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# Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites

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## SUMMARY

**Homeotic (*Hox*) genes regulate the identity of structures along the anterior-posterior axis of most animals. The low DNA-binding specificities of Hox proteins have raised the question of how these transcription factors selectively regulate target gene expression. The discovery that the Extradenticle (Exd)/Pbx and Homothorax (Hth)/Meis proteins act as cofactors for several Hox proteins has advanced the view that interactions with cofactors are critical to the target selectivity of Hox proteins. It is not clear, however, to what extent Hox proteins also regulate target genes in the absence of cofactors. In *Drosophila melanogaster*, the Hox protein Ultrabithorax (Ubx) promotes haltere development and suppresses wing development by selectively repressing many genes of the wing-patterning hierarchy, and this activity requires neither Exd nor Hth function. Here, we show that Ubx directly regulates a flight appendage-specific *cis*-regulatory element of the *spalt* (*sal*) gene. We find that multiple monomer Ubx-binding sites are required to completely repress this *cis*-element in the haltere, and that individual Ubx-binding sites are sufficient to mediate its partial repression. These results suggest that Hox proteins can directly regulate target genes in the absence of the cofactor Extradenticle. We propose that the regulation of some Hox target genes evolves via the accumulation of multiple Hox monomer binding sites. Furthermore, because the development and morphological diversity of the distal parts of most arthropod and vertebrate appendages involve Hox, but not Exd/Pbx or Hth/Meis proteins, this mode of target gene regulation appears to be important for distal appendage development and the evolution of appendage diversity.**

Key words: Serial homology, Ultrabithorax, Hox protein, Appendages, evolution, *Drosophila melanogaster*

## INTRODUCTION

Many animal body plans are constructed of serially repeated structures that develop from initially equivalent fields of cells during embryogenesis. Serially homologous structures often attain different morphologies and functions within a single animal and changes in their number, form and function mark many important evolutionary differences between taxa (Carroll et al., 2001). Understanding this basic modular aspect of animal design and evolution requires detailed knowledge of the genetic and developmental mechanisms that regulate the formation and identity of body parts. In arthropods and vertebrates, selector genes play central roles in these processes and encode transcription factors that possess the distinct ability to direct the formation of specific tissues, such as the eye or heart and the differentiation of homologous body parts, such as segments, vertebrae and appendages. Selector proteins are proposed to regulate the expression of numerous target genes within regulatory networks that control the development of these tissues (Guss et al., 2001; Halder et al., 1998; Mann and Morata, 2000; Weatherbee et al., 1998).

One class of selector genes, the Hox genes, encode homeodomain-containing proteins that regulate regional identity along the anteroposterior axis in animals and differentiate the identities of serially homologous structures such as vertebrae (Burke et al., 1995; Cohn and Tickle, 1999) and segments and appendages in arthropods (Abzhanov and Kaufman, 2000; Averof and Akam, 1995; Averof and Patel, 1997; Carroll et al., 1995; Grenier et al., 1997; Kaufman et al., 1990; Lewis, 1978; Panganiban et al., 1995; Rogers et al., 1997; Warren et al., 1994). Elucidating the mechanisms of Hox protein function is therefore critical to understanding the development and diversification of serially homologous structures. However, many facets of Hox target gene regulation are not well understood. Only a few direct genetic targets of Hox regulation have been identified, including *cis*-regulatory elements involved in Hox gene auto- and cross-regulation (Appel and Sakonju, 1993; Beachy et al., 1993; Beachy et al., 1988; Bergson and McGinnis, 1990; Chan et al., 1994; Dessain et al., 1992; Ferretti et al., 2000; Frasch et al., 1995; Gould et al., 1997; Grieder et al., 1997; Haerry and Gehring, 1996; Jacobs et al., 1999; Li and McGinnis, 1999; Maconochie et al., 1997; Malicki et al., 1992; Pinsonneault et al., 1997; Popperl et al., 1995; Regulski et al., 1991; Thuringer et al., 1993; Zeng

et al., 1994) and the regulation of the *Drosophila* genes *Distal-less* (*Dll*) (Vachon et al., 1992), *decapentaplegic* (*dpp*) (Capovilla and Botas, 1998; Chan et al., 1994; Manak et al., 1994), *teashirt* (McCormick et al., 1995), *forkhead* (Ryoo and Mann, 1999) and *apterous* (Capovilla et al., 2001). A key issue arising from studies of these targets and of DNA-binding by Hox proteins is the relatively low DNA-binding specificities of Hox proteins (Ekker et al., 1994). Hox proteins generally bind a six base pair DNA sequence containing a TAAT core (Ekker et al., 1991) that occurs with high frequency throughout animal genomes (about once every kilobase pair), including the *cis*-regulatory elements of many genes, suggesting that there are many potential targets for Hox proteins.

How is Hox target selectivity achieved? In other words, how does a particular Hox protein recognize and regulate a subset of target genes among a much larger number of potential targets within a genome? One 'widespread binding' model proposes that the binding of Hox proteins to multiple monomer sites increases their occupancy of *cis*-regulatory elements, perhaps co-operatively (Biggin and McGinnis, 1997). In this scenario, numerous Hox binding sites within *cis*-regulatory elements would be required for targets to be regulated by Hox proteins. Alternatively, a 'co-selective binding' model proposes that Hox proteins could regulate *cis*-elements through co-operative interactions with protein cofactors that increase Hox protein DNA-binding affinities for larger compound binding sites (Biggin and McGinnis, 1997).

Many studies have demonstrated that complexes of Hox proteins and Extradenticle (Exd), a DNA-binding cofactor of the PBC family that also includes the vertebrate Pbx and nematode Ceh-20 proteins, are required to directly regulate several target genes (reviewed by Mann and Affolter 1998; Mann and Morata, 2000). Complexed with PBC cofactors, Hox proteins bind to a compound DNA sequence with greater DNA-binding affinity and specificity and hence, increased selectivity (Chan et al., 1994; Chan et al., 1997; Chang et al., 1995; Mann and Affolter, 1998; Ryoo and Mann, 1999; van Dijk and Murre, 1994). MEIS family homeodomain proteins (Hth, Meis and Prep) promote the nuclear localization of PBC proteins and increase the DNA-binding specificities and/or affinities of Hox/PBC complexes (Abu-Shaar et al., 1999; Berthelsen, 1999; Berthelsen et al., 1998; Ferretti et al., 2000; Jacobs, 1990; Mann and Affolter, 1998; Mann and Morata, 2000; Mercader et al., 1999; Rieckhof et al., 1997; Ryoo et al., 1999; Shanmugam et al., 1999; Vlachakis et al., 2001). Thus, there exists a great deal of support for the 'co-selective binding' model for Hox protein selectivity.

However, the fact that most target genes that have been analyzed require PBC proteins for their regulation has led, in our view, to perhaps an overemphasis on the primacy of Hox/PBC interactions in determining the selectivity of Hox proteins. Some studies have suggested that Hox proteins may act through widespread binding to monomer sites (Appel and Sakonju, 1993), while other studies have shown that some Hox-regulated targets are controlled independently of Exd (Pederson et al., 2000; Pinsonneault et al., 1997). Moreover, many Hox-regulated structures in *Drosophila* do not require the activity of either Exd or Homothorax (Hth) for their proper development, most notably the distal appendages (Abu-Shaar and Mann, 1998; Casares and Mann, 1998; Gonzalez-Crespo et al., 1998; Gonzalez-Crespo and Morata, 1996; Mann and Morata, 2000; Rauskolb et al., 1995; Wu and Cohen, 1999), including the haltere (Azpiazu and Morata, 1998; Azpiazu and Morata, 2000; Casares and Mann, 2000; González-Crespo and Morata, 1995). Furthermore, distal appendage development in other arthropods (Gonzalez-Crespo and Morata, 1996) and in vertebrates (Capdevila et al., 1999; Gonzalez-Crespo et al., 1998; Mercader et al., 1999; Mercader et al., 2000; Selleri et al., 2001) requires neither PBC nor MEIS function. Because Hox regulation of gene networks is critical to distal appendage development and does not involve PBC cofactors, the question of whether or how Hox proteins act selectively in the absence of PBC cofactors has particular importance in understanding the genetic mechanisms underlying the development and diversification of distal appendage morphologies and functions in arthropods and tetrapods.

Here, we focus on the regulation of haltere identity by Ultrabithorax in *Drosophila* as a model to understand how Hox proteins selectively regulate specific subsets of target genes and the differentiation of serial homologs in the absence of PBC/MEIS cofactors. We analyze the regulation of several genes that are repressed in the haltere by Ubx and show that the *spalt* (*sal*) gene is cell-autonomously repressed by Ubx in flight appendages. We demonstrate that the repression of a flight appendage-specific *cis*-regulatory element of *sal* in the haltere is directly regulated through multiple Ubx binding sites. In addition, we show that individual Ubx binding sites within this element contribute to, but are not sufficient for its complete repression in the haltere. These findings suggest that Hox proteins can act through multiple monomer binding sites in the absence of known cofactors. We propose that the evolution of some Hox target genes involved the gradual accumulation of multiple Hox monomer binding sites within *cis*-regulatory elements.

## MATERIALS AND METHODS

### Clonal analysis and immunohistochemistry

Clones expressing UbxIa protein were generated by heat induction of FLP recombinase (Xu et al., 1993) at 34°C for 20 minutes at 75-99 hours after egg laying (AEL) in flies of the genotype hsFLP122; Act5C promoter-FRT-CD2-FRT-GAL4/*vg*Q; UAS-UbxIa/+ (Castelli-Gair et al., 1994; Pignoni and Zipursky, 1997; Struhl and Basler, 1993). Third instar imaginal discs were later dissected, fixed and immunostained at 116-140 hours AEL using methods previously described (Halder et al., 1998). Ubx protein was detected using a mouse monoclonal anti-Ubx antibodies FP3.83 or FP6.87 (Kelsh et al., 1994) (gifts from R. White), the anti-Kn and anti-Sal antibodies were generously provided by Michèle Crozatier (Crozatier and Vincent, 1999) and Rosa Barrio (de Celis et al., 1996), respectively and the anti-β-galactosidase antibodies are commercially available (Molecular Probes and Promega). Embryos were stained as described by Galant and Carroll (Galant and Carroll, 2002).

