age, that *Hox* proteins primarily affect regulatory genes, especially transcription factors. While this concept was based on the knowledge of 24 *Hox* targets (Table 1), a recent study now identified thousands of *Hox* response genes and showed that 13% code for realisator proteins.[27] A similar result was also obtained by a study performed in vertebrates.[98] This result lends support for the concept postulated by Garcia-Bellido more than 30 years ago[48] and showed, for the first time, that *Hox* proteins regulate morphogenesis at least in part through the regulation of terminal differentiation genes.[27] Most of these realisator genes likely act redundantly in general cellular processes required in many cells, which probably has precluded their discovery by genetic approaches. This highlights one of the advantages of genomic approaches, namely the ability to identify targets irrespective of their molecular nature. Conversely, in many cases, it will be very difficult to elucidate the in vivo function of the identified realisators by reverse genetics.

Each *Hox* protein specifies distinct morphological features within segments, and understanding how this specificity is achieved has been one of the major goals since the discovery of *Hox* genes. So far, there has been only a single study in which the effects of different *Hox* proteins under the same experimental conditions were tested.[27] One of the major findings is that many of the identified *Hox* downstream genes are primarily affected by a single *Hox* protein, implying that there is tremendous specificity in target gene regulation, despite the similarities in in vitro DNA binding. Moreover, there was a clear trend for distinct regulatory interactions in those cases where downstream genes were regulated by more than one *Hox* protein. These genes were likely to be affected in a similar manner when targeted by *Hox* proteins specifying segments with similar morphologies, whereas they were more often regulated in the opposite direction when targeted by *Hox* proteins functioning in different body parts.[27] The authors therefore concluded that the diversification of segments is achieved through the regulation of unique downstream genes on the one hand and through the differential regulation of shared downstream genes on the other hand.

The issue of *Hox* protein specificity on the transcriptome was not only reflected in the identification of a large portion of unique *Hox* downstream genes, but also in the observation that the majority of genes are primarily regulated at only one of the two developmental stages analyzed. The authors suggested that this might be achieved by an extensive interaction of *Hox* proteins with the regulatory environment that they are embedded in. Support for this notion not only comes from previous studies showing that regulation of *Hox* target genes is dependent on the context,[28,29,91,100] but more so from recent studies, which have provided direct evidence that *Hox* proteins gain the ability to regulate their target genes in a context-specific manner in vivo by interaction with known cell- and/or tissue-specific transcription factors, so-called collaborators.[40,42]

Taken together, transcriptomic approaches so far have been very informative about the number and nature of *Hox* response genes, but many questions about the mechanisms of regulatory interactions as well as the function of downstream genes remain open.

Large-scale identification of Hox response elements: in silico and in vivo approaches

