# Pleiotropic functions of a conserved insect-specific Hox peptide motif

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Development 132, 5261-5270
Published by The Company of Biologists 2005
doi:10.1242/dev.02146

*Accepted 4 October 2005*

## Summary

**The proteins that regulate developmental processes in animals have generally been well conserved during evolution. A few cases are known where protein activities have functionally evolved. These rare examples raise the issue of how highly conserved regulatory proteins with many roles evolve new functions while maintaining old functions. We have investigated this by analyzing the function of the 'QA' peptide motif of the Hox protein Ultrabithorax (Ubx), a motif that has been conserved throughout insect evolution since its establishment early in the lineage. We precisely deleted the QA motif at the endogenous locus via allelic replacement in *Drosophila melanogaster*. Although the QA motif was originally characterized as involved in the repression of limb formation, we have found that it is highly pleiotropic. Curiously, deleting the QA motif had strong effects in some tissues while barely affecting others, suggesting that QA function is preferentially required for a subset of Ubx target genes. QA deletion homozygotes had a normal complement of limbs, but, at reduced doses of *Ubx* and the *abdominal-A* (*abd-A*) Hox gene, ectopic limb primordia and adult abdominal limbs formed when the QA motif was absent. These results show that redundancy and the additive contributions of activity-regulating peptide motifs play important roles in moderating the phenotypic consequences of Hox protein evolution, and that pleiotropic peptide motifs that contribute quantitatively to several functions are subject to intense purifying selection.**

Key words: Ultrabithorax, Distal-less, Limb, Hox, Evolution, Peptide motif, Trichome, Abdominal tergite, Postnotal, Laterotergite, Gene targeting, Allelic replacement, Homologous recombination

## Introduction

Morphology evolves through changes in the genetic regulatory networks that govern development (Carroll, 2005; Carroll et al., 2005; Davidson, 2001; Stern, 2000). In principle, regulatory networks and gene expression can evolve by the modification of *cis*-regulatory elements or by alterations in the activity or distribution of *trans*-acting factors. Transcription factors regulate gene expression in *trans* by directly binding to *cis*-regulatory elements. A group of transcription factors referred to as selector proteins regulates extensive batteries of target genes and can transform the identity of cells and tissues when misexpressed or misregulated (Garcia-Bellido, 1975; Mann and Carroll, 2002). These include cell-type-specific proteins, as well as proteins with broader realms of action, such as the region-specific Hox proteins. The need to properly regulate many target genes is expected to tightly constrain the evolution of selector proteins. Furthermore, selectors expressed in several different cell types and tissues throughout development are anticipated to be especially pleiotropic and highly constrained (Carroll, 2005; Carroll et al., 2005; Mann and Carroll, 2002; Stern, 2000).

Several studies have demonstrated strong sequence and functional conservation of selector proteins across phylum-level evolutionary distances (Halder et al., 1995; Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993). Highly conserved regions have tended to be those most relevant for protein function. For example, in Hox proteins, the DNA-binding homeodomain and the 'hexapeptide' or 'YPWM' motif that interacts with the Extradenticle (Exd) co-factor (Chang et al., 1995; Passner et al., 1999) are both well-conserved. However, most of the sequence of Hox proteins appears free to vary across phyla, suggesting that the specific amino acid residues in these regions contribute less to protein function. In several cases, selector protein functions have evolved (Galant and Carroll, 2002; Grenier and Carroll, 2000; Hanks et al., 1998; Lamb and Irish, 2003; Ronshaugen et al., 2002; Shiga et al., 2002) or evolution has co-opted selectors for derived functions (Alonso et al., 2001; Lohr and Pick, 2005; Lohr et al., 2001; Stauber et al., 2002). These cases have generally involved regions outside of the DNA-binding domain and have implicated synapomorphic (shared, derived) peptide motifs conserved in subsets of related taxa (Hsia and McGinnis, 2003).

One of the most provocative cases of selector protein evolution correlates the acquisition of limb repression capacity

by the central class Hox selector protein Ultrabithorax (Ubx) with the reduction of abdominal limb number in insects (Galant and Carroll, 2002; Grenier and Carroll, 2000; Ronshaugen et al., 2002). Whereas insect Ubx possesses strong limb repression capacity when ectopically expressed in *Drosophila melanogaster*, crustacean (*Artemia francisana*) and onychophoran (*Acanthokara kaputensis*) Ubx do not. Sequences in the C terminus of Ubx are responsible for much of this functional divergence. *A. franciscana* Ubx possesses putative casein kinase II sites that modulate activity, whereas all insect Ubx orthologs contain the highly conserved C-terminal 'QA' motif required for full Ubx repression activity (Gebelein et al., 2002; Ronshaugen et al., 2002). This QA motif is capable of conferring limb repression activity when grafted onto onychophoran Ubx (Galant and Carroll, 2002).

The sufficiency of the QA motif to confer limb repression capacity suggests that its acquisition during early insect evolution could have played an important role in the evolution of insects lacking adult abdominal limbs. However, little is known about the role of the QA motif in normal development. For example, is the QA motif required for abdominal limb repression in insects? Is this motif dedicated to limb repression, or is it pleiotropic? What would be the phenotypic consequence of removing such a conserved part of an integral patterning gene?

To characterize the genetic and phenotypic role of the QA peptide motif of Ubx, we have precisely deleted this motif at the endogenous *Ubx* locus via allelic replacement in *D. melanogaster* (Rong et al., 2002). The effects of deleting the QA motif were strong in some tissues but barely detectable in others. This finding of differential pleiotropy suggests that peptide motifs in selector proteins can conditionally modulate selector activity and need not be uniformly pleiotropic across all tissues. We also find the requirement for the QA motif for limb repression to be dose dependent and partially redundant with the Abdominal-A (Abd-A) Hox protein, suggesting that redundancy and the additive contributions of peptide motifs play important roles in modulating the phenotypic consequences of selector protein evolution.