### DNase I footprinting and electromobility shift analyses

DNaseI footprinting analysis of the *sal* 1.1 element was performed with the Ubx homeodomain (a generous gift from Phil Beachy) using methods previously described (Halder et al., 1998). Sites that were protected by Ubx in the *sal* 1.1 element and their mutant counterparts (listed in Fig. 2B) were further analyzed by electromobility shift assays on 20 base pair double-stranded oligonucleotides containing individual sites centered within its native flanking DNA sequences. These oligo probes were radioactively labeled by end-filling in two T

overhangs on the 5′ and 3′ ends with [α-$^{32}$P]dATP using the Klenow fragment. The labeled probes were incubated for 30 minutes at room temperature (RT) with 0, 1.1, 3.3, 10, 30, or 90 ng of the Ubx homeodomain in 20 mM Hepes pH 7.5, 200 mM KCl, 0.25 mM EDTA, 1 mM DTT, 100 μg/ml BSA and 8% glycerol. They were then run on a 5% polyacrylamide gel (19:1 bis:acrylamide) at 150 V in 0.5× TBE for 2 hours. The gels were dried and exposed to XOMAT-AR X-ray film (Kodak). After developing the film, the concentration of Ubx at which half-maximal binding occurred to each wild-type probe was compared to its mutant counterpart to verify that the mutations decreased Ubx affinity for the probe to the level of non-specific DNA sequences without a TAAT core sequence (at least a ten-fold decrease).

### *sal* reporter constructs

Mutant variants of the *sal* 1.1 and 328 mutant elements were each created by using primers carrying appropriate base pair changes (listed in Fig. 2B) in two rounds of PCR, one round with mutant primers to amplify overlapping mutant DNA fragments individually and a second with flanking primers to amplify across all the fragments and generate the entire element. The mutant elements were cloned into the *hsp-lacZ-CaSpeR* reporter plasmid (Nelson and Laughon, 1993), their sequences verified by sequence analysis and they were subsequently used to produce transgenic fly lines. All primer sequences and further details are available upon request. The *sal* 1.1 element sequence is submitted in GenBank (accession no. AF46408712). β-galactosidase activity driven by the various wild-type and mutant elements was detected with X-gal (Halder et al., 1998) and observed to be consistent among at least three independent transgenic lines.

### Generation of phenotypes produced by ectopic Ubx expression

The construct used to drive Ubx YAAA mutant protein expression was created by amplifying two overlapping DNA fragments from a UAS-UbxIa cDNA (Castelli-Gair et al., 1994) using primers carrying mutations that change the YPWM motif in Ubx to YAAA in one round of PCR (primer sequences available upon request), amplifying across the two overlapping mutant fragments with flanking primers in a second round of PCR and cloning the final PCR product into *pUAST* (Brand and Perrimon, 1993). The Ubx YAAA mutant cDNA was confirmed by sequence analysis. Ectopic expression of the Ubx and Ubx YAAA proteins was produced by crossing female flies carrying the arm$^{11}$-Gal4 driver (available from the Bloomington fly stock facility) and the *Dll*304 embryonic limb enhancer β-galactosidase reporter gene (Vachon et al., 1992) to males carrying a UAS-UbxIa or UAS-Ubx YAAA transgene. The progeny from these crosses were either collected as embryos at 0-20 hours AEL and stained with antibodies (Panganiban et al., 1995) or their cuticles prepared 24-48 hours AEL (Roberts, 1998).

### Electromobility shift analyses

Gel shift analyses were performed with a radiolabeled oligonucleotide probe of the sequence 5′ TTAGCGATGATTAATTGCCTCCTT 3′ with in vitro transcribed and translated full-length Exd, Ubx and Ubx YPWM-YAAA proteins as previously published (Galant and Carroll, 2002). When indicated, 3 μl of Ubx protein and/or 1 μl of Exd were used in gel shift reactions.

## RESULTS

### Ubx acts cell autonomously to repress several target genes in the haltere

We have previously shown that many *cis*-regulatory elements and genes, including the *vestigial* quadrant enhancer (*vg*Q) and the vein patterning genes *Drosophila Serum Response Factor* (*DSRF*)/*blistered* and *sal*, exhibit wing-specific expression patterns, but a complete absence of expression within the developing haltere field because they are repressed by Ubx (Weatherbee et al., 1998). Because both cell-autonomous and non cell-autonomous regulation of some target genes by Ubx has been observed (Shashidhara et al., 1999; Weatherbee et al., 1998), this repression could be mediated directly by Ubx or indirectly through its repression of upstream activators of these target genes, including important signaling molecules (Weatherbee et al., 1998). In order to elucidate the mechanism of Ubx action in the developing haltere, we performed additional genetic experiments to identify Ubx-regulated genes that would be most amenable to molecular analysis.

Previous studies analyzed the responses of potential Ubx-regulated target genes t**o** broad, ectopic expression of Ubx in wings and in large, loss-of-function *Ubx* mutant clones in halteres (Shashidhara et al., 1999; Weatherbee et al., 1998). This can make it difficult to distinguish between cell-autonomous (potentially direct) and non cell-autonomous (indirect) effects. We reasoned that the expression of Ubx in small, discrete groups of cells would minimize the effects of non cell-autonomous regulation of target genes by Ubx. We monitored the autonomy of the effects of Ubx in small clones of cells that ectopically express Ubx on the expression of several downstream targets in third instar wing imaginal disks. Reporter gene expression driven by the *vg*Q enhancer was cell-autonomously repressed in Ubx-expressing clones close to the dorsal-ventral (DV) boundary in the wing (Fig. 1A). This result suggests that the *vg*Q enhancer may be a direct target of Ubx repression and indicates that Ubx regulates the *vg*Q enhancer at two levels, because Ubx also represses an unidentified signal emanating from the DV boundary in the haltere that affects vgQ enhancer activation (Shashidhara et al., 1999). We also found that the *DSRF* gene is regulated by Ubx at more than one level. *knot* (*kn*), a Hedgehog signaling-responsive gene that encodes a COE family transcription factor is required for the activation of *DSRF* along the anteroposterior (AP) boundary of the developing wing (Vervoort et al., 1999). Neither DSRF nor Kn is expressed in the developing haltere pouch (Weatherbee et al., 1998) (data not shown). In small clones of ectopic, Ubx-expressing cells located on the AP boundary, the repression of Kn by Ubx was cell-autonomous (Fig. 1B). It follows then that the repression of DSRF along the AP boundary of the wing disk by Ubx is achieved at least in part through Kn (i.e. is indirect). However, because DSRF is expressed in a broader domain than that of Kn (Montagne et al., 1996; Vervoort et al., 1999), DSRF expression may also be directly repressed in the haltere by Ubx.

In contrast to *vg* and *DSRF*, *sal* appears to be regulated by Ubx solely in a direct manner. The two known upstream activators of *sal*, the Dpp signaling pathway (Lecuit et al., 1996; Nellen et al., 1996) and the Vestigial (Vg)/Scalloped (Sd) flight appendage selector protein complex (Guss et al., 2001), are both active in the wing and haltere (Weatherbee et al., 1998). Previous work has shown while other Dpp-regulated genes, such as *optomotor-blind/bifid*, are expressed in the haltere, the *sal* gene is repressed and thus selectively regulated by Ubx. Clones of cells that ectopically expressed Ubx and were located within the Sal expression domain in the wing exhibited cell-autonomous repression of Sal (Fig. 1C). Because

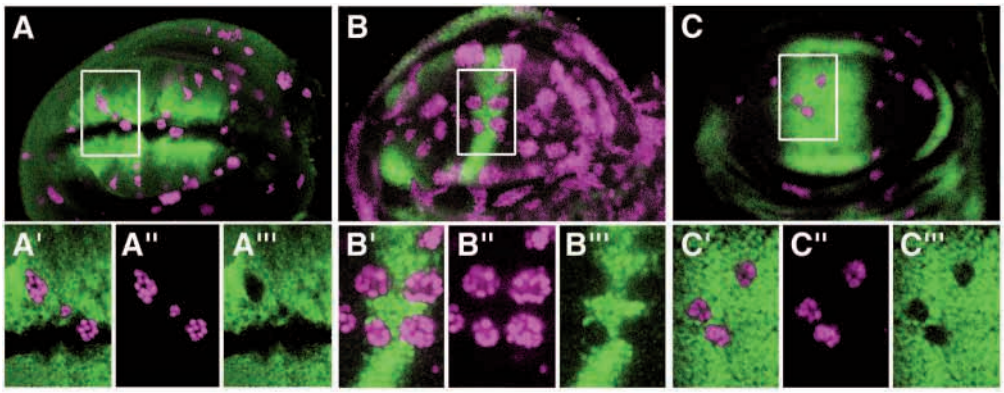


**Fig. 1.** Ubx cell-autonomously regulates several target genes in the wing. (A-C) Confocal photomicrographs of third instar wing discs that express various potential targets of Ubx regulation (green) and bear clones of cells ectopically expressing Ubx (purple). (A) The *vg*Q enhancer is cell autonomously repressed by Ubx in clones close to the DV boundary. (A′-A‴) Close-up views of the area boxed in A showing (A′) β-galactosidase expression driven by the *vg*Q enhancer and expression of Ubx together, (A″) Ubx alone, and (A‴) *vg*Q enhancer-driven reporter gene expression alone. (B) Ubx cell-autonomously represses Kn on the AP boundary. (B′-B‴) High-magnification views of the area boxed in B showing the expression of (B′) Ubx and Kn together, (B″) Ubx alone, and (B‴) Kn alone. (C) Sal is cell-autonomously repressed by Ubx. (C′-C‴) Close-up views of the area boxed in C showing the expression of (C′) Ubx and Sal together, and (C″) Ubx and (C‴) Sal alone. In each panel, ventral is to the top and anterior is to the left.