An essential aspect for our mechanistic understanding of *Hox*-dependent processes is the identification of direct targets versus downstream genes that are controlled via intermediate factors. The transcriptome datasets described thus far include both direct and indirect targets and so far there are only three published studies, which aim to identify *Hox* response elements on a genome-wide scale.[27,41,101] In principle, two complementing strategies have been used: in silico by searching for genomic regions that are enriched in transcription-factor-binding sites using computational tools,[27,41] and in vivo by identifying DNA fragments associated with transcription factors using chromatin immunoprecipitation (ChIP).[101] The bioinformatics detection of *Hox* response elements is hampered by the fact that individual *Hox* proteins have rather poorly defined binding sequences, which in addition occur very frequently in the genome. However, two distinct computational approaches have been applied to the identification of HREs in *Drosophila*. To enhance the stringency of the search criteria, the first study was based the observation that *Hox* proteins can bind to their target sequences in association with the cofactors Exd and Hth.[102] In this scenario, distinct *Hox*–Exd–Hth complexes recognize and select specific DNA sequences depending on the *Hox* protein included in the complex. Based on this model, Ebner and colleagues[41] searched the *D. melanogaster* genome for Lab–Exd heterodimer-binding sequences within 40 base pairs of an Hth consensus site.[41] Although they identified 40 putative target sequences for the Lab–Exd–Hth complex, only a single gene (*CG11339)* in the vicinity of the identified binding sites showed a Lab-like expression pattern. However, when the predicted Lab response element was tested in vivo, it did not show the expected enhancer activity. Interestingly, another DNA fragment nearby the *CG11339* transcription unit, which was not predicted to be bound by Lab, was able to drive reporter gene expression in Lab-expressing cells, despite the fact that a putative Lab-binding site within this enhancer was highly divergent from the consensus binding sequence. While the results of Ebner and colleagues[41] might not seem very encouraging with regards to the reliability of computational identification of HREs, they highlight two important issues for in silico strategies: First, in vivo *Hox* binding sites might be more divergent than anticipated and therefore, the stringency of the initial motif search might have been to high. And second, the cooperative binding of *Hox* proteins with dedicated cofactors of the Exd-type might not be of such a general importance for *Hox* target gene regulation as previously anticipated. Thus, the findings of Ebner and colleagues[41] should, in our view, not be considered as a failure of in silico approaches to predict HREs. There are meanwhile many examples of the successful in silico prediction of regulatory elements.[103–107] On the contrary, it could be very well argued that the findings of Ebner and colleagues[41] underline the peculiarity of the cooperative/co-selective binding model to explain the *Hox* paradox and support the possibility that collaboration might be indeed the more general mode of *Hox* proteins to select and regulate their target genes. And to be even a little provocative: Exd might assist *Hox* proteins in target gene regulation not only by increasing the DNA-binding specificity of *Hox* proteins through cooperate binding, but perhaps more so by acting, like other *Hox* collaborators, in a combinatorial fashion with *Hox* proteins. In this context, the outcome of an improved in silico search using less stringent Lab- and Exd-binding sequences and a more relaxed spacing of these sites would be very interesting.

In recent years, it has been realised that a combination of in silico prediction and in vivo approaches has a higher success rate in identifying in vivo functional regulatory elements than approaches only based on computational calculations.[108–110] This has been successfully integrated in the strategy used by Hueber and colleagues.[27] After a detailed analysis of all known HREs, which also included enhancers regulated independently of the Exd/Hth input, the main requirement for their *in silico* search was an accumulation of Hox-binding sites within a limited stretch of DNA sequence. In addition, for a *Hox* response element to be considered, it had to pass a sequence conservation filter across four *Drosophila* species. And finally, in contrast to the approach of Ebner and colleagues,[41] the parameters of the search were optimized and validated using in vivo results from transcript profiling experiments. By applying this approach, Hueber and colleagues[27] were able to identify a large number of putative response elements for the *Hox* protein Dfd. Two findings indicate that these may constitute true target sequences for

Dfd: first, all of the predicted Dfd response elements contained several conserved binding sites for other transcription factors, a known prerequisite for functional enhancer elements.[107] In the light of the collaboration model, these sites could represent interaction sites for collaborating transcription factors, which needs further experimental proof. Second, many elements have been tested experimentally by in vitro analysis and in all cases showed that they were specifically bound by Dfd, whereas Ubx, a *Hox* protein specifying trunk identity, was not able to interact with these enhancers. Meanwhile, some of the enhancers were also tested in the embryo, showing that all of them function in a Hox-dependent manner in vivo (Bezdan D, Schäder N, Piediotta M, Hent S, Lohmonn I, unpublished data. Thus, less-stringent sequence requirements and the incorporation of in vivo data seem to increase the power of computational methods for predicting HREs.

The only study using an in vivo approach for the large-scale identification of *Hox* response elements was performed by McCabe and Innis[101] in 2005. Here, genomic fragments bound by the *Hox* protein *HOX*A13, misexpressed in embryonic fibroblast cells, where isolated using ChIP. DNA fragments were eluted, cloned and 5% of the clones were sequenced.[101] To verify the identified fragments, the authors analyzed expression changes of nearby genes in response to *HOXA*13 activity and studied putative enhancers in reporter gene assays. Only seven new high-confidence HREs passed all requirements, which might be explained by the fact that this analysis was performed in tissue culture and thus in an environment deprived of other factors assisting *Hox* proteins in target gene regulation.

Taken together, it is obvious that further experimentation is required to identify HREs and thus the genes directly regulated by *Hox* proteins on a genome-wide scale. Due to the limited amount of data and in particular the lack of in vivo analysis of the identified enhancers, it is so far impossible to draw general conclusions on the mechanisms of how *Hox* proteins achieve specificity in target gene regulation. One of the major challenges on our way to deducing more general rules for *Hox*–DNA interactions will be the integration of data derived from diverse experiments, including more traditional gene by gene methods, to cover all aspects of *Hox* protein activity.