## Materials and methods

### Construction of the $Ubx^{QA}$ allele

Using standard molecular techniques and P1 clone DS03126 (Martin et al., 1995) as a template, we altered four nucleotides (C1096T, C1102T, C1108T and A1111G of the *Ubx* isoform Ib coding region; bp 8569, 8563, 8557 and 8554, respectively, of DS03126) of a 10,875 bp fragment of *Ubx* bound by unique *Aat*II and *Xma*I sites (bp 14,604 and 3730, respectively, of DS03126). We also inserted an 18 bp *I-Sce*I site (TAGGGATAACAGGGTAAT) 840 bp downstream of the desired changes, between bp 7715 and 7714 of DS03126. The four nucleotide changes introduced three in-frame premature stop codons and a novel *Avr*II site and are predicted to truncate all Ubx isoforms immediately following the UbdA peptide motif (starting at amino acid residue 366 of Ubx isoform Ib). In the final $Ubx^{\Delta QA}$ allele, these four changes were retained, while the *I-Sce*I site was not (Fig. 1). The *I-Sce*I insertion also introduced a novel *Xba*I site in conjunction with existing genomic sequence. The resulting 10,893 bp modified fragment of *Ubx* was subcloned into the gene targeting vector pTV2 (Rong et al., 2002); transformed into the germline of *D. melanogaster*; and mobilized via transposition to X, 2nd and CyO chromosomes.

Several lines containing the above construct were crossed to $y^1$ *w; P{ry*$^{+t7.2}$*=70FLP}11 P{v*$^{+t1.8}$*=70I-SceI}2B noc*$^{Sco}$/CyO, $S^2$ virgin females, and Flp and I-SceI were induced by heat-shocking 0- to 3-day-old progeny for 1 hour at 38°C (Rong and Golic, 2000). F1 virgins with mosaic germlines (non-CyO, two or three per vial) were then crossed to $w^{1118}$*; P{ry*$^{+t7.2}$*=70FLP}10* males and Flp was induced by heat-shocking 0- to 3-day-old progeny. Owing to concerns about potential difficulty activating $w^{hs}$ in the eye from a location within or near the *Bithorax Complex (BX-C)* (Bender and Hudson, 2000), developing larvae and pupae were further heat-shocked every 3 days until eclosion. Progeny were screened for non-mosaic red eyes, which were regarded as putative insertions (Rong and Golic, 2001).

Six putative insertions were obtained from 828 vials, four of which mapped to the 3rd chromosome. Only targeted duplications that retained one complete copy of $Ubx^+$ and a partial duplication of the $Ubx^{\Delta QA}$ allele [a subset of Class II events (Rong and Golic, 2000)] were useful for subsequent reduction. Candidate lines were screened via PCR and restriction digestion for introduction of the novel *Avr*II site and loss of the novel *Xba*I site (which should have been eliminated during the repair of the double-strand break at the *I-Sce*I site). Insertions were also screened to ensure the presence of a copy of $Ubx^+$ with no *Avr*II site and to ensure both junctions with vector backbone were intact. Only two independent Class II insertions fit all these criteria; one insertion line (*III65A*) was selected for reduction to single copy (Fig. 1B).

*III65A* males were crossed to $w^{1118}$*; P{v*$^{+t1.8}$*=hs-I-CreI.R}1A* $Sb^1$/TM6 virgin females, and 0- to 3-day-old progeny were heat-shocked for 1 hour at 36°C (Rong et al., 2002). F1 mosaic male progeny were crossed to *w;* TM3, *Sb*/TM6B, $Antp^{Hu}$*, Tb* virgin females. Single F2 males with white eyes were recovered as TM6B-balanced stocks for analysis; each reduction line was, thus, isogenous for its 3rd chromosome. Reduction homozygotes were analyzed via PCR and restriction digestion as above, and only those retaining either unduplicated $Ubx^+$ or $Ubx^{\Delta QA}$ alleles were analyzed further. To confirm that the alleles contained no artifactual mutations, we mapped the putative crossover points of three $Ubx^+$ (lines *A31*, *B2* and *C3*) and four $Ubx^{\Delta QA}$ (lines *A30*, *B1*, *C4* and *E7*) reduction alleles by sequencing overlapping PCR products from the entire manipulated region of *Ubx* and surrounding sequence (see Table S1 in the supplementary material). This assay was possible because of the presence of several well-spaced non-coding single nucleotide polymorphisms between the targeted chromosome and the source of the P1 clone. As each reduction is easily explained by a small odd number of crossovers, the alternative single-strand annealing hypothesis (Dolezal et al., 2003; Rong et al., 2002), where each difference between strands is retained or lost randomly, may not mechanistically explain the reduction events. Primer sequences, PCR conditions and further information are available upon request.

