Evolution of a transcriptional repression domain in an insect Hox protein

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Homeotic (*Hox*) genes code for principal transcriptional regulators of animal body regionalization[1]. The duplication and divergence of *Hox* genes, changes in their regulation, and changes in the regulation of Hox target genes have all been implicated in the evolution of animal diversity[2–4]. It is not known whether Hox proteins have also acquired new activities during the evolution of specific lineages. Amino-acid sequences outside the DNA-binding homeodomains of Hox orthologues diverge significantly. These sequence differences may be neutral with respect to protein function, or they could be involved in the functional divergence of Hox proteins and the evolutionary diversification of animals. Here, we identify a transcriptional repression domain in the carboxy-terminal region of the *Drosophila* Ultrabithorax (Ubx) protein. This domain is highly conserved among Ubx orthologues in other insects, but is absent from Ubx in other arthropods and onychophorans. The evolution of this domain may have facilitated the greater morphological diversification of posterior thoracic and anterior abdominal segments characteristic of modern insects.

Functional comparisons of Hox orthologues have largely focused on their highly conserved homeodomain sequences and have demonstrated their functional interchangeability between species[5–9]. For example, like *Drosophila* Ubx (DUbx), ectopic expression in *Drosophila melanogaster* of the Ubx protein from an onychophoran (OUbx) (Onychophora being a sister group to Arthropoda) induces transformations of the antenna to leg and the wing to haltere; it also induces ectopic activation of a *decapentaplegic* embryonic midgut enhancer[10]. This indicates that OUbx can perform some of the same molecular and developmental functions as DUbx. However, unlike DUbx, OUbx is unable to transform segmental identity of the embryonic ectoderm from a thoracic to an abdominal identity or to repress the DUbx-regulated target gene *Distal-less* (*Dll*). These functional differences between Ubx orthologues map outside of the homeodomain[10].

The differences between DUbx and OUbx could be due either to the aggregate divergence of sequences along the length of the proteins, or to the presence of one or more discrete functional motifs that arose in the insects or were lost in the onychophorans, some time after the separation of their lineages from a common ancestor more than 520 million years (Myr) ago. To better delimit when during evolution the functional difference among Ubx orthologues may have arisen, we cloned full-length *Ubx* orthologues from two phylogenetically intermediate taxa, the red flour beetle, *Tribolium castaneum* (TcUbx), and the butterfly *Junonia coenia* (JcUbx). Alignment of their amino-acid sequences with DUbx and OUbx revealed several domains that were conserved among all four Ubx orthologues, including the MXSXFE, NGYK and YPWM motifs amino-terminal to the homeodomain; the homeodomain itself; and the 'Ubd-A' peptide, a motif also shared with the abdominal A protein, which is C-terminal to the homeodomain (Figure 1). We surmised that sequences shared by the four Ubx orthologues probably contribute to functional similarities among them.

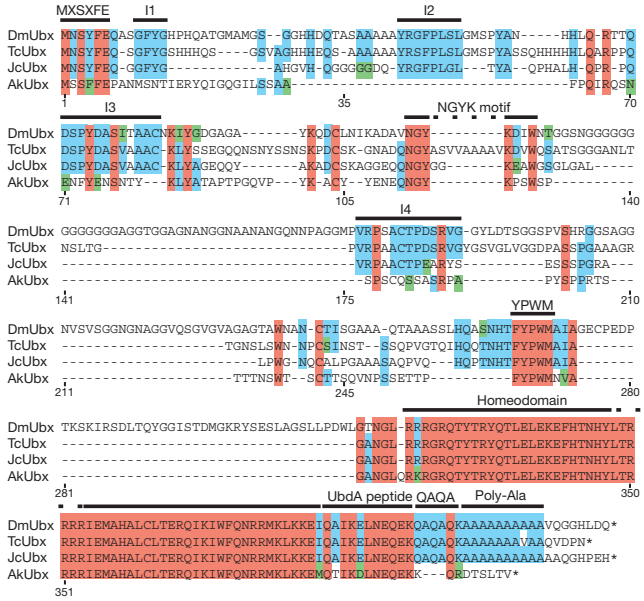

Figure 1 Several protein motifs are shared among Ubx orthologues. Aligned amino-acid sequences are: DmUbx from the fruit fly *Drosophila melanogaster*, TcUbx from the beetle *Tribolium castaneum*, JcUbx from the butterfly *Junonia coenia*, and AkUbx from the onychophoran *Akanthokara kaputensis*. Amino acids shaded in red are shared among all four Ubx orthologues, those shaded in blue are shared by at least three, and residues shaded in green are functionally similar to the others at the same position. Dashes indicate sequence gaps and asterisks are translation stops.