Conclusions and future directions

Although large-scale approaches are extremely important for a comprehensive understanding of all aspects of *Hox* gene function, only few such studies exist despite the widespread availability of many useful tools, such as expression profiling, or ChIP-on-chip experiments. Thus, the field so far suffers from a limitation of available data, which together with the enormous complexity of *Hox* protein activity makes it difficult to reconstruct the regulatory networks orchestrated by them. In addition, *Hox* output largely depends on the regulatory context within every cell, in the sense that other transcription factors dictate the "When", "Where" and "How" of *Hox* target gene regulation. Consequently, *Hox*-modulated regulatory networks will change dramatically during development. Thus, in order to understand those networks and their dynamic behaviour, we suggest a more-refined large-scale identification of direct and indirect *Hox* target genes and of active HREs in consecutive developmental stages using genome-wide approaches, like microarray expression profiling and ChIP-on-chip or ChIP-Seq experiments. In addition, data from other resources (like ArrayExpress, modENCODE, BDGP in situ database) need to be incorporated, since it is now realised that many other transcription factors will assist *Hox* proteins in target gene regulation. With new large-scale datasets and innovative mechanistic studies becoming available, the new millenium certainly is an exciting time for the *Hox* field. If we succeed in integrating results generated by large-scale in vivo, in vitro and in silico strategies, we might be able to decipher the mysteries of *Hox* activity that have caught the imagination of developmental biologists ever since the discovery of homeotic transformation by Bateson in 1894.

Acknowledgments

We thank Jan U. Lohmann, Daniela Bezdan, Aurelia L. Fuchs, Petra Stöbe and Zongzhao Zhai for critically reading the manuscript and helpful comments.

References

1. Duboule D, Dolle P. 1989. The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. EMBO J 8:1497–1505.
2. Graham A, Papalopulu N, Krumlauf R. 1989. The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell 57:367–378.
3. Pearson JC, Lemons D, McGinnis W. 2005. Modulating *Hox* gene functions during animal body patterning. Nature Genetics Reviews 6:893–904.
4. Mann RS, Morata G. 2000. The developmental and molecular biology of genes that subdivide the body of Drosophila. Annu Rev Cell Dev Biol 16:243–271.
5. McGinnis W, Krumlauf R. 1992. Homeobox genes and axial patterning. Cell 68:283–302.
6. Lewis EB. 1978. A gene complex controlling segmentation in Drosophila. Nature 276:565–570.
7. Kaufman TC, Seeger MA, Olsen G. 1990. Molecular and genetic organization of the Antennapedia gene complex of Drosophila melanogaster. Adv Genet 27:309–362.
8. Bateson W. 1894. Materials for the study of variation: treated with special regard to discontinuity in the origin of species. Cambridge: Cambridge Press.
9. Beeman RW, Stuart JJ, Haas MS, Denell RE. 1989. Genetic analysis of the homeotic gene complex (HOM-C) in the beetle Tribolium castaneum. Dev Biol 133:196–209.
10. Krumlauf R. 1994. *Hox* genes in vertebrate development. Cell 78:191–201.
11. Kmita M, Tarchini B, Zakany J, Logan M, Tabin CJ, et al. 2005. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. Nature 435:1113–1116.
12. Hafen E, Levine M, Gehring WJ. 1984. Regulation of Antennapedia transcript distribution by the Bithorax complex in Drosophila. Nature 307:287–289.