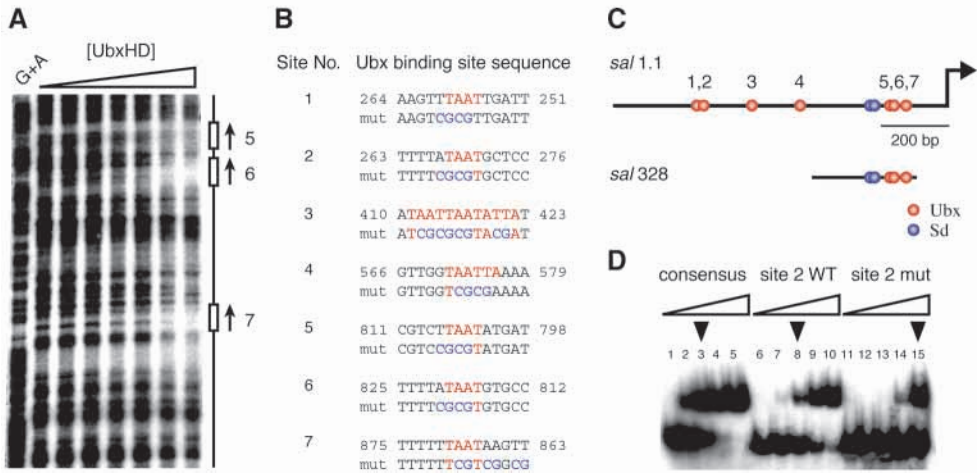


**Fig. 2.** Ubx binds to seven sites in a flight appendage-specific *cis*-regulatory element of *sal*. (A) DNase I footprinting of the *sal* 328 *cis*-regulatory element reveals three sites protected by Ubx homeodomain and is representative of footprinting of the entire *sal* 1.1 element. A G+A sequencing ladder is shown in the first lane, and DNase I digestions incubated with increasing concentrations of Ubx homeodomain from 0 to 90 ng, in three-fold increments, are shown in subsequent lanes. Sites numbered 5-7 are schematized to the right of the lanes and are represented by boxes; their orientation is indicated by the arrows. (B) A list of the sequences of the seven sites bound by Ubx in DNaseI footprinting assay showing 14 base pairs centered on the TAAT core sequence (highlighted in red). The numbers indicate the position of the sites within the 1.1 kb *sal* element. Below each Ubx binding site is the altered sequence (mut) that was introduced in the mutant *sal* elements to abolish the ability of Ubx to bind specifically to the site. The altered base pairs are highlighted in blue. (C) A schematic representation of the *sal* elements. The blue circles indicate Sd binding sites identified by Guss et al. (Guss et al., 2001), and red circles represent the seven Ubx binding sites identified by footprinting. We note that the *sal* 1.1 element contains other TAAT sites that were not footprinted by Ubx. (D) Gel shifts of oligos containing a Ubx consensus binding site (lanes 1-5), Ubx binding site 2 from the *sal* 1.1 element (lanes 6-10) or its mutant variant (lanes 11-15) using Ubx homeodomain protein indicate that the mutant variant of site 2 exhibits an approximate ten-fold decrease in affinity for Ubx. The open triangle indicates increasing concentrations of Ubx homeodomain, ranging from 0 to 30 ng in three-fold increments. The black arrowheads indicate the lane for each oligo in which binding of Ubx is closest to half-maximal.

both the *vg*Q enhancer and *DSRF* are regulated by Ubx at multiple levels, the analysis of their direct regulation by Ubx in vivo is complicated by these indirect effects. Therefore, we focused our molecular analyses on the regulation of the *sal* gene by Ubx.

## Sal is a direct target of Ubx in flight appendages

*sal* expression in the wing is regulated by a discrete *cis*-regulatory element that is directly activated by the Vg/Sd selector protein complex and is also bound by Mothers-against-dpp, a transcriptional effector of Dpp signaling (Guss et al., 2001). Both this 1.1 kb element (*sal* 1.1) and a smaller sub-element contained within it (*sal* 328) drove reporter gene expression in third instar wing discs, but no significant expression in haltere discs (see Fig. 3A,B,E,F,). Hence, these *sal* regulatory elements, like Sal, are repressed in the haltere and thus may be directly regulated by Ubx.

To test whether *sal* could be a direct target of Ubx repression, we searched for potential Ubx binding sites within the *sal* 1.1 element by DNaseI footprinting analysis with purified Ubx homeodomain protein. We identified seven regions protected by Ubx (a representative set of footprints is shown in Fig. 2A). Five of these regions contained one consensus TAAT core site (Fig. 2B, sites 1,2,5-7), one region contained two core sites (Fig. 2B, site 4) and one contained three core sites (Fig. 2B, site 3). The nucleotide sequences

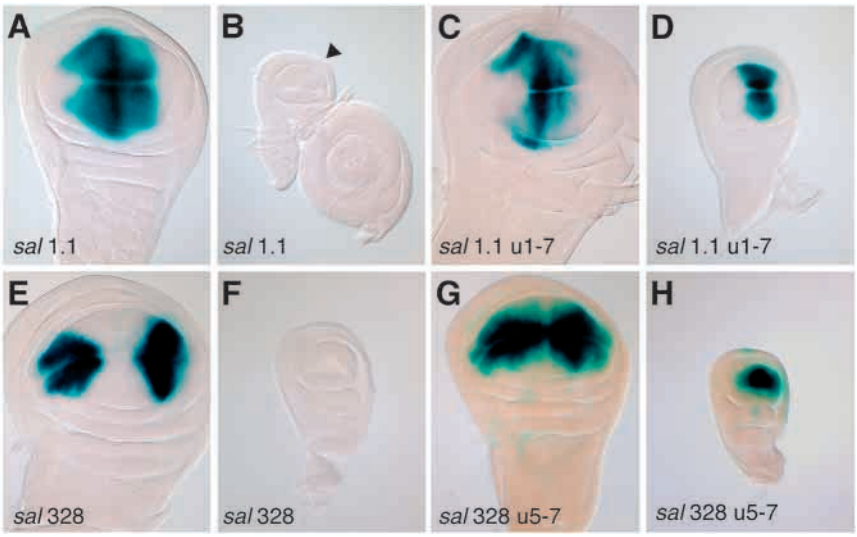


**Fig. 3.** (A-H) The *sal* flight appendage-specific *cis*-regulatory element is directly repressed in the haltere by Ubx. Nomarski photomicrographs of third larval instar wing (A,C,E,G) and haltere (B,D,F,H) imaginal disks assayed for β-galactosidase activity driven by various elements in transgenic animals carrying reporter constructs. In these panels, anterior is to the left and ventral is to the top. (A) The *sal* 1.1 element drives reporter activity in the wing field straddling AP boundary. (B) No *sal* 1.1 element driven reporter activity is seen in the haltere (arrowhead). (C) Mutation of all seven Ubx binding sites in the *sal* 1.1 u1-7 element does not alter the pattern of reporter activity in the wing. Therefore, the abilities of *trans*-activating factors required to activate the *sal* 1.1 element have not been affected by the mutations. (D) Mutating all seven Ubx binding sites in the *sal* 1.1 u1-7 element results its dramatic derepression in the haltere, as indicated by its ability to drive strong β-galactosidase activity in a pattern very similar to that in the wing. (E) The *sal* 328 element drives wing-specific reporter activity in a pattern complementary to that of the *sal* 1.1 element. (F) No reporter activity driven by this element is present in the haltere. (G) The mutant *sal* 328 u5-7 element in which the three Ubx binding sites were abolished drives β-galactosidase activity in the wing in a pattern largely similar to that driven by the wild-type *sal* 328 element. We note that reporter activity driven by the mutant element is expanded towards the AP boundary compared to that driven the wild-type one. This may have occurred because the Ubx binding site mutations in the *sal* 328 u5-7 element also affected binding sites for other transcription factors that regulate it. (H) The mutant *sal* 328 u5-7 element drives reporter activity very strongly in the haltere.

flanking the TAAT core site in all seven regions of Ubx binding in the *sal* 1.1 element showed characteristics of sites bound by Ubx in vitro (Ekker et al., 1991; Ekker et al., 1994). The sites protected by Ubx were scattered throughout the *sal* 1.1 element and three of these were located within the *sal* 328 element (Fig. 2C). The presence of these Ubx binding sites in the *sal* elements supports the possibility that *sal* may be directly repressed in vivo by Ubx.

If repression of the *sal* 1.1 and *sal* 328 elements in the haltere is directly mediated by Ubx binding to these sites, then abolishing the binding sites should result in derepression of reporter gene expression driven by the mutant elements in the haltere. To test this, we altered all seven of the Ubx binding sites within the *sal* 1.1 element and the three sites in the *sal* 328 element (shown in Fig. 2B) by introducing mutations that abolished the specific binding of Ubx to them (Ekker et al., 1994). We performed electromobility gel shift analyses on short oligonucleotide probes containing individual wild-type sites footprinted by Ubx or their mutant variants to verify that the affinity of Ubx protein for each mutant site was reduced to that of Ubx for non-specific DNA sequences (lacking a TAAT core sequence), at least ten-fold lower for each site than its wild-type counterpart (a representative example is shown in Fig. 2D). We then reintroduced these elements with mutated Ubx binding sites into a *lacZ* reporter gene vector and assayed β-galactosidase activity in developing wing and haltere imaginal disks from transgenic *Drosophila*. We observed that the mutant *sal* 1.1 element lacking all seven Ubx binding sites (*sal* 1.1 u1-7) now strongly drove β-galactosidase expression in the haltere along the anteroposterior boundary in a pattern similar to that in the wing (i.e. it was derepressed) (compare Fig. 3C,D). Similarly, the mutant *sal* 328 element in which three Ubx binding sites were altered (*sal* 328 u5-7) drove strong reporter gene expression in the haltere (Fig. 3G,H). We conclude that repression of the *sal* 1.1 and *sal* 328 elements in the haltere requires some or all of the Ubx binding sites we identified in vitro and that these elements are direct targets of Ubx repression.