### Outcrossing

We introduced X and 2$^{nd}$ chromosomes from *Oregon-R* (G. Boekhoff-Falk laboratory stock) and *WI129* (Kopp et al., 2003) lines into several reduction lines and a $Sb^{sbd}$ $Ubx^{6.28}$ *e* line containing a null allele of *Ubx* to assess the effects of genetic background in a controlled manner. We used standard genetic manipulations with balancer chromosomes to prevent recombination and track chromosomes. Thus, each stock created had X and 2$^{nd}$ chromosomes from either *Oregon-R* or *WI129* (referred to as genetic background below), isogenous experimental 3rd chromosomes generated when its *Ubx* allele was created, and unknown Y and 4th chromosomes (which were expected to contribute little to phenotype). The chromosome containing $Sb^{sbd}$ $Ubx^{6.28}$ *e* was treated in the same manner, except that it was balanced with TM6B, $Antp^{Hu}$*, Tb*. Homozygotes from lines *A31* ($Ubx^+$), *B2* ($Ubx^+$), *A30* ($Ubx^{\Delta QA}$), *B1* ($Ubx^{\Delta QA}$) and *E7* ($Ubx^{\Delta QA}$) were quantified in the *WI129* background, and crosses between the above *Ubx* alleles and the above $Ubx^-$ chromosome were used to obtain $Ubx^-/Ubx^+$ and $Ubx^{\Delta QA}/Ubx^-$ adults for quantification. Lines *B2* ($Ubx^+$), *C3* ($Ubx^+$), *A30* ($Ubx^{\Delta QA}$), *B1* ($Ubx^{\Delta QA}$) and *E7* ($Ubx^{\Delta QA}$)

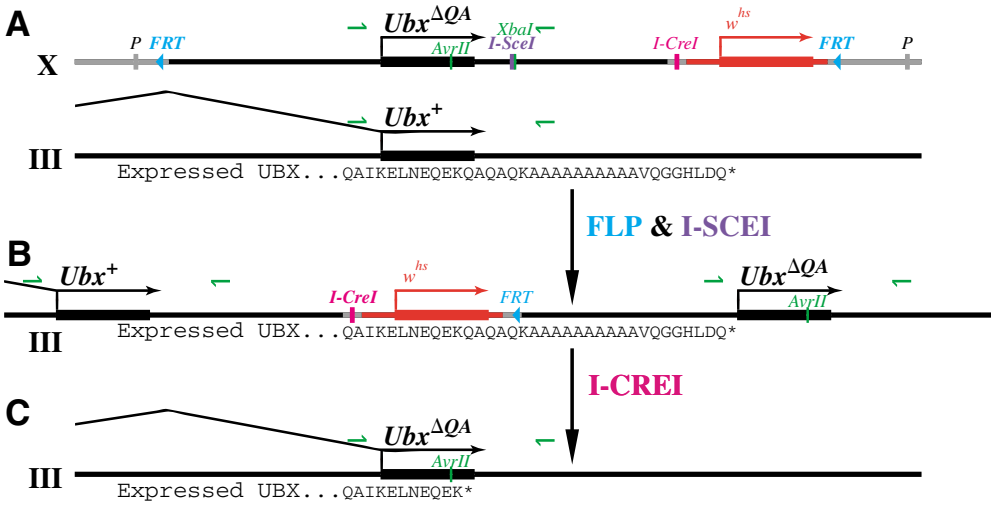


**Fig. 1.** Two-step construction of the $Ubx^{\Delta QA}$ allele. (A) An X chromosome insertion of the *P* element construct containing the desired changes to *Ubx* and the necessary sequences for allelic replacement (Rong et al., 2002) was used to manipulate the endogenous *Ubx* locus on the 3rd chromosome. Only the 3′ end of *Ubx*, which is also the edge of the *BX-C*, is shown. The scale is approximate; the targeting construct contained 10,893 bp between the vector cloning sites. At each step, the expressed polypeptide sequence of Ubx following the homeodomain is shown. Each step was catalyzed by crossing to flies containing the appropriate heat-shock transgenes and heat-shocking the progeny (see Materials and methods) (Rong et al., 2002). (B) The first step created a targeted duplication of the 3′ end of the *Ubx* locus that contained one complete and expressed $Ubx^{+}$ allele and a partial unexpressed $Ubx^{\Delta QA}$ allele. (C) The second step catalyzed the reduction of this duplication to a single copy, creating both full-length $Ubx^{\Delta QA}$ alleles and control $Ubx^{+}$ alleles in independent reduction events. Only the recovery of an $Ubx^{\Delta QA}$ allele is shown. Black indicates sequences with homology to *Ubx*; gray indicates vector or unknown sequences; red indicates marker $w^{hs}$ gene for screening; blue indicates site-specific recombinase Flp and *FRT* targets (triangles); purple indicates homing endonuclease I-SceI and *I-Sce*I target (bar); pink indicates homing endonuclease I-CreI and *I-Cre*I targets (bars); green indicates PCR primers (half arrows) and restriction sites (bars) used in preliminary screens of putative targeted duplication and reduction events. Ultimately, the entire region was sequenced from alleles selected for study.

were quantified in the *Oregon-R* background, and crosses between the above *Ubx* alleles and the above $Ubx^{-}$ chromosome were used to obtain $Ubx^{-}/Ubx^{+}$ and $Ubx^{\Delta QA}/Ubx^{-}$ adults for quantification. All flies were raised on sugar food at 25°C with 70-80% humidity on a 12-hour light cycle.

### Quantification and photography of adult phenotype

The number of bristles on both halteres was recorded and averaged for each adult. Likewise, the number of bristles on both half-laterotergites was recorded and averaged for the same adults. To calculate the number of A1 tergite bristle rows, we averaged four readings for each of the same adults: the number of rows on each of the two most lateral columns and the column with the highest number of rows present between the lateral column and the dorsal midline for each side. All statistical analyses were performed using Mstat v. 4.01 (http://mcardle.oncology.wisc.edu/mstat/). Unless otherwise indicated, all statistical tests were two-sided Wilcoxon rank sum tests. To maximize the amount of tissue in focus, several focal planes of representative samples were photographed and digitally combined using Syncroscopy Auto-Montage Pro according to the manufacturer's instructions (Cambridge, UK). Legs were photographed using dark-field microscopy (Stern and Sucena, 2000).