13. Struhl G. 1982. Genes controlling segmental specification in the Drosophila thorax. Proc Natl Acad Sci USA 79:7380–7384.
14. Gonzalez-Reyes A, Morata G. 1990. The developmental effect of overexpressing a Ubx product in Drosophila embryos is dependent on its interactions with other homeotic products. Cell 61: 515–522.
15. Mann RS, Hogness DS. 1990. Functional dissection of Ultrabithorax proteins in D. melanogaster. Cell 60:597–610.
16. Regulski M, McGinnis N, Chadwick R, McGinnis W. 1987. Developmental and molecular analysis of Deformed: a homeotic gene controlling Drosophila head development. EMBO J 6:767–777.
17. Scott MP, Weiner AJ. 1984. Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax, and fushi tarazu loci of Drosophila. Proc Natl Acad Sci USA 81:4115–4119.
18. McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring WJ. 1984. A conserved DNA sequence in homoeotic genes of the Drosophila Antennapedia and bithorax complexes. Nature 308:428–433.
19. Scott MP, Tamkun JW, Hartzell GW 3rd. 1989. The structure and function of the homeodomain. Biochim Biophys Acta 989:25–48.
20. Krasnow MA, Saffman EE, Kornfeld K, Hogness DS. 1989. Transcriptional activation and repression by Ultrabithorax proteins in cultured Drosophila cells. Cell 57:1031–1043.
21. Desplan C, Theis J, O'Farrell PH. 1988. The sequence specificity of homeodomain-DNA interaction. Cell 54:1081–190.
22. Carr A, Biggin MD. 1999. A comparison of in vivo and in vitro DNA-binding specificities suggests a new model for homeoprotein DNA binding in Drosophila embryos. EMBO J 18:1598–1608.
23. Walter J, Dever CA, Biggin MD. 1994. Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in Drosophila embryos. Genes Dev 8:1678–1692.
24. Li X, Murre C, McGinnis W. 1999. Activity regulation of a *Hox* protein and a role for the homeodomain in inhibiting transcriptional activation. EMBO J 18:198–211.
25. Biggin MD, McGinnis W. 1997. Regulation of segmentation and segmental identity by Drosophila homeoproteins: the role of DNA binding in functional activity and specificity. Development 124:4425–4433.
26. Ekker SC, Jackson DG, von Kessler DP, Sun BI, Young KE, et al. 1994. The degree of variation in DNA sequence recognition among four Drosophila homeotic proteins. EMBO J 13:3551–3560.
27. Hueber SD, Bezdan D, Henz SR, Blank M, Wu H, et al. 2007. Comparative analysis of *Hox* downstream genes in Drosophila. Development 134:381–392.
28. Grienenberger A, Merabet S, Manak J, Iltis I, Fabre A, et al. 2003. TGF-? signaling acts on a *Hox* response element to confer specificity and diversity to *Hox* protein function. Development 130:5445–5455.
29. Bondos SE, Tan XX. 2001. Combinatorial transcriptional regulation: the interaction of transcription factors and cell signaling molecules with homeodomain proteins in Drosophila development. Crit Rev Eukaryot Gene Expr 11:145–171.
30. Graba Y, Aragnol D, Pradel J. 1997. Drosophila *Hox* complex downstream targets and the function of homeotic genes. Bioessays 19:379–388.
31. Hombria JC, Lovegrove B. 2003. Beyond homeosis–HOX function in morphogenesis and organogenesis. Differentiation 71:461–476.
32. Pradel J, White RA. 1998. From selectors to realizators. Int J Dev Biol 42:417–421.
33. Cobb J, Duboule D. 2005. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development 132:3055–3067.
34. Hedlund E, Karsten SL, Kudo L, Geschwind DH, Carpenter EM. 2004. Identification of a Hoxd10-regulated transcriptional network and combinatorial interactions with Hoxa10 during spinal cord development. J Neurosci Res 75:307–319.
35. Williams TM, Williams ME, Kuick R, Misek D, McDonagh K, et al. 2005. Candidate downstream regulated genes of HOX group 13 transcription factors with and without monomeric DNA binding capability. Dev Biol 279:462–480.