### Individual Ubx binding sites contribute to but are not sufficient for complete repression of *sal* in the haltere

*Cis*-regulatory elements in *Drosophila* display a wide range of both the number and composition of Hox binding sites that contribute to their regulation, from an individual Hox/PBC compound binding site that can mediate a detectable level of target gene regulation (Vachon et al., 1992; White et al., 2000), to a very large number of apparently monomer Hox binding sites (Appel and Sakonju, 1993), to a combination of both compound Hox/Exd and Hox monomer binding sites (Capovilla and Botas, 1998; Capovilla et al., 1994; Chan et al., 1994; Manak et al., 1994; Sun et al., 1995). The diversity of Hox binding sites within *cis*-regulatory elements raises the question of whether Hox-regulation is mediated by a net effect through multiple binding sites or primarily through a single monomer or compound site? For most Hox-regulated elements the number and the nature of binding sites sufficient for regulation by Hox proteins is unknown and this is of primary importance in understanding the regulation of Hox target genes and their evolution. The properties of Ubx-regulation of the *sal* 328 *cis*-regulatory element presented an opportunity to investigate the scope of the ability of individual Ubx binding sites to contribute to repression.

We examined whether each of the individual Ubx binding sites contribute to repression of the *sal* 328 element in the haltere in two ways. First, we examined the necessity of each of the three binding sites for repression by mutating each one singly and examining β-galactosidase activity driven by *lacZ*

reporters for each of these elements in developing wings and halteres. Abolishing specific Ubx binding to sites 5 (Fig. 4A, right) and 6 (Fig. 4B, right) in the *sal* 328 element resulted in the ability of these single site mutant *sal* 328 elements to drive significant reporter activity above background levels in the haltere (i.e. partial derepression) while abolishing site 7 did not (Fig. 4C, right). Therefore, both Ubx binding sites 5 and 6 are necessary for complete repression of the *sal* 328 element, while Ubx binding site 7 is not.

Our second approach was to examine the degree to which these single sites were sufficient to impart repression of the *sal* 328 element in the haltere by mutating pairwise combinations of two Ubx binding sites at a time, leaving one site intact within each mutant element. We then assayed β-galactosidase activity driven by each of these elements. All three elements containing a single Ubx binding site drove reporter gene expression in haltere disks (Fig. 4D,E,F), indicating that no single Ubx binding site within the *sal* 328 element is sufficient to impart full repression by Ubx. However, we also observed that none of the single Ubx binding site elements was expressed at the same level in the haltere as was the triple mutant construct (compare Fig. 4D,E,F with Fig. 3H). These necessity and sufficiency tests demonstrate that each individual Ubx binding site contributes to the repression of the *sal* 328 element and that complete repression is a net effect of all three sites. Among the three Ubx binding sites, site 5 appeared to mediate the strongest level of repression of the *sal* 328 element (Fig. 4D). Because *Drosophila* haltere development does not require any activity of the Hox cofactor Exd and single Ubx binding sites appear to contribute to, but are insufficient for complete repression of, the *sal* 328 element by Ubx, we conclude that Ubx regulates this target *cis*-regulatory element in the absence of Exd via multiple monomer binding sites.

### Ubx regulates other target genes independently of Exd

The ability of Ubx to regulate genes in the haltere independently of Exd raised the question of whether Ubx can do so elsewhere during development, especially in tissues where Exd is present. Co-crystal structures of partial Hox/PBC complexes demonstrate that interactions between these two proteins are mediated via contacts between a YPWM peptide that is located upstream of Hox protein homeodomains and very highly conserved among Hox proteins and a hydrophobic pocket in the PBC protein (Passner et al., 1999; Piper et al., 1999). Many studies have shown that the YPWM motif is essential for Hox/PBC interactions (Chang et al., 1995; Johnson et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995) and both X-ray crystallographic and other biochemical experiments indicate that the tryptophan residue is critical for complex formation (Neuteboom et al., 1995; Passner et al., 1999; Phelan et al., 1995; Piper et al., 1999). To create a Ubx protein that should not interact with Exd, we mutated the YPWM motif in the Ubx protein to the sequence YAAA, a sequence that incorporates several mutations that have been shown to abolish the interaction between several Hox proteins and Exd (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995; Shanmugam et al., 1997). Interestingly however, this protein still displayed significant (but slightly reduced) cooperative binding with Exd to a compound Ubx/Exd site in vitro (Fig. 5A). Since this result was not expected based on the literature, we also tested another mutant version of the YPWM motif (AAAM). This protein also bound cooperatively with Exd to a compound site (data not shown). These results suggest that mutation of the YPWM alone is not sufficient to eliminate Ubx/Exd interactions in vitro in the presence of a DNA binding site. We note that the original report of Ubx/Exd interactions detected this interaction with a form of Ubx protein that lacked the YPWM motif (Chan et al., 1994), suggesting that other residues in the Ubx protein must also interact with Exd. Furthermore, it should be pointed out that co-crystallographic study of Ubx/Exd was performed with a form of Ubx that lacked most of the N terminus and all of the C terminus of the protein (Passner et al., 1999), so additional contacts that might involve these residues would not have been observable. Lastly, we note that yeast two-hybrid experiments that indicated a requirement for the YPWM motif were performed in the absence of target DNA sequences (Johnson et al., 1995). Based upon our data, it appears that Ubx and Exd monomers, when brought into proximity by the presence of a compound binding site, may still interact in vitro.

We tested the activity of the YAAA mutant Ubx protein by ectopically expressing it during *Drosophila* embryogenesis. Ectopic expression of wild-type Ubx during embryogenesis transforms third thoracic (T3) segmental identity to that of the first abdominal (A1) segment (compare Fig. 5B with 5C) (Mann and Hogness, 1990). However, ectopic expression of the Ubx YPWM→YAAA mutant protein transformed T3 segmental identity to that of A2 (Fig. 5C). Importantly, in an *exd* zygotic mutant, Ubx specifies A2 segmental identity and when ectopically expressed in an *exd* mutant background, Ubx induces transformations of thoracic segmental identities to that of A2 (Peifer and Wieschaus, 1990). Because Ubx specifies A1 identity in the presence of Exd, but A2 segmental identity in its absence, our results suggest that the A2 segmental identity transformation induced by the Ubx YAAA protein may be the result of Ubx action that is independent of Exd. This raises the possibility that the YAAA mutation affects the activity of the Ubx/Exd complex more so than in vitro binding to compound sites.

In order to ascertain whether specific target genes are regulated by this mutant Ubx protein, we examined reporter gene expression driven by a *Dll* embryonic limb enhancer. In the abdomen, this enhancer is directly repressed by Ubx (Vachon et al., 1992) and it has recently been proposed that this is mediated by a Ubx/Exd compound binding site (White et al., 2000). Ectopic expression of wild-type Ubx protein in the embryonic ectoderm repressed the *Dll* enhancer (Fig. 5E,F) (Chan and Mann, 1993; White et al., 2000). We also observed that ectopic expression of Ubx YAAA strongly repressed *Dll* (Fig. 5G). Previous work has shown that abolishing the YPWM motif in the Hox protein Labial caused both an increase in in vitro DNA-binding affinity and hyperactivity in the regulation of a target *cis*-regulatory element in vivo (Chan et al., 1996). We tested this possibility for our Ubx YAAA mutant, but observed no difference between the binding affinities of the Ubx and Ubx YAAA proteins for an oligonucleotide bearing the compound Ubx/Exd binding site from the *Dll* enhancer in electromobility gel shift assays (data not shown). Together, these results suggest that a Ubx protein with an altered YPWM motif may act independently of

Exd to regulate target genes but the in vitro data indicates that caution is necessary concerning whether this mutation affects Ubx binding to targets and/or Ubx activity in vivo.

## DISCUSSION

We have shown that three targets of Ubx in the haltere, the *vg*Q enhancer, *kn* and *sal*, are cell-autonomously regulated by Ubx and that the *sal* gene is directly repressed by Ubx. This repression is mediated by multiple Ubx binding sites in a flight appendage-specific *sal cis*-regulatory element in which each individual Ubx binding site contributes to the overall complete repression of gene expression. Because the development of the haltere does not require PBC or MEIS protein activity, we suggest that Ubx is acting through simple monomer sites. We further provide evidence that Ubx may regulate other target genes independently of the Hox DNA-binding cofactor Exd in tissues other than the haltere. These results show that Hox target gene selectivity can be achieved through several Hox monomer binding sites without requiring either PBC or MEIS protein activity. This has important implications regarding the mechanisms underlying the regulation and evolution of Hox target genes and the development and diversification of serially homologous structures.

### The regulation of Hox targets independent of Exd: Monomer Hox binding sites are required to mediate target gene regulation

The low DNA-binding specificities of Hox proteins in vitro have been a long standing problem in determining how various Hox proteins selectively regulate target genes in vivo. This challenge has been further compounded because relatively few direct Hox targets have been identified. Molecular studies of Hox target genes have been limited to several auto- and cross-regulated *Hox* gene *cis*-regulatory elements (Appel and Sakonju, 1993; Beachy et al., 1993; Beachy et al., 1988; Bergson and McGinnis, 1990; Chan et al., 1997; Dessain et al., 1992; Ferretti et al., 2000; Frasch et al., 1995; Gould et al., 1997; Grieder et al., 1997; Haerry and Gehring, 1996; Jacobs et al., 1999; Li and McGinnis, 1999; Maconochie et al., 1997; Malicki et al., 1992; Pinsonneault et al., 1997; Popperl et al., 1995; Regulski et al., 1991; Thuringer et al., 1993; Zeng et al., 1994) and to only six other target gene *cis*-regulatory elements known in *Drosophila* (Capovilla and Botas, 1998; Capovilla et al., 2001; Chan et al., 1994; Manak et al., 1994; McCormick et al., 1995; Pederson et al., 2000; Ryoo and Mann, 1999; Vachon et al., 1992). The regulation of all but two of these elements has been shown to involve Hox/PBC complexes that, compared to Hox proteins alone, exhibit an increase both in DNA-binding affinity and the size of the binding sites that they occupy in *cis*-regulatory elements. Target genes regulated in vivo by Hox/PBC protein complexes generally require a single ten base pair compound binding site consisting of an Exd binding site neighboring a Hox binding site for their regulation (Chan et al., 1996; Chan et al., 1997; Grieder et al., 1997; Jacobs et al., 1999; Popperl et al., 1995; Ryoo and Mann, 1999; Ryoo et al., 1999; White et al., 2000). In addition, several of these elements also require a MEIS binding site for their activation (Berthelsen et al., 1998; Jacobs et al., 1999; Ryoo et al., 1999). Altogether, a Hox/PBC compound binding site and a MEIS binding site span at least sixteen base pairs and will occur rarely at random (see below). Hence, Hox/PBC/MEIS interactions bestow upon Hox proteins a much greater capacity to select among many potential target genes within animal genomes.