In contrast, sequences shared by the insect Ubx orthologues but not by OUbx might account for functional differences between DUbx and OUbx. Insect-restricted sequences include four regions N-terminal to the homeodomain (I1–I4), a peptide motif (QAQAQK), and an extended run of alanine residues C-terminal to the homeodomain (Fig. 1). To determine whether the presence of these sequences correlates with DUbx functions, we analysed the activity of TcUbx *in vivo*. Ectopic expression of TcUbx throughout the embryonic ectoderm induced the same phenotypes as those induced by DUbx: transformation of segmental identity from thoracic to abdominal (Fig. 2a–c), and repression of the activity of a *lacZ* reporter gene driven by the *Dll*304 embryonic limb enhancer (*Dll*304–*lacZ*), an element that is directly regulated by DUbx in *Drosophila*[11] (Fig. 2f–h; frequency $f = 100\%$, repression activity RA = 80%; see Methods for definitions). OUbx did not exhibit either of these activities (Fig. 2d, j; $f = 0\%$, RA = 0%). This indicates that the evolution of sequences required for these functions arose in the Ubx protein before the divergence of Coleoptera and Diptera, about 200–250 Myr ago, and after their divergence from Onychophora.

To identify protein sequences responsible for the functional differences between the insect Ubx orthologues and OUbx, we generated chimaeric Ubx proteins between DUbx and OUbx and ectopically expressed them in *Drosophila*. Several chimaeric proteins in which different OUbx sequences were replaced with those from DUbx N-terminal to the homeodomain were, like OUbx, completely unable to transform thoracic larval cuticle to abdominal identity or to repress *Dll*304–*lacZ* ($f = 0\%$; data not shown). However, replacement of the short OUbx sequence C-terminal to the Ubd-A peptide with just the 24-amino-acid sequence C-terminal to the Ubd-A peptide from DUbx (including the QAQAQK motif and poly-alanine stretch, QA), resulted in a chimaeric protein (O/QA) that was competent both to transform thoracic segments to

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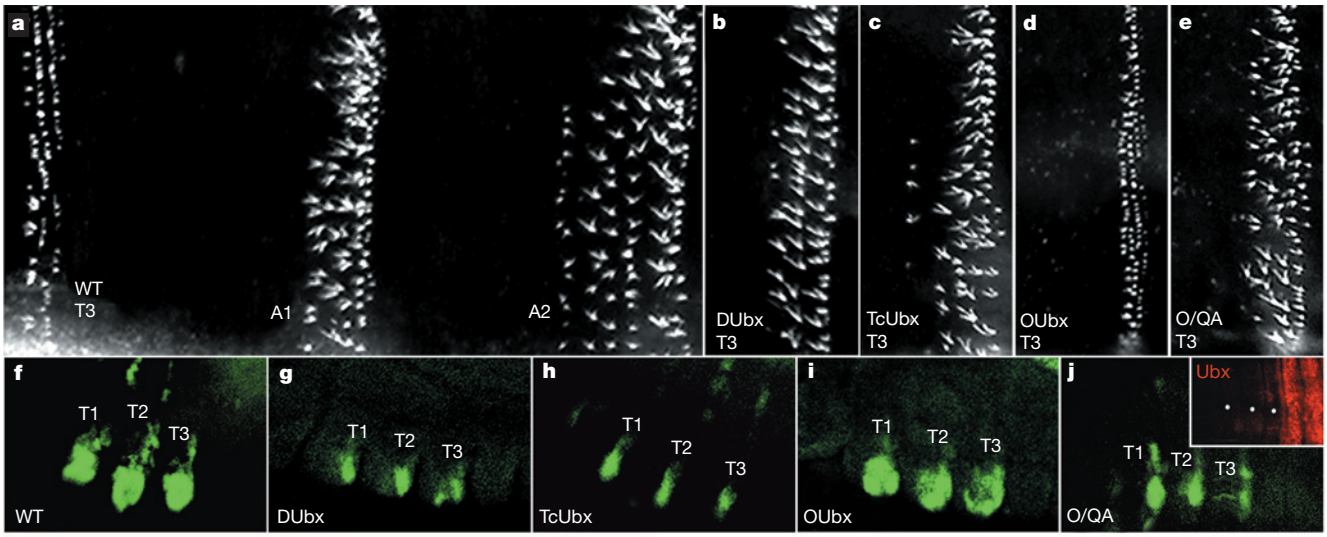