36. Mastick GS, McKay R, Oligino T, Donovan K, Lopez AJ. 1995. Identification of target genes regulated by homeotic proteins in Drosophila melanogaster through genetic selection of Ultrabithorax protein-binding sites in yeast. Genetics 139:349–363.
37. Mann RS. 1995. The specificity of homeotic gene function. Bioessays 17:855–863.
38. Mann RS, Affolter M. 1998. *Hox* proteins meet more partners. Curr Opin Genet Dev 8:423–429.
39. Mahaffey JW. 2005. Assisting *Hox* proteins in controlling body form: are there new lessons from flies (and mammals)? Curr Opin Genet Dev 15:422–429.
40. Gebelein B, McKay DJ, Mann RS. 2004. Direct integration of *Hox* and segmentation gene inputs during Drosophila development. Nature 431:653–659.
41. Ebner A, Cabernard C, Affolter M, Merabet S. 2005. Recognition of distinct target sites by a unique Labial/Extradenticle/Homothorax complex. Development 132:1591–1600.
42. Walsh CM, Carroll SB. 2007. Collaboration between Smads and a *Hox* protein in target gene repression. Development 134:3585–3592.
43. Manak JR, Mathies LD, Scott MP. 1994. Regulation of a decapentaplegic midgut enhancer by homeotic proteins. Development 120:3605–3619.
44. Vachon G, Cohen B, Pfeifle C, McGuffin ME, Botas J, et al. 1992. Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene Distalless. Cell 71:437–450.
45. Graba Y, Aragnol D, Laurenti P, Garzino V, Charmot D, et al. 1992. Homeotic control in Drosophila; the scabrous gene is an in vivo target of Ultrabithorax proteins. EMBO J 11:3375–3384.
46. Strutt DI, White RA. 1994. Characterisation of T48, a target of homeotic gene regulation in Drosophila embryogenesis. Mech Dev 46:27–39.
47. Heuer JG, Li K, Kaufman TC. 1995. The Drosophila homeotic target gene centrosomin (cnn) encodes a novel centrosomal protein with leucine zippers and maps to a genomic region required for midgut morphogenesis. Development 121:3861–3876.
48. Garcia-Bellido A. 1975. Genetic control of wing disc development in Drosophila. Ciba Found Symp 0:161–182.
49. Percival-Smith A, Teft WA, Barta JL. 2005. Tarsus determination in Drosophila melanogaster. Genome 48:712–721.
50. Zeng C, Pinsonneault J, Gellon G, McGinnis N, McGinnis W. 1994. Deformed protein binding sites and cofactor binding sites are required for the function of a small segment-specific regulatory element in Drosophila embryos. EMBO J 13:2362–2377.
51. Appel B, Sakonju S. 1993. Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in Drosophila embryos. EMBO J 12:1099–1109.
52. Lohmann I, McGinnis N, Bodmer M, McGinnis W. 2002. The Drosophila *Hox* gene Deformed sculpts head morphology via direct regulation of the apoptosis activator reaper. Cell 110:457–466.
53. Hinz U, Wolk A, Renkawitz-Pohl R. 1992. Ultrabithorax is a regulator of b3 tubulin expression in the Drosophila visceral mesoderm. Development 116:543–554.
54. Gould AP, White RA. 1992. Connectin, a target of homeotic gene control in Drosophila. Development 116:1163–1174.
55. Li K, Kaufman TC. 1996. The homeotic target gene centrosomin encodes an essential centrosomal component. Cell 85:585–596.
56. Bello BC, Hirth F, Gould AP. 2003. A pulse of the Drosophila *Hox* protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. Neuron 37:209–219.
57. Hu N, Castelli-Gair J. 1999. Study of the posterior spiracles of Drosophila as a model to understand the genetic and cellular mechanisms controlling morphogenesis. Dev Biol 214:197–210.
58. Lovegrove B, Somoes S, Rivas ML, Sotillos S, Johnson K, et al. 2006. Co-ordinated control of cell adhesion, cell polarity and cytoskeleton underlies *Hox* induced organogenesis in Drosophila. Curr Biol 16:2206–2216.
59. Malicki J, Schughart K, McGinnis W. 1990. Mouse Hox-2.2 specifies thoracic segmental identity in Drosophila embryos and larvae. Cell 63:961–967.

60. Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, et al. 1994. Homeodomain-DNA recognition. Cell 78:211–223.
61. McGinnis N, Kuziora MA, McGinnis W. 1990. Human Hox-4.2 and Drosophila Deformed encode similar regulatory specificities in Drosophila embryos and larvae. Cell 63:969–976.
62. Kuziora MA, McGinnis W. 1989. A homeodomain substitution changes the regulatory specificity of the Deformed protein in Drosophila embryos. Cell 59:563–571.
63. Lin L, McGinnis W. 1992. Mapping functional specificity in the Dfd and Ubx homeo domains. Genes Dev 6:1071–1081.
64. Gibson G, Schier A, LeMotte P, Gehring WJ. 1990. The specificities of Sex combs reduced and Antennapedia are defined by a distinct portion of each protein that includes the homeodomain. Cell 62:1087–1103.
65. Zeng W, Andrew DJ, Mathies LD, Horner MA, Scott MP. 1993. Ectopic expression and function of the Antp and Scr homeotic genes: the N terminus of the homeodomain is critical to functional specificity. Development 118:339–352.
66. Keleher CA, Goutte C, Johnson AD. 1988. The yeast cell-type-specific repressor alpha 2 acts cooperatively with a non-cell-type-specific protein. Cell 53:927–936.
67. Taghli-Lamallem O, Gallet A, Leroy F, Malapert P, Vola C, et al. 2007. Direct interaction between Teashirt and Sex combs reduced proteins, via Tsh's acidic domain, is essential for specifying the identity of the prothorax in Drosophila. Dev Biol 307:142–151.
68. Johnson FB, Parker E, Krasnow MA. 1995. Extradenticle protein is a selective cofactor for the Drosophila homeotics: role of the homeodomain and YPWM amino acid motif in the interaction. Proc Natl Acad Sci USA 92:739–743.
69. Chan SK, Jaffe L, Capovilla M, Botas J, Mann RS. 1994. The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with Extradenticle, another homeoprotein. Cell 78:603–615.
70. Popperl H, Bienz M, Studer M, Chan SK, Aparicio S, et al. 1995. Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon Exd/Pbx. Cell 81:1031–1042.
71. van Dijk MA, Murre C. 1994. Extradenticle raises the DNA binding specificity of homeotic selector gene products. Cell 78:617–624.
72. Rieckhof GE, Casares F, Ryoo HD, Abu-Shaar M, Mann RS. 1997. Nuclear translocation of Extradenticle requires Homothorax, which encodes an Extradenticle-related homeodomain protein. Cell 91:171–183.
73. Chan SK, Mann RS. 1996. A structural model for a homeotic protein-extradenticle-DNA complex accounts for the choice of HOX protein in the heterodimer. Proc Natl Acad Sci USA 93:5223–5228.
74. Phelan ML, Featherstone MS. 1997. Distinct HOX N-terminal arm residues are responsible for specificity of DNA recognition by HOX monomers and HOX.PBX heterodimers. J Biol Chem 272:8635–8643.
75. Shen WF, Chang CP, Rozenfeld S, Sauvageau G, Humphries RK, et al. 1996. *Hox* homeodomain proteins exhibit selective complex stabilities with Pbx and DNA. Nucleic Acids Res 24:898–906.
76. Chan SK, Ryoo HD, Gould A, Krumlauf R, Mann RS. 1997. Switching the in vivo specificity of a minimal Hox-responsive element. Development 124:2007–2014.
77. Gebelein B, Culi J, Ryoo HD, Zhang W, Mann RS. 2002. Specificity of Distalless repression and limb primordia development by abdominal *Hox* proteins. Dev Cell 3:487–498.
78. Ryoo HD, Mann RS. 1999. The control of trunk *Hox* specificity and activity by Extradenticle. Genes Dev 13:1704–1716.
79. Joshi R, Passner JM, Rohs R, Jain R, Sosinsky A, et al. 2007. Functional specificity of a *Hox* protein mediated by the recognition of minor groove structure. Cell 131:530–543.
80. Knoepfler PS, Kamps MP. 1995. The pentapeptide motif of *Hox* proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. Mol Cell Biol 15:5811–5819.
81. Passner JM, Ryoo HD, Shen L, Mann RS, Aggarwal AK. 1999. Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. Nature 397:714–719.
82. Merabet S, Saadaoui M, Sambrani N, Hudry B, Pradel J, et al. 2007. A unique Extradenticle recruitment mode in the Drosophila *Hox* protein Ultrabithorax. Proc Natl Acad Sci USA 104:16946–16951.