Recent studies of Hox-regulated target genes have focused largely upon Hox/PBC/MEIS protein complexes and their binding sites. The question we address here is how Hox proteins selectively regulate the expression of target genes independently of these co-factors. Specifically, we examine the potential contribution of single Hox monomer binding sites to the repression of Hox target genes, which we suggest has been largely underestimated. We have shown that several Ubx binding sites individually mediate partial repression of the *sal* 328 element in the haltere (Fig. 4D-F) and that the additive contribution of three sites completely represses *sal* expression (Fig. 3B,F). Because these sites are apparently simple monomer sites and the activities of Exd and Hth are not genetically required for Ubx function in the development of the haltere (Azpiazu and Morata, 1998; Azpiazu and Morata, 2000; Casares and Mann, 2000; González-Crespo and Morata, 1995), Ubx repression of the *sal* element is clearly independent of Exd or Hth. Our results suggest that Hox proteins do regulate the activity of *cis*-elements through single monomer binding sites without requiring either of the currently identified DNA-binding Hox protein cofactors and these provide support for the 'widespread binding' model for Hox protein selectivity (Biggin and McGinnis, 1997).

We have not ruled out the possible existence of unidentified DNA-binding cofactors that may be required for Ubx to repress target genes. One would predict that such a DNA-bin**d**ing cofactor of Ubx would bind to similar flanking sequences that are shared among Ubx binding sites within a *cis*-regulatory element, as is the case for Ubx/Exd composite sites. However, we do not observe any shared motifs outside of the core Ubx binding site sequences among sites 5, 6 and 7 (Fig. 2B). Rather, we believe that the evidence points toward the ability of Hox proteins to mediate Hox repression through monomer binding sites without requiring other DNA-binding cofactors. Three previous studies of Hox-regulated *cis*-elements have demonstrated or implicated the ability of apparent individual Hox monomer binding sites to contribute to target gene regulation. In a remarkable example, the *Antp* gene contains as many as 41 Ubx binding sites in its P2 *cis*-regulatory element, a large number of which are required for its repression (Appel and Sakonju, 1993). In another case, in addition to two Exd binding sites and a Hox/Exd compound binding site that is required to regulate the *dpp* midgut enhancer, seven Ubx/Abdominal-A (Abd-A) monomer binding sites also contribute to target gene regulation (Capovilla and Botas, 1998; Capovilla et al., 1994). Furthermore, Pederson et al. (Pederson et al., 2000) have shown that Deformed may regulate a target independently of Exd. Here, we have provided evidence that individual Ubx monomer binding sites contribute to and that just three sites are sufficient for, the complete repression of the *sal* 328 element (Fig. 4D-F). Therefore, Hox repression of *cis*-regulatory elements may be realized through the net, additive effect of Hox proteins binding to multiple monomer sites that individually mediate weak regulation.

Hox regulation of the activity of *cis*-regulatory elements through multiple monomer binding sites may operate through a

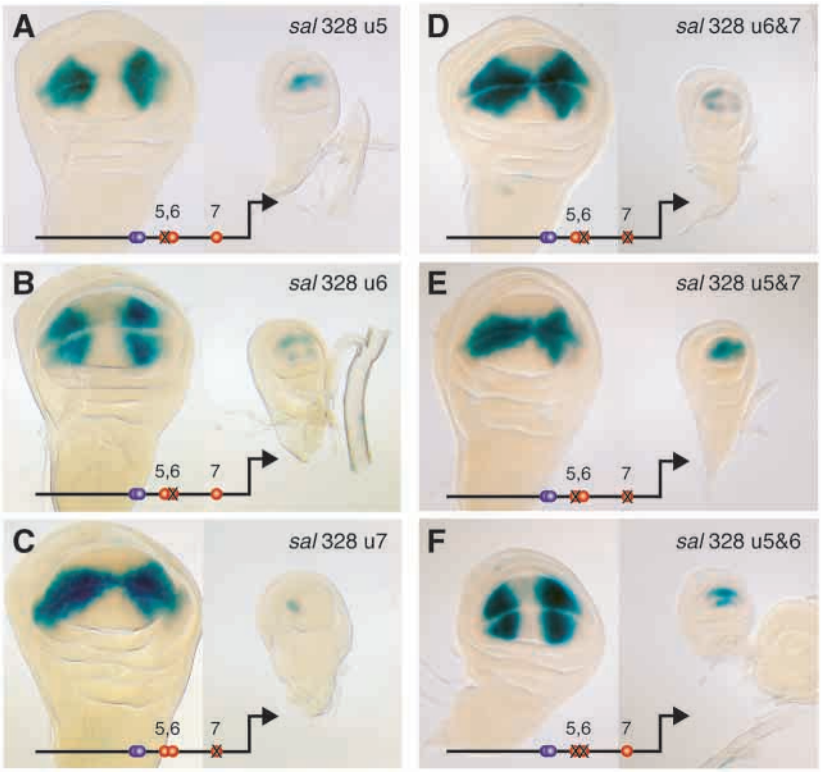


**Fig. 4.** Multiple Ubx binding sites are necessary for complete repression of a *sal* flight appendage *cis*-regulatory element and individual sites are sufficient to mediate its partial repression in the haltere. (A-F) Nomarski photomicrographs of third instar wing (left) and haltere (right) imaginal discs assayed for β-galactosidase activity driven by various Ubx binding site mutant *sal* 328 elements to test the necessity (A-C) and sufficiency (D-F) of individual Ubx binding sites. Schematic representations of the mutant *sal* 328 elements are displayed at the bottom of each panel. Binding sites for Ubx and Sd are indicated as in Fig. 2C, and mutant binding sites are designated by an 'X' over them. (A, left) The *sal* 328 u5 single binding site mutant element drives reporter activity in the wing in a pattern similar to that of the wild-type element (Fig. 2E). (A, right) The *sal* 328 u5 element drives reporter activity in the haltere, demonstrating that Ubx binding site 5 is necessary for complete repression of the *sal* 328 element. (B, left) The *sal* 328 u6 element drives reporter gene activity in the wing in a pattern similar to the wild-type element. (B, right) The *sal* 328 u6 element drives some reporter activity in the haltere, and it is weaker than the *sal* 328 u5 element (compare to A, right). Therefore, Ubx binding site 6 is necessary to completely repress the *sal 328* element, but binding site 5 mediates stronger repression. (C, left) The *sal* 328 u7 element drives β-galactosidase activity in the wing. Because this pattern is similar to that of the *sal* 328 u5-7 element (see Fig. 3G), it appears that the mutant Ubx binding site 7 is probably responsible for the difference in the reporter expression patterns driven in the wing by the *sal* 328 wild-type and *sal* 328 u5-7 elements, as well as other elements in which binding site 7 is mutant. (C, right) The *sal* 328 u7 element drives barely detectable levels of reporter activity. The small region of reporter activity has also been observed by overstaining of discs carrying the wild-type *sal* 328 element. Binding site 7 appears not to be necessary for complete repression of the *sal* 328 element. (D, left) The *sal 328* u6&7 mutant element bearing only Ubx site 5 drives reporter activity in the wing in a pattern that is similar to that of the wild-type *sal* 328 element (Fig. 2E), but it is expanded towards the AP boundary. (D, right) The sal 328 u6&7 element drives reporter activity in the haltere, but not to the level observed for the *sal* 328 u5-7 element (compare to Fig. 3H). Therefore, Ubx binding site 5 alone can mediate partial repression of the *sal* 328 element by Ubx. (E, left) The *sal 328* u5&7 element bearing only Ubx site 6 drives reporter activity in a pattern similar to that of the *sal* 328 u6&7 element in the wing. (E, right) The *sal* 328 u6&7 element drives reporter activity in the haltere at nearly the level observed for the triple mutant *sal* 328 u5-7 element. This indicates that Ubx binding site 6 alone can mediate only a small degree of repression of the *sal* 328 element. (F, left) The *sal* 328 u5&6 element bearing only Ubx site 7 drives β-galactosidase activity in the wing in a pattern very similar to that of the wild-type *sal* 328 element. (F, right) The *sal* 328 u5&6 element drives reporter activity in the haltere at a lower level than the *sal* 328 u5-7 element, but at a higher level than the *sal* u6&7 element. Therefore, Ubx binding site 7 can mediate partial repression of the *sal* 328 element in the haltere.

number of different modes. For instance, the presence of multiple sites may increase Hox-binding site occupancy in *cis*-elements either through cooperative interactions between Hox proteins (Beachy et al., 1993) or by increasing the probability that a site is bound by a Hox protein by virtue of a large number of sites (i.e. the more Hox binding sites, the greater the probability that any one site is bound by a Hox protein at a given time). Because Hox/PBC/MEIS compound sites also serve to increase binding site occupancy by Hox proteins, these mechanisms may serve essentially equivalent functional roles in regulating *cis*-elements. Hox proteins may also regulate some of their targets without requiring PBC or MEIS cofactors by binding to Hox monomer sites that are positioned in specific sequence contexts, such as their proximity to other activator or repressor binding sites within *cis*-regulatory elements. For instance, among the three Ubx binding sites within the *sal* 328 element, Ubx binding site 5 is the closest to the Sd binding sites that are required for the activation of this *cis*-regulatory element (Fig. 2C) and it appears to mediate the strongest repression (Fig. 4D).

Repression by Ubx could be mediated by a number of mechanisms, including steric hindrance of Sd or other transcriptional activators from binding to the *sal* 328 element or by affecting local chromatin structure. We have recently localized a motif at the C terminus of the Ubx protein that is involved in repression activity (Galant and Carroll, 2002). This motif may potentiate repression activity through interactions with components of the basal or activated transcriptional machinery, or by recruiting corepressors. Such interactions may be sufficient to account for the ability of single monomer binding sites to affect gene regulation.