Figure 2 Localization of a repression domain in DUbx. **a**, Denticle belts in wild-type (WT) ventral cuticle. Note that the third thoracic (T3) denticle belt normally has only two or three rows of hairs, the first abdominal segment (A1) has four, and the second abdominal segment (A2) has six. The abdominal denticle belts are also arranged in a trapezoidal pattern, whereas the T1 rows are not. **b**–**e**, T3 denticle-belt phenotypes induced by ectopic expression of different Ubx orthologues. **b**, Ectopic expression of DUbx transforms T3 segmental identity to that of A1 ($f = 100\%$). **c**, **d**, Ectopic expression of TcUbx mediates the same transformation ($f = 100\%$) (**c**), whereas OUbx does not ($f = 0\%$) (**d**). **e**, Ectopic expression of the chimaeric protein O/QA transforms T3 segmental identity to that of A1. **f**, *Dll*304–*lacZ* activity in the three thoracic segments of a wild-type *Drosophila* embryo. **g**–**j**, The same views of reporter activity in flies ectopically expressing various Ubx orthologues or chimaeric proteins. **g**, DUbx represses *Dll* *Dll*304–*lacZ* expression, although some residual reporter activity is typically observed[27]. **h**, **i**, TcUbx represses *Dll*304–*lacZ* expression as DUbx does (**h**), whereas OUbx does not (**i**). **j**, Ectopic expression of the O/QA chimaera represses *Dll*304–*lacZ* ($f = 100\%$, RA = 50%), although not as well as DUbx ($f = 100\%$, RA = 100%) or TcUbx ($f = 100\%$, RA = 80%). Inset, endogenous DUbx expression is unaffected by ectopic O/QA expression. Ubx expression is shown in the same region from the embryo in **j**, and the white dots in the inset indicate the position of the text labels in the panel. Ubx is normally expressed faintly in posterior T2 and more strongly in T3 and A1. In all panels, anterior is to the left. The phenotypes observed for each construct were consistent among at least three independent lines.

abdominal identity (Fig. 2e; $f = 100\%$), and to repress *Dll*304–*lacZ* (Fig. 2j; $f = 100\%$, RA = 50%). Importantly, ectopic expression of O/QA did not affect endogenous DUbx expression (Fig. 2j, inset). Therefore, the O/QA gain-of-function phenotypes observed are due solely to the activity of the O/QA chimaeric protein. Furthermore, simple deletion of the OUbx C terminus had no effect on the *in vivo* activity of OUbx (data not shown), indicating that the OUbx C terminus does not mask an OUbx repression activity, as is the case in a crustacean Ubx orthologue, shown in an accompanying paper[12]. These results indicate that the residues critical in differentiating DUbx function from that of OUbx are located in the C terminus of DUbx and are sufficient to impart repression activity on the otherwise inactive OUbx orthologue.