83. Galant R, Walsh CM, Carroll SB. 2002. *Hox* repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. Development 129:3115–3126.
84. Robertson LK, Bowling DB, Mahaffey JP, Imiolczyk B, Mahaffey JW. 2004. An interactive network of zinc-finger proteins contributes to regionalization of the Drosophila embryo and establishes the domains of HOM-C protein function. Development 131:2781–2789.
85. Liang Z, Biggin MD. 1998. Eve and ftz regulate a wide array of genes in blastoderm embryos: the selector homeoproteins directly or indirectly regulate most genes in Drosophila. Development 125:4471–4482.
86. Capovilla M, Brandt M, Botas J. 1994. Direct regulation of decapentaplegic by Ultrabithorax and its role in Drosophila midgut morphogenesis. Cell 76:461–475.
87. Regulski M, Dessain S, McGinnis N, McGinnis W. 1991. High-affinity binding sites for the Deformed protein are required for the function of an autoregulatory enhancer of the Deformed gene. Genes Dev 5:278–286.
88. Pinsonneault J, Florence B, Vaessin H, McGinnis W. 1997. A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. EMBO J 16:2032–2042.
89. Marty T, Vigano MA, Ribeiro C, Nussbaumer U, Grieder NC, et al. 2001. A HOX complex, a repressor element and a 50 bp sequence confer regional specificity to a DPP-responsive enhancer. Development 128:2833–2845.
90. McCormick A, Core N, Kerridge S, Scott MP. 1995. Homeotic response elements are tightly linked to tissue-specific elements in a transcriptional enhancer of the teashirt gene. Development 121:2799–2812.
91. Zaffran S, Kuchler A, Lee HH, Frasch M. 2001. Biniou (FoxF), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in Drosophila. Genes Dev 15:2900–2915.
92. Mohit P, Makhijani K, Madhavi MB, Bharathi V, Lal A, et al. 2006. Modulation of AP and DV signaling pathways by the homeotic gene Ultrabithorax during haltere development in Drosophila. Dev Biol 291:356–367.
93. Hersh BM, Nelson CE, Stoll SJ, Norton JE, Albert TJ, et al. 2007. The UBX-regulated network in the haltere imaginal disc of D. melanogaster. Dev Biol 302:717–727.
94. Ferrell CM, Dorsam ST, Ohta H, Humphries RK, Derynck MK, et al. 2005. Activation of stem-cell specific genes by HOXA9 and HOXA10 homeodomain proteins in CD34+ human cord blood cells. Stem Cells 23:644–655.
95. Lei H, Wang H, Juan AH, Ruddle FH. 2005. The identification of Hoxc8 target genes. Proc Natl Acad Sci USA 102:2420–2424.
96. Martinez-Ceballos E, Chambon P, Gudas LJ. 2005. Differences in gene expression between wild type and Hoxa1 knockout embryonic stem cells after retinoic acid treatment or leukemia inhibitory factor (LIF) removal. J Biol Chem 280:16484–16498.
97. Shen J, Wu H, Gudas LJ. 2000. Molecular cloning and analysis of a group of genes differentially expressed in cells which overexpress the Hoxa-1 homeobox gene. Exp Cell Res 259:274–283.
98. Rohrschneider MR, Elsen GE, Prince VE. 2007. Zebrafish Hoxb1a regulates multiple downstream genes including prickle1b. Dev Biol 309:358–372.
99. Schwab K, Hartman HA, Liang HC, Aronow BJ, Patterson LT, et al. 2006. Comprehensive microarray analysis of Hoxa11/Hoxd11 mutant kidney development. Dev Biol 293:540–554.
100. Bondos SE, Tan XX, Matthews KS. 2006. Physical and genetic interactions link *Hox* function with diverse transcription factors and cell signaling proteins. Mol Cell Proteomics 5:824–834.
101. McCabe CD, Innis JW. 2005. A genomic approach to the identification and characterization of HOXA13 functional binding elements. Nucleic Acids Res 33:6782–6794.
102. Ryoo HD, Marty T, Casares F, Affolter M, Mann RS. 1999. Regulation of *Hox* target genes by a DNA bound Homothorax/Hox/Extradenticle complex. Development 126:5137–5148.
103. Rajewsky N, Vergassola M, Gaul U, Siggia ED. 2002. Computational detection of genomic cis-regulatory modules applied to body patterning in the early Drosophila embryo. BMC Bioinformatics 3:30.