## The diversification of Hox-differentiated serial homologs: quantitative to qualitative regulation

The evolution of Hox target genes has accompanied a major evolutionary trend of diversification of animal body plans and

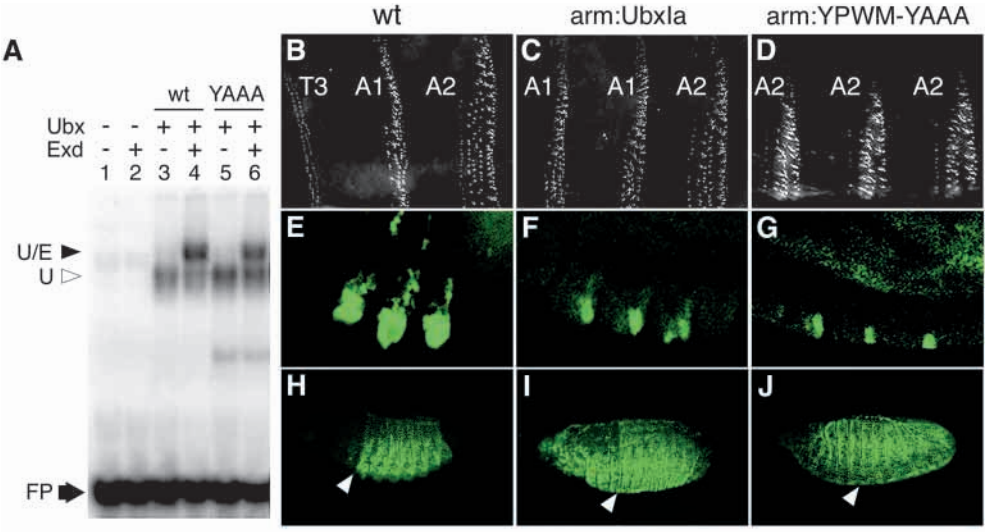


**Fig. 5.** The activity of a Ubx protein lacking the YPWM Exd-interaction motif. (A) Electromobility shift analysis of an oligonucleotide probe bearing a consensus composite Ubx/Exd binding site using wild-type and YPWM-YAAA mutant Ubx proteins with and without Exd. Exd alone does not bind the oligo (lane 2). Together with Exd, wild-type Ubx protein binds with higher affinity than Ubx alone (compare lanes 3 and 4). Surprisingly, the Ubx YPWM-YAAA mutant protein also exhibits an increased binding affinity for a composite Ubx/Exd binding site when complexed with Exd (compare lanes 5 and 6), although not as great an increase as exhibited by wild-type Ubx and Exd (20% less) (compare lanes 4 and 6). The Ubx YAAA mutant protein alone exhibits a slight increase in binding to the probe than the wild-type protein (~1.5-fold) (compare lanes 3 and 5). The closed arrowhead indicates the position of shifts due to Ubx/Exd complexes, the open arrowhead indicates Ubx shifts, and the arrow indicates the position of free probe. (B-D) Dark-field photomicrographs of cuticle preparations showing ventral denticle belts in the third thoracic segment (T3) and the first and second abdominal segments (A1 and A2, respectively), from left to right. Segmental identities are indicated next to each of the three denticle belts in each panel. (E-G) Confocal photomicrographs of the three thoracic segments in embryos carrying a β-galactosidase reporter *trans*-gene driven by the *Dll* embryonic limb enhancer and stained with anti-β-galactosidase antibody (green). (H-J) Confocal photomicrographs of embryos stained for Ubx protein (green). White arrowheads indicate the boundary between A1 and T3. Ectopic expression of the Ubx proteins is driven by arm$^{11}$-Gal4. In all images, anterior is to the left and ventral is down. (B) In wild-type larvae, the T3, A1 and A2 denticle belts each have distinct morphologies. The T3 denticle belt comprises two rows of small hairs, the A1 denticle belt comprises four rows of larger hairs, and the A2 denticle belt comprises six rows arranged in a trapezoidal shape. (C) The ectopic expression of wild-type Ubx protein transforms T3 segmental identity to that of A1. Thus, wild-type Ubx specifies A1 segmental identity. (D) Ectopically expressing the Ubx YAAA mutant protein induces segmental identity transformations to A2 in the T3 and A1 segments. Therefore, the Ubx YAAA mutant protein specifies A2 segmental identity, a phenotype consistent with the inability of this protein to physically interact with Exd. (E) The *Dll*304 embryonic limb enhancer drives reporter gene expression in the three limb primordia in wild-type embryos. (F) Ecoptic expression of Ubx strongly represses the *Dll*304 enhancer. (G) Ectopic expression of the Ubx YAAA mutant protein similarly represses the *Dll*304 enhancer, indicating that an interaction between Ubx and Exd is not required to repress an embryonic target gene. (H) In a wild-type embryo, the anterior boundary of Ubx expression is posterior T2. (I) Ectopic expression of wild-type UbxIa protein occurs anterior to its normal anterior boundary in thoracic and head segments. (J) The Ubx YAAA protein is ectopically expressed at levels similar to ectopic UbxIa (I).

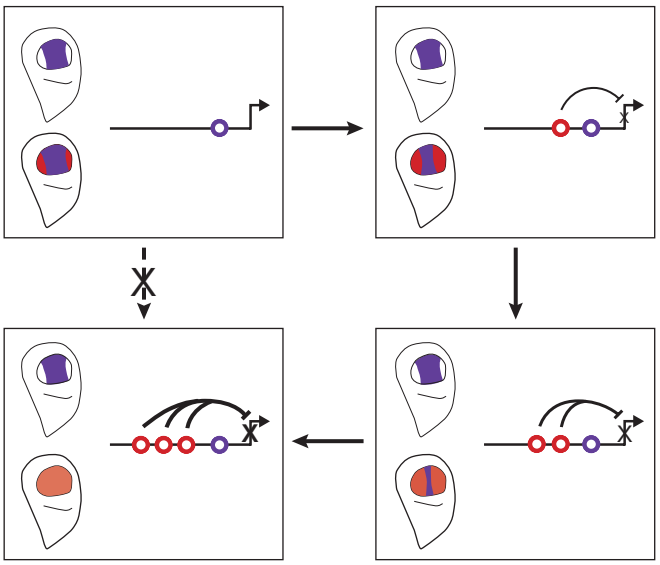

**Fig. 6.** The evolution of Hox target gene regulation by the stepwise accumulation of monomer binding sites. Represented are two serially homologous structures, one of which is under Hox control (shaded red). The schematic of a *cis*-regulatory element is shown to the right of each pair of serial homologs. Its expression is indicated by the blue pattern in each structure and is mediated through a binding site (blue circle) for a transcriptional activator. Our model posits that the repression of a target gene by a Hox protein begins with the evolution of a single Hox monomer binding site (red circle) that can mediate partial repression of the activity of a *cis*-regulatory element. If the binding site becomes fixed, then directional selection for a further decrease in gene activity can fix additional Hox monomer sites that increase the repression of the *cis*-regulatory element and eventually lead to its qualitative repression. Thus, the qualitative regulation of Hox target genes evolves in a gradual, stepwise fashion and not all at once (the pathway indicated by the crossed out, dotted arrow).

morphologies (reviewed by Carroll et al., 2001). Several studies have demonstrated that changes in Hox target gene regulation are correlated with morphological differences among homologous body regions (Belting et al., 1998; Kopp et al., 2000; Palopoli and Patel, 1998; Warren et al., 1994; Weatherbee et al., 1999). For example, it has been postulated that the serially homologous fore- and hindwings of the four-winged ancestor of Dipterans had largely identical morphologies and presumably largely identical gene

expression patterns and that during the evolution of the Dipteran haltere from this ancestral state, a subset of genes involved in flight appendage development became repressed by Ubx (Weatherbee et al., 1999). Elucidating the molecular mechanisms by which Hox proteins selectively regulate *cis*-elements is therefore key to understanding how Hox target genes may have evolved and contributed to such events.

Our findings that individual Hox monomer binding sites can contribute to Hox target gene regulation and that as few as three sites are sufficient to mediate complete repression suggest a simple, but potentially very important mechanism that may underlie Hox target gene evolution. We propose that the evolution of the Hox-repression of a target gene involves the stepwise accumulation of Hox monomer binding sites within *cis*-regulatory elements and progresses from initially quantitative differences in the activity of a *cis*-element to a qualitative difference (i.e. full repression) (Fig. 6). Our scenario is as follows: (i) point mutations can easily give rise to a single monomer Hox binding site that mediates partial repression of the activity of a *cis*-regulatory element in a tissue that expresses a given Hox protein (Fig. 6, red shading); (ii) if the Hox binding site becomes fixed under selection for a decreased level of gene activity in this tissue, then (iii) directional selection on the activity of a *cis*-regulatory element can fix additional sites that further repress it; and (iv) the evolution of several Hox monomer binding sites in the *cis*-element can eventually lead to its full repression and thus a qualitative difference in gene expression.

This scenario suggests that Hox target genes can evolve gradually through multiple intermediate steps. We favor this view because of the greater probability of functional sites arising as monomers and because smaller, gradual changes in gene regulation are likely to be less deleterious to development than larger ones. The rate at which target gene *cis*-elements can evolve regulation by Hox proteins is determined by the number and composition of the binding sites required for Hox proteins to regulate the activity of *cis*-regulatory elements. Hox monomer binding sites (TAAT[g/t][g/a]) should occur approximately once every 500 base pairs, on average. However, high affinity Hox/PBC binding sites (TGATNNAT[g/t][g/a]) should occur around 16 times less frequently (≈1/8,200 base pairs). If composite Hox/PBC and MEIS binding sites were minimally required to mediate Hox-regulation, then no fewer than 16 specific base pairs would need to evolve within a *cis*-regulatory element and the occurrence of combinations of a high affinity Hox/PBC binding site and a high affinity MEIS binding site (CTGTCA) located within 20 base pairs of each other is more than 800 times less probable (≈1/420,000 base pairs) than the occurrence of Hox monomer binding sites. These probabilities indicate that a Hox monomer binding site is much more likely to arise de novo than either a Hox/PBC compound site or a Hox/PBC/MEIS trimeric binding site. In this manner, the low DNA-binding specificities of Hox proteins may actually facilitate the evolution of new binding sites. The yeast alpha2 homeodomain repressor protein has a similarly low DNA-binding specificity and this results in its ability to repress target genes in vivo through many different DNA sequences (Smith and Johnson, 1994). Because Hox monomer sites appear to be functional, selection can favor the evolution of higher affinity Hox/PBC composite and Hox/PBC/MEIS trimeric sites through the sequential modification of monomer sites.