### Embryonic and larval phenotypic characterization

First larval stage cuticles were prepared essentially as described previously (Stern and Sucena, 2000). Denticle belts were photographed using dark-field microscopy, and KOs were photographed using phase-contrast microscopy. Embryos were stained for Distal-less (Dll) as described previously (Panganiban et al., 1995). $Df(3R)Ubx^{109}$ (Lewis, 1963), a deficiency deleting all of *Ubx* and *abd-A* (Bender et al., 1983), was used in $Ubx^{-}$ $abd\text{-}A^{-}$ assays.

### Sub-epidermal adult leg tissue

Adults and dead pupae ($Ubx^{\Delta QA}/Ubx^{-}$ and especially $Ubx^{\Delta QA}/Ubx^{-}$ $abd\text{-}A^{-}$ often died as pupae) were dissected, and their A1 cavity was examined for sub-epidermal leg tissue. Only growths with clearly identifiable bristles were counted as leg tissue and were removed and photographed using bright-field microscopy (Stern and Sucena, 2000). Rare (1.6%; 8/490) cases where the positions of legs were shifted towards the posterior were not counted as producing ectopic A1 leg tissue because no additional leg tissue was formed.

## Results

### Construction of the $Ubx^{\Delta QA}$ allele

In principle, the two-step gene targeting and allelic replacement methodology developed for *D. melanogaster* allows any desired change to be introduced into an otherwise wild-type genome without leaving any trace of the manipulation (Rong et al., 2002). We introduced a mutation that converted the first codon of the QA motif to a premature stop codon into the *Ubx* locus of *D. melanogaster*. This change precisely deleted the QA motif and the eight additional C-terminal residues that are poorly conserved, which left RNA length, splicing sites and the remainder of the coding region unmodified.

The scheme for creating the targeted mutation is shown in Fig. 1. The targeting construct was first randomly inserted into the genome on the X chromosome (Fig. 1A). In the first 'targeting' step, Flp and I-SceI catalyzed the excision and subsequent insertion of the construct into the *Ubx* locus via homologous recombination. Rarely, this produced a partial duplication of the *Ubx* locus with one complete wild-type $Ubx^{+}$ copy and the 3′ end of a mutant $Ubx^{\Delta QA}$ copy [specifically, a targeted 'Class II' (Rong and Golic, 2000) insertion that retained the introduced mutations only in the 3′ duplicated copy; Fig. 1B]. Two such insertions were recovered after screening 828 vials.

In the second 'reduction' step, I-CreI efficiently created a double-strand break between the duplicated regions of *Ubx*, which was then repaired via homologous recombination to

leave either a single unaltered $Ubx^{+}$ allele or a single mutant $Ubx^{\Delta QA}$ allele with the premature stop codon deleting the QA motif (Fig. 1C). After verification by PCR, restriction digestion and sequencing, one insertion meeting the criteria shown in Fig. 1B was reduced to several single-copy $Ubx^{+}$ and $Ubx^{\Delta QA}$ alleles (Fig. 1C). We screened and confirmed the molecular identity and fidelity of several alleles using a PCR and restriction digestion assay. Ultimately, the entire region covered by the targeting construct was sequenced for at least three independently generated reduction lines for both the $Ubx^{+}$ and $Ubx^{\Delta QA}$ alleles, and only those with the desired changes were analyzed further.

## The $Ubx^{\Delta QA}$ allele is pleiotropic

While the QA motif was originally characterized for its role in limb repression (Galant and Carroll, 2002; Ronshaugen et al., 2002), analysis of the homozygous $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ phenotype revealed the QA motif to be highly pleiotropic and involved in several Ubx functions. As will be described below in detail, we found a strong requirement for QA function in some tissues and much less of a requirement in others.

*Ubx* most strongly influences the development of metathoracic (T3) and first abdominal segment (A1) structures. *Ubx* loss-of-function mutations transform these tissues towards more anterior identities (Bender et al., 1983; Lewis, 1963; Lewis, 1978), while ectopic expression of Ubx in anterior tissues transforms them toward T3 or A1 identity (Mann and Hogness, 1990). In dipterans, *Ubx* is required to sculpt the T3 hindwing into a reduced, balloon-shaped haltere. Modest reductions in *Ubx* activity result in increased haltere size and ectopic bristles, making this tissue the most obvious phenotypic readout of reduced *Ubx* activity. Reduction of *Ubx* to a single genetic dose in heterozygotes for null alleles ($Ubx^{-}/Ubx^{+}$) results in halteres that are about twice the volume of wild type and often have multiple ectopic bristles (Fig. 2A,C).