The C terminus of DUbx and the YPWM peptide motif located N-terminal to the homeodomain have been implicated in mediating interactions between DUbx and the Hox cofactor Extradenticle (Exd)[13–15]. Together, the two proteins form a complex with an increased DNA-binding affinity and regulate several embryonic enhancers[13,16,17], including repression of *Dll*304–*lacZ*[18]. It is possible that OUbx does not possess C-terminal residues crucial for mediating an interaction with *Drosophila* Exd (DExd), thus preventing its ability to repress *Dll*. To investigate this possibility, we examined the DNA- and Exd-interacting ability of Ubx orthologues and chimaeric proteins. As expected, DExd alone did not bind to a DNA probe containing a Hox/Exd composite site (Fig. 3a, lane 2), and neither DUbx nor OUbx alone bound to the probe very well (Fig. 3a, lanes 3 and 5). However, *Drosophila* Ubx and Exd together exhibited a much higher DNA-binding affinity (Fig. 3a, lane 4). Significantly, OUbx and DExd also bound the DNA with a high affinity (Fig. 3a, compare lane 6 with lanes 2 and 5), indicating that onychophoran Ubx and DExd indeed interact, as do the O/QA chimaeric protein and DExd (Fig. 3a, lane 8). Thus, the inability of OUbx to repress *Dll*

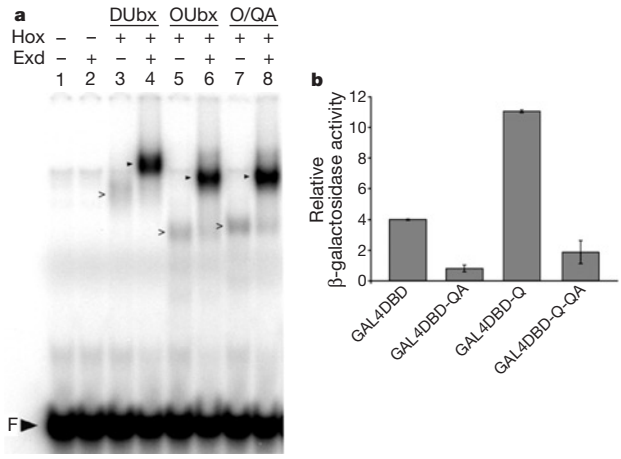

Figure 3 The QA domain is a repression domain. **a**, OUbx interacts with DExd on DNA. Electromobility gel shift analysis was performed with an oligonucleotide probe carrying a composite Exd/Hox binding site. DExd alone does not bind the probe very well (compare lane 2 to the lysate-alone control in lane 1), and DUbx binds DNA weakly (lane 3), but together they bind with high affinity (lane 4). Likewise, OUbx does not bind DNA with high affinity (lane 5), but exhibits a much higher affinity when mixed with DExd (lane 6). The chimaeric O/QA protein shows the same DNA-binding characteristics as the other Ubx orthologues (compare lanes 7 and 8). Open arrowheads indicate Hox–DNA complexes, whereas filled arrowheads indicate Hox–Exd–DNA complexes. The larger arrowhead (F) points to the free probe in each lane. **b**, β-galactosidase activities mediated by various GAL4DBD fusion proteins relative to basal transcription (that is, the level of reporter activity in the absence of any GAL4 plasmid is set to 1.0). The DUbx QA domain completely represses transcription driven by the GAL4DBD protein alone, and reduces activation mediated by GAL4DBD-Q by 91%. Relative values are the means of triplicates and the same results were observed in at least three separate experiments.

is not due to an inability to interact with DExd on target DNA regulatory elements.

Rather, these results and the ability of O/QA to repress *Dll*304–*lacZ* suggest that the C-terminal QA domain may be a repression domain. Poly-alanine-rich and glutamine/alanine-rich sequences have been found in many repression domains in several homeodomain proteins as well as other transcription factors[19–21]. These domains seem to mediate repression by interacting with the basal transcriptional machinery[20]. To test whether the DUbx QA domain has a similar activity, we examined its ability to repress transcription when fused to the yeast GAL4 DNA-binding domain (GAL4DBD) or to a chimaeric GAL4 protein that also bears a glutamine-rich activation domain from the *Drosophila* Bicoid protein (GAL4DBD-Q)[21]. In transfected *Drosophila* S2 cells, GAL4DBD mediated a fourfold increase in the relative activity of a UAS β-galactosidase reporter gene (Fig. 3b). When fused to GAL4DBD, the DUbx QA domain completely repressed activation of reporter gene expression relative to GAL4DBD alone; the GAL4DBD-Q protein mediated 11-fold relative activation, and the DUbxQA domain, when fused to GAL4DBD-Q, reduced activation by GAL4DBD-Q to only 1.9-fold relative reporter activity (a 91% reduction or 5.8-fold repression of GAL4DBD-Q activation) (Fig. 3b). The magnitude of repression mediated by the QA domain is comparable to that observed for repression domains from other transcriptional repressors. These assays show that the DUbx C-terminal QA domain is sufficient to repress transcription in *Drosophila* S2 cells, and, combined with its ability to confer repression activity on OUbx *in vivo* in *Drosophila*, demonstrate that the QA domain is a discrete repression domain.