Hox proteins have played a central role in the diversification of serially homologous structures. Subsets of serially homologous structures express different Hox proteins (e.g. regions of vertebrate hindbrains, vertebrae, insect flight appendages and arthropod body segments), thereby uncoupling the development of serial homologs and enabling them to follow independent evolutionary trajectories. This regulatory logic facilitated the evolution of morphological differences between thoracic and cervical vertebral identities among vertebrates (Burke et al., 1995; Cohn and Tickle, 1999), between wings and halteres in Dipterans (Warren et al., 1994; Weatherbee et al., 1998) and the vast diversity of appendage number, shape and function in arthropods (reviewed by Gellon and McGinnis, 1998; Carroll et al., 2001). We suggest that the accumulation of simple Hox protein binding sites within *cis*-regulatory elements that direct gene expression in serial homologs has played an important role in the evolution of morphological diversity. This is certainly the case in the distal appendages of arthropods and vertebrates where the activity of neither PBC nor MEIS proteins is required and is perhaps a general mechanism governing the development and evolution of Hox-regulated characters.

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## REFERENCES

**Abu-Shaar, M. and Mann, R. S.** (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* **125**, 3821-3830.

**Abu-Shaar, M., Ryoo, H. D. and Mann, R. S.** (1999). Control of the nuclear localization of Extradenticle by competing nuclear import and export signals. *Genes Dev.* **13**, 935-945.

**Abzhanov, A. and Kaufman, T.** (2000). Crustacean (malacostracan) Hox genes and the evolution of the arthropod trunk. *Development* **127**, 2239-2249.

**Appel, B. and 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.

**Averof, M. and Akam, M.** (1995). Insect-crustacean relationships: insights from comparative developmental and molecular studies. *Proc. R. Soc. Lond.* **347**, 293-303.

**Averof, M. and Patel, N. H.** (1997). Crustacean appendage evolution associated with changes in *Hox* gene expression. *Nature* **388**, 682-686.

**Azpiazu, N. and Morata, G.** (1998). Functional and regulatory interactions between Hox and extradenticle genes. *Genes Dev.* **12**, 261-273.

**Azpiazu, N. and Morata, G.** (2000). Function and regulation of homothorax in the wing imaginal disc of *Drosophila*. *Development* **127**, 2685-2693.

**Beachy, P., Varkey, J., Young, K., von Kessler, D., Sun, B. and Ekker, S.** (1993). Cooperative binding of an Ultrabithorax homeodomain protein to nearby and distant DNA sites. *Mol. Cell. Biol.* **13**, 6941-6956.

**Beachy, P. A., Krasnow, M. A., Gavis, E. R. and Hogness, D. S.** (1988). An *Ultrabithorax* protein binds sequences near its own and the *Antennapedia* P1 promoters. *Cell* **55**, 1069-1081.

**Belting, H.-G., Shashikant, C. S. and Ruddle, F. H.** (1998). Modification of expression and *cis*-regulation of *Hoxc8* in the evolution of diverged axial morphology. *Proc. Natl. Acad. Sci. USA* **95**, 2355-2360.

**Bergson, C. and McGinnis, W.** (1990). An autoregulatory enhancer element of the *Drosophila* homeotic gene *Deformed*. *EMBO J.* **9**, 4287-4297.
**Berthelsen, J.** (1999). The subcellular localization of PBX1 and EXD proteins depends on nuclear import and export signals and is modulated by association with PREP1 and HTH. *Genes Dev.* **13**, 946-953.
**Berthelsen, J., Zappavigna, V., Mavilio, F. and Blasi, F.** (1998). Prep1, a novel functional partner of Pbx proteins. *EMBO J.* **17**, 1423-1433.
**Biggin, M. D. and 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.
**Brand, A. and Perrimon, N.** (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
**Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C.** (1995). *Hox* genes and the evolution of vertebrate axial morphology. *Development* **121**, 333-346.
**Capdevila, J., Tsukui, T., Rodriquez Esteban, C., Zappavigna, V. and Izpisua Belmonte, J.** (1999). Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* **4**, 839-849.
**Capovilla, M. and Botas, J.** (1998). Functional dominance among *Hox* genes: repression dominates activation in the regulation of Dpp. *Development* **125**, 4949-4957.
**Capovilla, M., Brandt, M. and Botas, F.** (1994). Direct regulation of decapentaplegic by Ultrabithorax and its role in Drosophila midgut morphogenesis. *Cell* **76**, 461-475.
**Capovilla, M., Kambris, Z. and Botas, J.** (2001). Direct regulation of the muscle-identity gene *apterous* by a Hox protein in the somatic mesoderm. *Development* **128**, 1221-1230.
**Carroll, S., Weatherbee, S. and Langeland, J.** (1995). Homeotic genes and the regulation and evolution of insect wing number. *Nature* **375**, 58-61.
**Carroll, S. B., Grenier, J. K. and Weatherbee, S. D.** (2001). *From DNA to Diversity: Molecular Genetics and the Evolution of Animal Design.* Malden: Blackwell Scientific.
**Casares, F. and Mann, R.** (2000). A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*. *Development* **127**, 1499-1508.
**Casares, F. and Mann, R. S.** (1998). Control of antennal versus leg development in *Drosophila*. *Nature* **392**, 723-726.
**Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M.** (1994). Dissecting the temporal requirements for homeotic gene function. *Development* **120**, 1983-1985.
**Chan, S., Popperl, H., Krumlauf, R. and Mann, R.** (1996). An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.* **15**, 2476-2487.
**Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J. and Mann, S.** (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with Extradenticle, another homeoprotein. *Cell* **78**, 603-615.
**Chan, S. K. and Mann, R. S.** (1993). The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. *Genes Dev.* **7**, 796-811.
**Chan, S. K., Ryoo, H. D., Gould, A., Krumlauf, R. and Mann, R. S.** (1997). Switching the in vivo specificity of a minimal Hox-responsive element. *Development* **124**, 2007-2014.
**Chang, C.-P., Shen, W.-F., Rozenfeld, S. and Lawrence, H. J.** (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.* **9**, 663-674.
**Cohn, M. J. and Tickle, C.** (1999). Developmental basis of limblessness and axial patterning in snakes. *Nature* **399**, 474-479.
**Crozatier, M. and Vincent, A.** (1999). Requirement for the *Drosophila* COE transcription factor Collier in formation of an embryonic muscle: transcriptional response to notch signalling. *Development* **126**, 1495-1504.
**de Celis, J., Barrio, R. and Kafatos, F.** (1996). A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis. *Nature* **381**, 421-424.
**Dessain, S., Gross, C. T., Kuziora, M. A. and McGinnis, W.** (1992). *Antp*-type homeodomains have distinct DNA-binding specificities that correlate with their different regulatory functions in embryos. *EMBO J.* **11**, 991-1002.
**Ekker, S., Young, K., von Kessler, D. and Beachy, P.** (1991). Optimal DNA sequence recognition by the *Ultrabithorax* homeodomain of *Drosophila*. *EMBO J.* **10**, 1179-1189.
**Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A.** (1994). The degree of variation in DNA sequence recognition among four *Drosophila* homeotic proteins. *EMBO J.* **13**, 3551-3560.
**Ferretti, E., Marshall, H., Popperl, H., Macoachle, M., Krumlauf, R. and Blasi, F.** (2000). Segmental expression of Hoxb2 in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. *Development* **127**, 155-166.
**Frasch, M., Chen, X. and Lufkin, T.** (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and *Drosophila*. *Development* **121**, 945-974.
**Galant, R. and Carroll, S. B.** (2002). Evolution of a novel transcripitional repression domain in the Ultrabithorax Hox protein in insects. *Nature* **415**, 848-849.
**Gellon, G. and McGinnis, W.** (1998). Shaping animal body plans in development and evolution by modulation of *Hox* expression patterns. *BioEssays* **20**, 116-125.
**Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez-A., C., Mann, R. S. and Morata, G.** (1998). Antagonism between *extradenticle* function and Hedgehog signalling in the developing limb. *Nature* **394**, 196-200.
**Gonzalez-Crespo, S. and Morata, G.** (1996). Genetic evidence for the subdivision of the arthropod limb into coxopodite and telopodite. *Development* **122**, 3921-3928.
**González-Crespo, S. and Morata, G.** (1995). Control of *Drosophila* adult pattern by *extradenticle*. *Development* **121**, 2117-2125.
**Gould, A., Morrison, A., Sproat, G., White, R. A. H. and Krumlauf, R.** (1997). Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping Hox expression patterns. *Genes Dev.* **11**, 900-913.
**Grenier, J., Garber, T., Warren, R., Whitington, P. and Carroll, S.** (1997). Evolution of the entire arthropod *Hox* gene set predated the origin and radiation of the onychophoran/arthropod clade. *Curr. Biol.* **7**, 547-553.
**Grieder, N., Marty, T., Ryco, H., Mann, R. and Affolter, M.** (1997). Synergistic activation of a Drosophila enhancer by HOM/EXD and DPP signaling. *EMBO J.* **16**, 7402-7410.
**Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. and Carroll, S. B.** (2001). Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164-1167.
**Haerry, T. and Gehring, W.** (1996). Intron of the mouse Hoxa-7 gene contains conserved homeodomain binding sites that can function as an enhancer element in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **93**, 13884-13889.
**Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W. J.** (1998). *Eyeless* initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development* **125**, 2181-2191.
**Jacobs, D.** (1990). Selector genes and the Cambrian radiation of Bilateria. *Proc. Natl. Acad. Sci. USA* **87**, 4406-4410.
**Jacobs, Y., Schnabel, C. and Cleary, M.** (1999). Trimeric association of Hox and TALE homeodomain proteins mediates Hoxb2 hindbrain enhancer activity. *Mol. Cell* **19**, 134-142.
**Johnson, F. B., Parker, E. and Krasnow, M. A.** (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.
**Kaufman, T. C., Seeger, M. A. and Olsen, G.** (1990). Molecular and genetic organization of the Antennapedia gene complex of *Drosophila melanogaster*. *Adv. Genet.* **27**, 309-362.
**Kelsh, R., Weinzierl, R., White, R. and Akam, M.** (1994). Homeotic gene expression in the locust *Schistocerca:* An antibody that detects conserved epitopes in Ultrabithorax and abdominal-A genes. *Dev. Genet.* **15**, 19-31.
**Knoepfler, P. and Kamps, M.** (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.
**Kopp, A., Duncan, I., Godt, D. and Carroll, S. B.** (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408**, 533-559.
**Lecuit, T., Brook, W., Ng, M., Calleja, M., Sun, H. and Cohen, S.** (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387-393.
**Lewis, E. B.** (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 565-570.
**Li, X. and McGinnis, W.** (1999). Activity regulation of Hox proteins, a mechanism for altering functional specificity in development and evolution. *Proc. Natl. Acad. Sci. USA* **96**, 6802-6807.
**Maconochie, M., Nonchev, S., Studer, M., Chan, S., Popperl, H., Sham, M., Mann, R. and Krumlauf, R.** (1997). Cross-regulation in the mouse