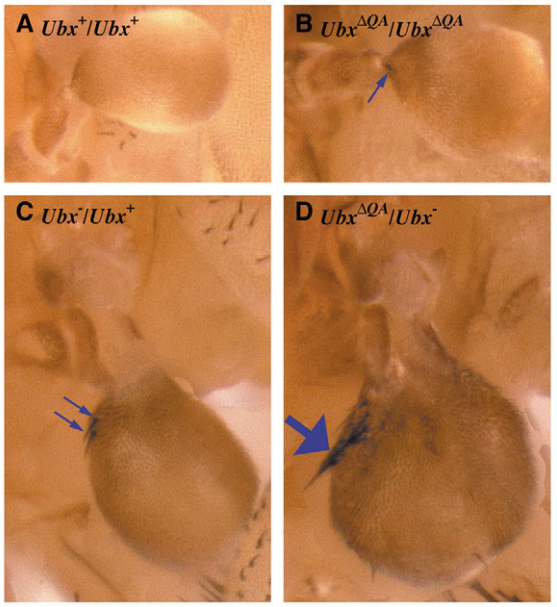


**Fig. 2.** The QA motif has a limited role in haltere development. Haltere genotypic series for both haltere size and number of ectopic bristles (small blue arrows, single; large blue arrow, large quantity): (A) $Ubx^{+}/Ubx^{+}$ > (B) $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ > (C) $Ubx^{-}/Ubx^{+}$ > (D) $Ubx^{\Delta QA}/Ubx^{-}$. $Ubx^{+}/Ubx^{+}$ halteres never had bristles, whereas $Ubx^{\Delta QA}/Ubx^{-}$ always had several bristles. In the *WI129* background (shown above), $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ flies had 0.86±0.73 bristles per haltere, whereas $Ubx^{-}/Ubx^{+}$ flies had 2.59±1.04 bristles per haltere ($P<10^{-17}$; $n$=90 and 60, respectively). In the *Oregon-R* background (not shown), $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ flies had 0.14±0.25 bristles per haltere, whereas $Ubx^{-}/Ubx^{+}$ flies had 0.78±0.50 bristles per haltere ($P<10^{-17}$; $n$=90 and 60, respectively). Anterior is leftwards, dorsal is upwards.

As the haltere is so sensitive to reductions in *Ubx* activity, we first examined the effect of deleting the QA motif here. Heterozygous $Ubx^{\Delta QA}/Ubx^{+}$ flies had no detectable phenotype, indicating the $Ubx^{\Delta QA}$ allele retained some activity and is not neomorphic or antimorphic. Surprisingly, homozygous $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ flies were viable and had an incompletely penetrant haltere phenotype of variable expressivity (Fig. 2B). $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ halteres generally had zero or one ectopic bristle emanating from the anterior, proximal region of the capitellum or, more rarely, from the anterior of the pedicel. Halteres often varied within a single animal, so some of the incomplete penetrance and variable expressivity was due to environmental or stochastic developmental factors. Halteres were slightly increased in size, and some were visually indistinguishable from the wild type. The haltere phenotype of hemizygous $Ubx^{\Delta QA}/Ubx^{-}$ flies was more severe (Fig. 2D), but did not approach the four-winged fly phenotype of a near-complete loss of *Ubx* activity in the haltere nor the phenotypes of strong homozygous *bithorax* (*bx*), *anterobithorax* (*abx*) or *postbithorax* (*pbx*) *Ubx* loss-of-function regulatory alleles (Bender et al., 1983; Lewis, 1963). Thus, with respect to the development of the haltere, the $Ubx^{\Delta QA}$ allele is a weak recessive hypomorph.

## The QA motif plays a partially redundant role in limb repression

Previous studies have suggested that the QA motif played an important role in limb repression (Galant and Carroll, 2002; Gebelein et al., 2002; Ronshaugen et al., 2002). However, $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ adults had a normal complement of limbs. This result suggested the QA motif was not strictly required for limb repression and seemed to agree with previous ectopic expression assays that found that deleting the QA motif caused only a slight reduction in the ability of Ubx to repress thoracic limb primordia and the associated sensory Keilin's organs (KOs) (Gebelein et al., 2002; Ronshaugen et al., 2002). It was possible that the $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ adult phenotype was milder than embryonic or larval phenotypes because the limb repression developmental program was more robust at later stages or because phenotypically extreme animals died early in development. Although $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ larvae had mild A1 denticle belt transformations towards intermediate T3/A1 identity, ectopic A1 KOs were never detected (Fig. 3A,B). Similarly, $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ embryos never produced ectopic limb primordia, as assessed by examination of the expression pattern of the appendage selector and marker protein Dll (Cohen, 1990) (Fig. 4A,B). Thus, the phenotype of $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ adults and embryos were both wild type with respect to abdominal limb repression.

Ectopic expression of Ubx is sufficient to repress thoracic limbs and transform segments to A1 identity (Mann and Hogness, 1990). However, the requirement of *Ubx* to pattern A1 is partially masked by the expression of its *Hox* paralog *abd-A*