104. Schroeder MD, Pearce M, Fak J, Fan H, Unnerstall U, et al. 2004. Transcriptional control in the segmentation gene network of Drosophila. PLoS Biol 2:E271.
105. Kel A, Konovalova T, Waleev T, Cheremushkin E, Kel-Margoulis O, et al. 2006. Composite Module Analyst: a fitness-based tool for identification of transcription factor binding site combinations. Bioinformatics 22: 1190–1197.
106. Berman BP, Nibu Y, Pfeiffer BD, Tomancak P, Celniker SE, et al. 2002. Exploiting transcription factor binding site clustering to identify cis-regulatory modules involved in pattern formation in the Drosophila genome. Proc Natl Acad Sci USA 99:757–762.
107. Berman BP, Pfeiffer BD, Laverty TR, Salzberg SL, Rubin GM, et al. 2004. Computational identification of developmental enhancers: conservation and function of transcription factor binding-site clusters in Drosophila melanogaster and Drosophila pseudoobscura. Genome Biol 5:R61.
108. Kim SY, Kim Y. 2006. Genome-wide prediction of transcriptional regulatory elements of human promoters using gene expression and promoter analysis data. BMC Bioinformatics 7:330.
109. Nilsson R, Bajic VB, Suzuki H, di Bernardo D, Bjorkegren J, et al. 2006. Transcriptional network dynamics in macrophage activation. Genomics 88:133–142.
110. Ramsey SA, Klemm SL, Zak DE, Kennedy KA, Thorsson V, et al. 2008. Uncovering a macrophage transcriptional program by integrating evidence from motif scanning and expression dynamics. PLoS Comput Biol 4:e1000021.
111. Capovilla M, Kambris Z, Botas J. 2001. Direct regulation of the muscle-identity gene apterous by a *Hox* protein in the somatic mesoderm. Development 128:1221–1230.
112. Chauvet S, Maurel-Zaffran C, Miassod R, Jullien N, Pradel J, et al. 2000. dlarp, a new candidate *Hox* target in Drosophila whose orthologue in mouse is expressed at sites of epithelium/mesenchymal interactions. Dev Dyn 218:401–413.
113. Graba Y, Gieseler K, Aragnol D, Laurenti P, Mariol MC, et al. 1995. DWnt-4, a novel Drosophila Wnt gene acts downstream of homeotic complex genes in the visceral mesoderm. Development 121:209–218.
114. Grieder NC, Marty T, Ryoo HD, Mann RS, Affolter M. 1997. Synergistic activation of a Drosophila enhancer by HOM/EXD and DPP signaling. EMBO J 16:7402–7410.
115. Jones B, McGinnis W. 1993. The regulation of empty spiracles by Abdominal-B mediates an abdominal segment identity function. Genes Dev 7:229–240.
116. Kremser T, Hasenpusch-Theil K, Wagner E, Buttgereit D, Renkawitz-Pohl R. 1999. Expression of the ? tubulin gene (? Tub60D) in the visceral mesoderm of Drosophila is dependent on a complex enhancer that binds Tinman and UBX. Mol Gen Genet 262:643–658.
117. Leemans R, Loop T, Egger B, He H, Kammermeier L, et al. 2001. Identification of candidate downstream genes for the homeodomain transcription factor Labial in Drosophila through oligonucleotide-array transcript imaging. Genome Biol 2: RESEARCH0015.
118. Pederson JA, LaFollette JW, Gross C, Veraksa A, McGinnis W, et al. 2000. Regulation by homeoproteins: a comparison of Deformed-responsive elements. Genetics 156:677–686.
119. Valerius MT, Patterson LT, Witte DP, Potter SS. 2002. Microarray analysis of novel cell lines representing two stages of metanephric mesenchyme differentiation. Mech Dev 110:151–164.
120. Zhao Y, Potter SS. 2001. Functional specificity of the Hoxa13 homeobox. Development 128:3197–3207.
121. Zhou B, Bagri A, Beckendorf SK. 2001. Salivary gland determination in Drosophila: a salivary-specific, fork head enhancer integrates spatial pattern and allows fork head autoregulation. Dev Biol 237:54–67.
122. Hersh BM, Carroll SB. 2005. Direct regulation of *knot* gene expression by Ultrabithorax and the evolution of cis-regulatory elements in *Drosophila*. Development 132:1567–1577.