It is possible that this repression domain evolved after the split of the *Drosophila* and onychophoran lineages, or was present in a common ancestor, but subsequently lost by onychophorans. To address these alternative models, we examined the phylogenetic distribution of the C-terminal repression domain. Alignment of part of helix 3 of the homeodomains and C-terminal sequences from a collection of Ubx orthologues revealed that the QAQA peptide motif is shared by all the arthropods except for *Artemia* (Fig. 4a). This is consistent with its presence in a common ancestor of arthropods and its loss in the *Artemia* lineage. Most notably, the insect Ubx orthologues share a remarkably conserved poly-alanine tract, which is absent from onychophoran and other Ubx orthologues, including that from Collembola, a sister taxon to the insects (Fig. 4a). The poly-alanine stretch thus seems to have arisen in the insects, after their divergence from the more basal hexapods (Fig. 4b) and its near-perfect conservation suggests that it is under strong stabilizing selection. The evolution of the poly-alanine motif in the Ubx protein in insects may have increased the repression potency of Ubx or given it a new mode of target gene repression. There must also be other repression domains within DUbx, because deletion of the QA domain reduces but does not abolish DUbx repression activity[12].

The evolution of this repression domain in Ubx demonstrates the acquisition of a new function within a Hox protein while maintaining its homeotic role. In an accompanying paper[12], the serine/threonine-rich C terminus of an *Artemia* Ubx orthologue is demonstrated to modulate the repression activity of *Artemia* Ubx as well as limb repression by DUbx when inserted in place of the QA domain. Replacement of Ser/Thr-rich residues with alanine converts *Artemia* Ubx to a strong repressor[12]. Taken together, and in light of the view that crustaceans and insects are sister taxa, these studies suggest that a C-terminal activity-modulating Ser/Thr domain in the Ubx protein of a common ancestor of crustaceans and insects was replaced with the QA repression domain during early insect evolution, and that this sequence has subsequently remained under strong selection. Two well-known examples of genes that evolved from *Hox* genes are the derivation of *fushi tarazu* from a central class *Hox* gene[22–24] and the evolution of *zerknullt* from a