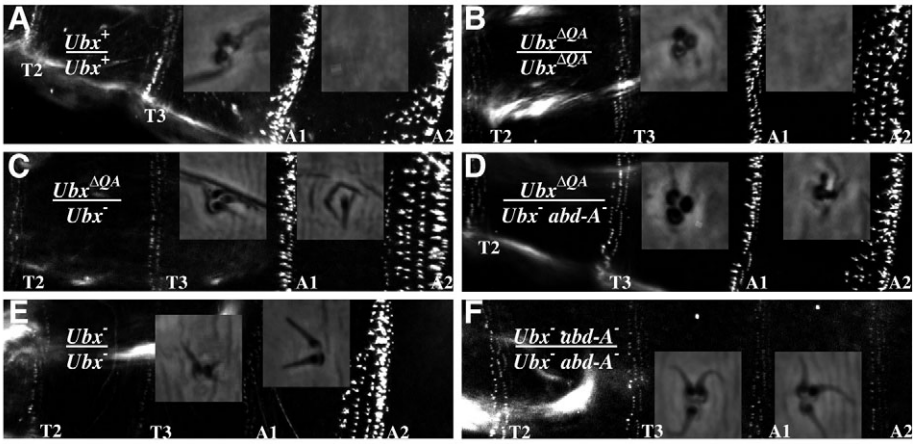


**Fig. 3.** Redundancy of the QA motif in larval limb repression. Ventral/lateral views of T2-A2 of first larval stage cuticles with KOs shown in insets (5×, lightened, T3 and A1 only). (A) *Ubx*$^{+}$/*Ubx*$^{+}$, (B) *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$, (C) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$, (D) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$, (E) *Ubx*$^{-}$/*Ubx*$^{-}$, (F) *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *adb-A*$^{-}$. Each thoracic segment has two three-bristle KOs. *Ubx*$^{+}$/*Ubx*$^{+}$ (A), *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$ (B), *Ubx*$^{-}$/*Ubx*$^{+}$ (not shown) and *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{+}$ *abd-A*$^{+}$ (not shown) larvae never had ectopic A1 KOs. *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ (C) larvae had up to two-bristle ectopic A1 KOs. *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$ (D) larvae had up to three-bristle ectopic A1 KOs. *Ubx*$^{-}$/*Ubx*$^{-}$ (E) larvae generally had two-bristle ectopic A1 KOs but occasionally had one-bristle KOs. *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *adb-A*$^{-}$ (F) larvae had full three-bristle ectopic KOs on A1 through A7. *Ubx*$^{+}$/*Ubx*$^{+}$ larvae (A) have thin, narrow thoracic ventral denticle belts. The A2-A7 denticle belts are broad and trapezoidal, while A1 denticles belts are somewhat less broad and trapezoidal. *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$ (B), *Ubx*$^{-}$/*Ubx*$^{+}$ (not shown), *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{+}$ *abd-A*$^{+}$ (not shown) larvae had mild transformations of the A1 denticle belts towards an intermediate T3/A1 identity. *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ (C) and *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$ (D) larvae had more severe transformations of the A1 denticle belts to intermediate T3/A1 identity. *Ubx*$^{-}$/*Ubx*$^{-}$ (E) larvae had complete transformations of the A1 denticle belts to T2 identity. *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *adb-A*$^{-}$ (F) larvae had complete transformations of the A1-A7 denticle belts to T2 identity. Anterior is leftwards.

in the posterior compartment (Karch et al., 1990). For example, *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *abd-A*$^{-}$ larvae formed full three-bristle ectopic KOs on A1 (Fig. 3F), but *Ubx*$^{-}$/*Ubx*$^{-}$ larvae formed only partial two-bristle ectopic KOs on A1 (Fig. 3E) (Lewis, 1978). Similarly, complete ectopic limb primordia formed in A1 of *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *abd-A*$^{-}$ embryos (Fig. 4F) (Simcox et al., 1991; Vachon et al., 1992), but only incomplete ectopic limb primordia formed in A1 of *Ubx*$^{-}$/*Ubx*$^{-}$ embryos (Fig. 4E). Moreover, fully formed ectopic A1 adult legs have been recovered only from individuals carrying strong *bithoraxoid*