HoxB complex: the expression of Hoxb2 in rhombomere 4 is regulated by Hoxb1. *Genes Dev.* **11**, 1885-1895.

**Malicki, J., Clianetti, L. C., Peschle, C. and McGinnis, W.** (1992). A human HOX4B regulatory element provides head-specific expression in Drosophila embryos. *Nature* **358**, 345-347.

**Manak, J. R., Mathies, L. D. and Scott, M. P.** (1994). Regulation of a decapentaplegic midgut enhancer by homeotic proteins. *Development* **120**, 3605-3619.

**Mann, R. S. and Affolter, M.** (1998). Hox proteins meet more partners. *Curr. Opin. Genet. Dev.* **8**, 423-429.

**Mann, R. S. and Hogness, D. S.** (1990). Functional dissection of Ultrabithorax proteins in D. melanogaster. *Cell* **60**, 597-610.

**Mann, R. S. and Morata, G.** (2000). The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Annu. Rev. Cell Dev. Biol.* **16**, 243-271.

**McCormick, A., Core, N., Kerridge, S. and Scott, M.** (1995). Homeotic response elements are tightly linked to tissue-specific elements in a transcriptional enhancer of the *teashirt* gene. *Development* **121**, 2799-2812.

**Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez, C. and Torres, M.** (1999). Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* **402**, 425-429.

**Mercader, N., Leonardo, E., Piedra, M., Martinez-A., C., Ros, M. and Torres, M.** (2000). Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development* **127**, 3961-3970.

**Montagne, J., Groppe, J., Guillemin, K., Krasnow, M., Gehring, W. and Affolter, M.** (1996). The *Drosophila* serum response factor gene is required for the formation of intervein tissue of the wing and is allelic to *blistered*. *Development* **122**, 2589-2597.

**Nellen, D., Burke, R., Struhl, G. and Basler, K.** (1996). Direct and long-range actions of a Dpp morphogen gradient. *Cell* **85**, 357-368.

**Nelson, H. and Laughon, A.** (1993). The DNA binding specificity of the Drosophila fushi tarazu protein: a possible role for DNA bending in homeodomain recognition. *Roux's Arch. Dev. Biol.* **202**, 341-354.

**Neuteboom, S., Peltenburg, L., van Dijk, M. and Murre, C.** (1995). The hexapeptide LFPWMR in Hoxb-8 is required for cooperative DNA binding with Pbx1 and Pbx2 proteins. *Proc. Natl. Acad. Sci. USA* **92**, 166-170.

**Palopoli, M. F. and Patel, N. H.** (1998). Evolution of the interaction between *Hox* genes and a downstream target. *Curr. Biol.* **8**, 587-590.

**Panganiban, G., Sebring, A., Nagy, L. and Carroll, S.** (1995). The development of crustacean limbs and the evolution of arthropods. *Science* **270**, 1363-1366.

**Passner, J., Ryoo, H., Shen, L., Mann, R. and Aggarwal, A.** (1999). Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature* **397**, 714-719.

**Pederson, J. A., LaFollette, J. W., Gross, G., Veraksa, A., McGinnis, W. and Mahaffey, J. W.** (2000). Regulation by homeoproteins: a comparison of Deformed-responsive elements. *Genetics* **156**, 677-686.

**Peifer, M. and Wieschaus, E.** (1990). Mutations in the *Drosophila* gene extradenticle affect the way specific homeo domain proteins regulate segmental identity. *Genes Dev.* **4**, 1209-1223.

**Phelan, M., Rambaldi, I. and Featherstone, M.** (1995). Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* **1995**, 3989-3997.

**Pignoni, F. and Zipursky, S.** (1997). Induction of *Drosophila* eye development by Decapentaplegic. *Development* **124**, 271-278.

**Pinsonneault, J., Florence, B., Vaessin, H. and McGinnis, W.** (1997). A model for *extradenticle* function as a switch that changes Hox proteins from repressors to activators. *EMBO J.* **116**, 2032-2042.

**Piper, D., Batchelor, A., Chang, C., Cleary, M. and Wolberger, C.** (1999). Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. *Cell* **96**, 587-597.

**Popperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R.** (1995). Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. *Cell* **81**, 1031-1042.

**Rauskolb, C., Smith, K., Peifer, M. and Wieschaus, E.** (1995). *extradenticle* determines segmental identities throughout development. *Development* **121**, 3663-3671.

**Regulski, M., Dessain, S., McGinnis, N. and 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.

**Rieckhof, G., Casares, F., Ryoo, H., Abu-Shaar, M. and Mann, R. S.** (1997). Nuclear translocation of Extradenticle requires *homothorax*, which encodes an Extradenticle-related homeodomain protein. *Cell* **91**, 171-183.

**Roberts, D. B.** (1998). *Drosophila: A Practical Approach.* New York: Oxford University Press.

**Rogers, B., Peterson, M. and Kaufman, T.** (1997). Evolution of the insect body plan as revealed by the Sex combs reduced expression pattern. *Development* **124**, 149-157.

**Ryoo, H. D. and Mann, R. S.** (1999). The control of trunk Hox specificity and activity by Extradenticle. *Genes Dev.* **13**, 1704-1716.

**Ryoo, H. D., Marty, T., Casares, F., Affolter, M. and Mann, R. S.** (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* **126**, 5137-5148.

**Selleri, L., Depew, M., Jacobs, Y., Chanda, S., Tsang, K., Cheah, K., Rubenstein, J., O'Gorman, S. and Cleary, M.** (2001). Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. *Development* **128**, 3543-3557.

**Shanmugam, K., Featherstone, M. and Saragovi, H.** (1997). Residues flanking the HOX YPWM motif contribute to cooperative interactions with PBX. *J. Biol. Chem.* **272**, 19081-19087.

**Shanmugam, K., Green, N., Rambaldi, I., Saragovi, H. and Featherstone, M.** (1999). PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX proteins. *Mol. Cell. Biol.* **19**, 7577-7588.

**Shashidhara, L., Agrawal, N., Bajpai, R., Bharathi, V. and Sinha, P.** (1999). Negative regulation of dorsoventral signaling by the homeotic gene Ultrabithorax during haltere development in *Drosophila*. *Dev. Biol.* **212**, 491-502.

**Smith, D. L. and Johnson, A. D.** (1994). Operator-constitutive mutations in a DNA sequence recognized by a yeast homeodomain. *EMBO J.* **13**, 2378-2387.

**Struhl, G. and Basler, K.** (1993). Organizing activity of wingless protein in *Drosophila*. *Cell* **72**, 527-540.

**Sun, B., Hursh, D. A., Jackson, D. and Beachy, P. A.** (1995). Ultrabithorax protein is necessary but not sufficient for full activation of decapentaplegic expression in the visceral mesoderm. *EMBO J.* **14**, 520-535.

**Thuringer, F., Cohen, S. and Bienz, M.** (1993). Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene. *EMBO J.* **12**, 2419-2430.

**Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M., Botas, J. and Cohen, S.** (1992). Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene. *Cell* **71**, 437-450.

**van Dijk, M. and Murre, C.** (1994). *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* **78**, 617-624.

**Vervoort, M., Crozatier, M., Valle, D. and Vincent, A.** (1999). The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the *Drosophila* wing. *Curr. Biol.* **9**, 632-639.

**Vlachakis, N., Choe, S. and Sagerstrom, C.** (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128**, 1299-1312.

**Warren, R., Nagy, L., Selegue, J., Gates, J. and Carroll, S.** (1994). Evolution of homeotic gene regulation and function in flies and butterflies. *Nature* **372**, 458-461.

**Weatherbee, S., Halder, G., Hudson, A., Kim, J. and Carroll, S.** (1998). Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Dev.* **10**, 1474-1482.

**Weatherbee, S. D., Nijhout, H. F., Grunert, L. W., Halder, G., Galant, R., Selegue, J. and Carroll, S.** (1999). *Ultrabithorax* function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol.* **9**, 109-115.

**White, R. A. H., Aspland, S. E., Brookman, J. J., Clayton, L. and Sproat, G.** (2000). The design and analysis of a homeotic response element. *Mech. Dev.* **91**, 217-226.

**Wu, J. and Cohen, S. M.** (1999). Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126**, 109-117.

**Xu, Y., Baldassare, M., Fisher, P., Rathburn, G., Oltz, E. M., Yancopoulos, G. D., Jessell, T. M. and Alt, F. W.** (1993). LH-2: A lim/homeodomain gene expressed in developing lymphocytes and neural cells. *Proc. Natl. Acad. Sci. USA* **90**, 227-231.

**Zeng, C., Pinsonneault, J., Gellon, G., McGinnis, N. and 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.