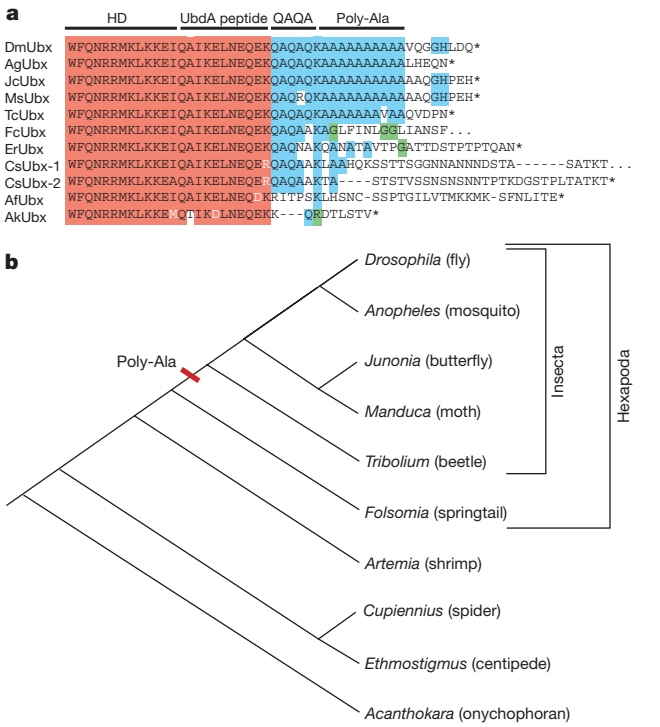

Figure 4 A repression domain containing poly-alanine evolved in the insect lineage. **a**, An alignment of part of helix 3 of the homeodomains and C-terminal amino-acid sequences from several Ubx orthologues. Red shading indicates residues conserved among the homeodomains and Ubd-A peptides. Identical amino acids are designated by black letters within the shading, whereas those similar in function are indicated by white letters. Amino acids identical to those in DUbx C-terminal to the Ubd-A peptide and shared by at least three sequences are shaded blue; those similar in function are shaded green. Sequence gaps are shown as dashes, and translation stops are asterisks. An ellipsis (. . .) indicates additional amino acids not shown. Note the presence of the poly-alanine motif in all insects but not in collembolan or other arthropod Ubx orthologues. **b**, The distribution of the poly-alanine domain is mapped onto an arthropod phylogeny (taken from ref. 30). The repression domain arose in the insect lineage after its split from the more basal hexapods. In **a**, the two-letter species designations used to name Ubx orthologues designate the following animals: Dm, *Drosophila melanogaster* (Diptera); Ag, *Anopholes gambiae* (Diptera); Jc, *Junonia coenia* (Lepidoptera); Ms, *Manduca sexta* (Lepidoptera); Tc, *Tribolium castaneum* (Coleoptera); Fc, *Folsomia candida* (Collembola); Er, *Ethmostigmus rubripes* (Myriapoda); Cs, *Cupiennius salei* (a chelicerate with two Ubx orthologues); Af, *Artemia franciscana* (Crustacea); Ak, *Acanthokara kaputensis* (Onychophora).

Hox3 predecessor[25], but in neither case has the protein retained its homeotic role.

The restricted phylogenetic distribution of the QAQAQK and poly-alanine repression motifs is especially intriguing in light of its correlation with the pattern of segmental diversity that evolved in the insects. Primitive hexapods such as collembolans possess abdominal limbs, and their posterior thoracic and anterior abdominal segments are not highly differentiated. More-derived winged insects, such as Diptera and Lepidoptera, have completely limbless adult abdomens. Their second and third thoracic segments, which bear wing appendages and legs, and their anterior abdomen, are highly differentiated. The evolution of the poly-alanine repression domain may have facilitated the diversification of these segments by further potentiating Ubx repression of target genes. □

Methods

Cloning of Ubx orthologues

Ubx from *Tribolium castaneum* and *Junonia coenia* was cloned by 5′ rapid amplification of cloned ends (RACE) with the AP1 primer (Clontech) and a primer targeted to the 3′ untranslated region of the *Ubx* gene[26] (details on request). The resulting polymerase chain reaction (PCR) product was cloned using a TA cloning kit (Invitrogen). Subsequent

sequencing of ligated products revealed two isoforms of *TcUbx*, a 'b' isoform with the amino-acid sequence DSMTF inserted at amino-acid position 213, and an 'a' isoform without it.

Ectopic expression of Ubx orthologues and chimaeras

UAS–*DUbxIa*[27] and UAS–*OUbx*[10] have been described previously. *TcUbxa* was cloned into pUAST[28]. UAS–O/QA was created by replacing *OUbx* nucleotide sequences coding for amino acids C-terminal to the Ubd-A motif (ending EQEK) with the corresponding sequences from *DUbxIa* (starting QAQA) by PCR and then cloning the chimaera into pUAST. The OUbx C-terminal deletion was constructed by deleting the last nine amino acids of OUbx using PCR and cloning into pUAST. The C-terminal replacement construct of OUbx by collembolan Ubx was also created by PCR and cloned into pUAST. Ubx orthologues and the chimaera were ectopically expressed using the arm–Gal4[11] driver, which was obtained from the Bloomington Fly Stock Center. Flies carrying this Gal4 driver, along with the *Dll*304 reporter[11], were crossed to flies carrying the appropriate UAS construct. Details of the generation of the chimaeric Ubx proteins involving N-terminal amino-acid sequences are available on request.