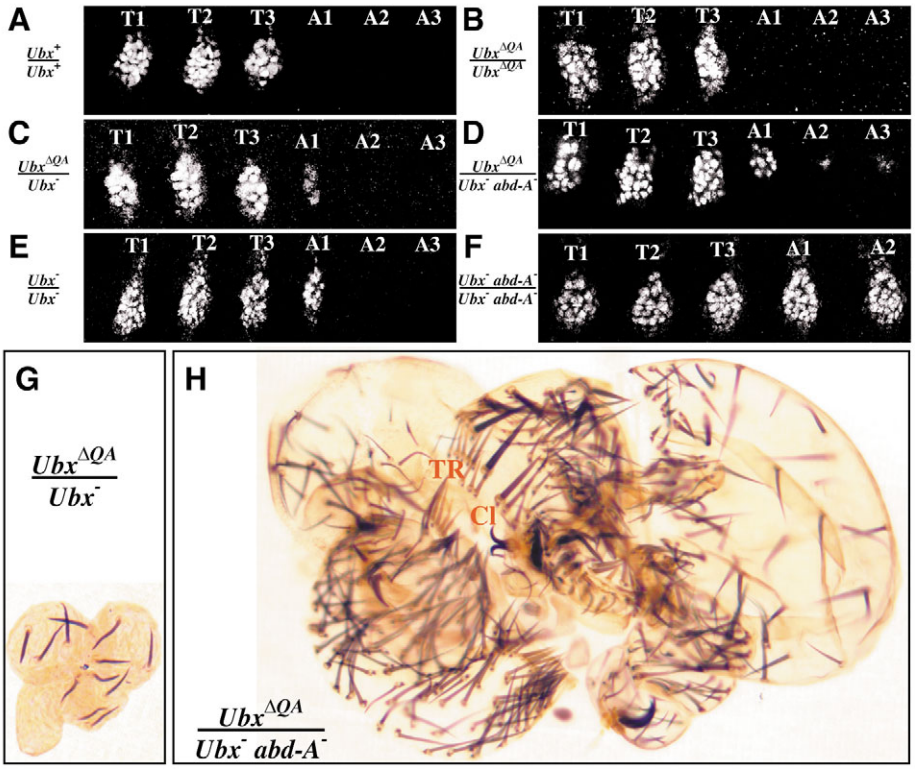


**Fig. 4.** Redundancy of the QA motif in the repression of limb selector expression. Dll expression in T1-A2 or A3 of stage 12 embryos. (A) *Ubx*$^{+}$/*Ubx*$^{+}$, (B) *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$, (C) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$, (D) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$, (E) *Ubx*$^{-}$/*Ubx*$^{-}$, (F) *Ubx*$^{-}$ *abd-A*$^{-}$/*Ubx*$^{-}$ *adb-A*$^{-}$. All genotypes tested had essentially non-overlapping phenotypes, except for those with no A1 Dll expression: *Ubx*$^{+}$/*Ubx*$^{+}$ (A), *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$ (B), and *Ubx*$^{-}$/*Ubx*$^{+}$ (not shown). At earlier stages, abdominal Dll expression in A1 was inconsistent; some *Ubx*$^{+}$/*Ubx*$^{+}$ occasionally expressed Dll in one or two cells in A1, while dose or activity reductions enhanced the number of cells affected (not shown). By stage 12, *Ubx*$^{+}$/*Ubx*$^{+}$ (A), *Ubx*$^{\Delta QA}$/*Ubx*$^{\Delta QA}$ (B) and *Ubx*$^{-}$/*Ubx*$^{+}$ (not shown) embryos did not express Dll in A1, suggesting Dll expression at this stage more accurately marks limb primordia. The Dll expression in A2 and A3 of *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$ embryos (D) was also present in *Ubx*$^{-}$ *adb-A*$^{-}$/*Ubx*$^{+}$ *abd-A*$^{+}$ embryos (not shown) up to segment A7 and suggests a lag in repression when *abd-A* dose is reduced as later stages no longer express this ectopic Dll. Anterior is leftwards, dorsal is upwards. (G) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ sub-epidermal A1 leg tissue found in 1.7% of adults and pupae (5/296); an extreme specimen is shown. (H) *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$ sub-epidermal A1 leg tissue found in 6.7% of adults and pupae (13/194); an extreme specimen with a well-developed claw (Cl) and transverse bristle rows (TR) is shown. In addition to producing more well-developed ectopic A1 leg tissue, the frequency difference between *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ and *Ubx*$^{\Delta QA}$/*Ubx*$^{-}$ *adb-A*$^{-}$ is significant ($\chi_1^2$=8.3, $P$<$10^{-2}$).

(*bxd*) loss-of-function regulatory alleles in $Ubx^{bxd}/Ubx^{-}$ *abd-A*$^{-}$ adults where the genetic doses of both *Ubx* and *abd-A* were reduced (Bender et al., 1983; Lewis, 1963). We wondered whether the lack of strict necessity for the QA motif in limb repression might be due to the additive contribution of other peptide motifs within Ubx and/or redundancy with Abd-A.

Manipulation of the dose level of *abd-A* and *Ubx* revealed a crucial role for the QA motif in imparting full limb repression capacity to Ubx. $Ubx^{\Delta QA}/Ubx^{-}$ larvae formed ectopic A1 KOs with up to two bristles, and $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ larvae formed ectopic A1 KOs with up to three bristles (Fig. 3C,D). $Ubx^{\Delta QA}/Ubx^{-}$ embryos also ectopically expressed Dll in a few cells in A1, whereas $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ embryos generated small but robust ectopic limb primordia of up to half the size of thoracic limb primordia (Fig. 4C,D). Comparison of the $Ubx^{\Delta QA}/Ubx^{-}$ embryos with $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ embryos (compare Fig. 3C and Fig. 4C with 3D and 4D) indicates that the QA motif is partially redundant with Abd-A.

Similarly, comparison of $Ubx^{-}/Ubx^{+}$ and $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ embryos with $Ubx^{\Delta QA}/Ubx^{-}$ embryos (Figs 3, 4) suggests that other peptide motifs within Ubx contribute to the repression of Dll at normal expression levels, which has also been inferred in previous studies (Gebelein et al., 2002; Ronshaugen et al., 2002). However, these motifs are not sufficient for full limb repression when both the QA motif is removed and the genetic dose is reduced in $Ubx^{\Delta QA}/Ubx^{-}$ embryos. In the accompanying manuscript (Tour et al., 2005), the quantitative contributions of two motifs, the YPWM motif and the highly conserved YRXFPLXL motif, are demonstrated. Additionally, our loss-of-function data are in agreement with the steep sigmoidal curves proposed (Tour et al., 2005) for Ubx activity and limb repression.

In rare instances, ectopic A1 limb primordia survived through metamorphosis and produced sub-epidermal adult leg tissue in A1 (Fig. 4G,H). In $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ flies, the leg tissue could undergo a great deal of differentiation such that most bristles had bracts and diverse morphologies, such as claws and transverse bristle rows (Fig. 4H). The extensive differentiation achieved suggests that this genotype allows the production of nearly complete abdominal legs.

## The QA motif is preferentially required in several tissues

Ectopic leg tissues may have failed to evert and remained sub-epidermal because they were blocked from doing so by the A1 pleurum, which formed normally in $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ flies. By contrast, the A1 ventral histoblast nests that form the ventral and lateral pleural epidermis (Madhavan and Madhavan, 1980) are deleted in $Ubx^{bxd}/Ubx^{-}$ *abd-A*$^{-}$ larvae capable of producing A1 legs (Frayne and Sato, 1991). The ability of $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ flies to form normal A1 pleural tissue, even as they failed to repress A1 limb formation, suggests that the QA motif might be preferentially required for a subset of tissues or target genes under Ubx control.

We reasoned that comparing the phenotype of $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ and $Ubx^{-}/Ubx^{+}$ adults in several tissues could provide a rigorous test of this hypothesis. The haltere phenotype indicated a clear genotypic series for *Ubx* activity in the haltere with respect to both size and bristle number: $Ubx^{+}/Ubx^{+} > Ubx^{\Delta QA}/Ubx^{\Delta QA} > Ubx^{-}/Ubx^{+} > Ubx^{\Delta QA}/Ubx^{-}$ (Fig. 2). If the QA motif were uniformly pleiotropic and similarly required across all tissues and Ubx targets, this genotypic series would hold true for all tissues examined. However, if the QA motif were differentially pleiotropic and preferentially required in a subset of tissues, the placement of $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ in the genotypic series might differ among tissues. This was, in fact, the case.