Assays of *Dll* repression by Ubx

Antibody staining of embryos was performed as previously described[10]. Expression of the *lacZ* reporter gene driven by *Dll*304–*lacZ* was monitored using a rabbit anti-β-galactosidase antibody (Molecular Probes). Ectopic expression of DUbxIa, OUbx or TcUbx was verified with an anti-Ubx/Abd-A antibody (FP6.87)[29], and the effect of ectopic OUbx expression on endogenous Ubx expression in *Drosophila* was visualized using an anti-Ubx antibody (FP3.38) that is specific to *Drosophila* Ubx (gift of R. A. H. White). Embryonic cuticles were prepared as described[10]. The frequency (*f*) refers to the percentage of embryos in which repression of *Dll*304–*lacZ* or segmental identity transformations were observed. The relative repression activity (RA) was calculated by measuring the area of *Dll*304–*lacZ* expression that was repressed in embryonic limb primordia relative to the activity of DUbx.

Protein expression and DNA-binding assays

All Ubx orthologues and O/QA were cloned into T7pLink[30] for protein production using the TNT T7 Quick Coupled Transcription/Translation System (Promega). EMSA was performed with 5 μl of mock lysate, 3 μl of TNT-produced DExd (a gift of R. Mann) and 2 μl of mock lysate, or 3 μl of TNT-produced Hox protein and 2 μl of either mock lysate or DExd, incubated with 1 μg poly-d(I-C) in 13 μl of gel shift buffer. A radiolabelled oligonucleotide probe containing an Exd/Hox compound site (TTAGCGATGATTTATTGCCTCCTT) was then added to a final concentration of 500 pM, and incubated for 30 min on ice. We loaded 15 μl of the EMSA reactions to undergo electrophoresis on a 6% polyacrylamide gel (37.5:1). To control for protein translation, protein products labelled with ^{35}S-methionine in TNT reactions were analysed on a 10% SDS polyacrylamide gel. All translation reactions, correcting for differing numbers of methionines, produced approximately the same yields.

Transfection plasmid construction

The 5X UAS–*lacZ* reporter and pPac-GAL4DBD plasmids were gifts of A. Laughon, pBluescript-GAL4DBD was a gift of G. Halder, and GAL4DBD-Q (previously[21] called Gal4-Q) was a gift of N. Dostatni. GAL4DBD-QA was constructed by amplifying the DUbx QA domain from a DUbx complementary DNA using PCR (primer sequences on request). The resulting product with purified, restriction digested, and ligated into pBluescript-GAL4DBD digested with the same restriction enzymes. The GAL4DBD-QA fusion was then isolated and ligated into pPac. GAL4DBD-Q-QA was constructed by PCR amplifying the DUbx QA domain. The PCR product was cloned in-frame into pPac-GALDBD-Q. Insertions of the correct orientation were identified by PCR. Both pPac-GAL4DBD-QA and pPac-GAL4DBD-Q-QA were verified by sequence analysis.

Tissue culture and transactivation assay

Each well of 7×10^5 *Drosophila* S2 cells was transfected with 1 μg of the UAS–*lacZ* reporter plasmid, 2 μg of sheared salmon sperm DNA, and either 1 μg of pPac producer plasmid or an additional 1 μg of salmon sperm DNA (for a total of 4 μg of DNA per transfection). After 48 h of growth after transfection, cells were collected and pelleted, washed once with 1× Dulbecco's phosphate-buffered saline (GibcoBRL), pelleted once again, and then lysed with 25 μl of X PBS and 0.1% NP-40. β-galactosidase activity was measured by mixing 10 μl of cell lysate with 90 μl of CPRG assay buffer. Reactions were allowed to proceed for 20 min at 25 °C, and then stopped with 900 μl of 10 mM Tris buffer and 1 mM EDTA. Ultraviolet absorbance at 574 nm was quantified with a spectrophotometer (Shimadzu UV160U). Relative activity is the ratio of the β-galactosidase activity measured for a given GAL4DBD variant to background activity from UAS–*lacZ*. Indicated relative values are the means of three independent experiments, and the same trends were observed in at least three separate trials.

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Competing interests statement

The authors declare that they have no competing financial interests.

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