In addition to its role in reducing the dipteran T3 hindwing to a haltere, Ubx represses the formation of other tissues derived from the dorsal T3 disc, such that the adult dorsal mesothoracic (T2) structures nearly abut the adult dorsal A1 structures (anterior histoblast-derived tergite) with only a thin band of T3 laterotergite separating them (Fig. 5A). This function is most clearly illustrated by the phenotype of the four-winged fly, which has nearly all of its dorsal thorax duplicated because of the complete transformation of the dorsal T3 disc to dorsal T2 identity (Lewis, 1963). More moderate laterotergite transformations with extensive ectopic 'postnotal' tissue (named for the dorsal and medial position of the ectopic tissue relative to the notum of the haltere; not named for the postnotum, which is T2 tissue) are characteristic of homozygotes and hemizygotes for strong $Ubx^{pbx}$ and $Ubx^{bxd}$ alleles but have not been described in $Ubx^{-}/Ubx^{+}$ flies (Bender et al., 1983; Lewis, 1963). We found that $Ubx^{-}/Ubx^{+}$ adults only very rarely developed limited postnotal tissue (Fig. 5B), but $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ adults had moderate transformations of the T3 laterotergite towards T2 identity with up to three ectopic bristles per half-laterotergite (Fig. 5C). This result suggests a specific requirement for the QA motif in this region of the

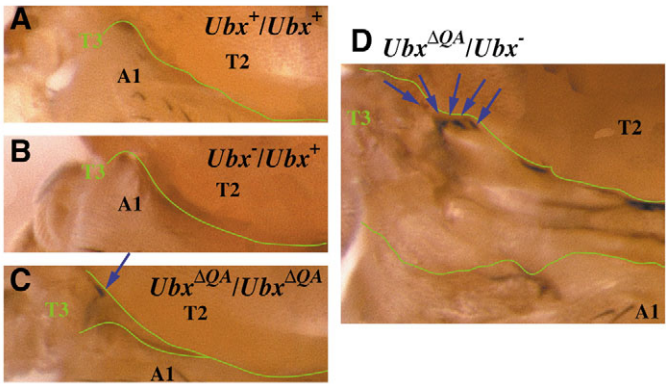


**Fig. 5.** The QA motif is preferentially required for repression of postnotal tissue. Postnotal genotypic series for T3 laterotergite transformation towards T2 thorax as assessed by number of ectopic bristles (blue arrows): (A) $Ubx^{+}/Ubx^{+}$ > (B) $Ubx^{-}/Ubx^{+}$ > (C) $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ > (D) $Ubx^{\Delta QA}/Ubx^{-}$. The T3 laterotergite (green line) is normally a thin band of tissue separating T2 and A1 but is expanded in mutants lacking the QA motif (surrounded by green lines marking border). The junction of the haltere and the laterotergite is marked with a green 'T3' at the edge of the line. Anterior is upwards, lateral is leftwards and the dorsal midline is rightwards. (A) $Ubx^{+}/Ubx^{+}$ T3 half-laterotergites never had bristles, while $Ubx^{\Delta QA}/Ubx^{-}$ (D) always had bristles (up to a dozen). In the *Oregon-R* background (shown above), $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ T3 half-laterotergites (C) had 0.972±0.592 bristles, whereas $Ubx^{-}/Ubx^{+}$ T3 half-laterotergites (B) had 0.017±0.091 bristles ($P<10^{-20}$; $n$=90 and 60, respectively). In the *WI129* background (not shown), $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ T3 half-laterotergites had 0.167±0.290 bristles, whereas $Ubx^{-}/Ubx^{+}$ T3 half-laterotergites had 0.000±0.000 bristles ($P<10^{-5}$; $n$=90 and 60, respectively).

dorsal T3 disc, a requirement further supported by the extensive postnotal tissue in $Ubx^{\Delta QA}/Ubx^{-}$ adults with up to a dozen ectopic bristles per half-laterotergite (Fig. 5D). The reversal of the order of the $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ and $Ubx^{-}/Ubx^{+}$ genotypes in the haltere and postnotal region (compare Fig. 2B,C with Fig. 5C,B) was consistent in different genetic backgrounds and, thus, allows us to reject the null hypothesis that the QA motif plays uniform pleiotropic roles in favor of the differential pleiotropy hypothesis.

$Ubx^{\Delta QA}/Ubx^{-}$ adults also had severely reduced A1 tergites (Fig. 6D), which were nearly absent in extreme cases. Similar A1 tergite reductions occur in homozygotes or hemizygotes of strong $Ubx^{bxd}$ alleles (Bender et al., 1983), stemming from a failure of the anterior dorsal histoblast nests to form (Frayne and Sato, 1991). In many $Ubx^{\Delta QA}/Ubx^{-}$ adults, the combination of an extremely reduced A1 tergite and a partial transformation of the T3 laterotergite toward an intermediate T2/T3 postnotal identity created an indistinct boundary between the dorsal thorax and abdomen (Fig. 5D, Fig. 6D). When *Ubx* activity was less strongly reduced, the average number of bristle rows in adult A1 tergites was clearly dependent on *Ubx* (Fig. 6), but the trait was complex and quantitative. $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ adults had a slight reduction in bristle row number when compared with $Ubx^{-}/Ubx^{+}$ adults (Fig. 6B,C). Collectively, the phenotypes in these tissues suggest an integral role for the QA motif of Ubx in patterning dorsal T3 and A1 tissues, and the morphological boundary between adult thoracic and abdominal segments.

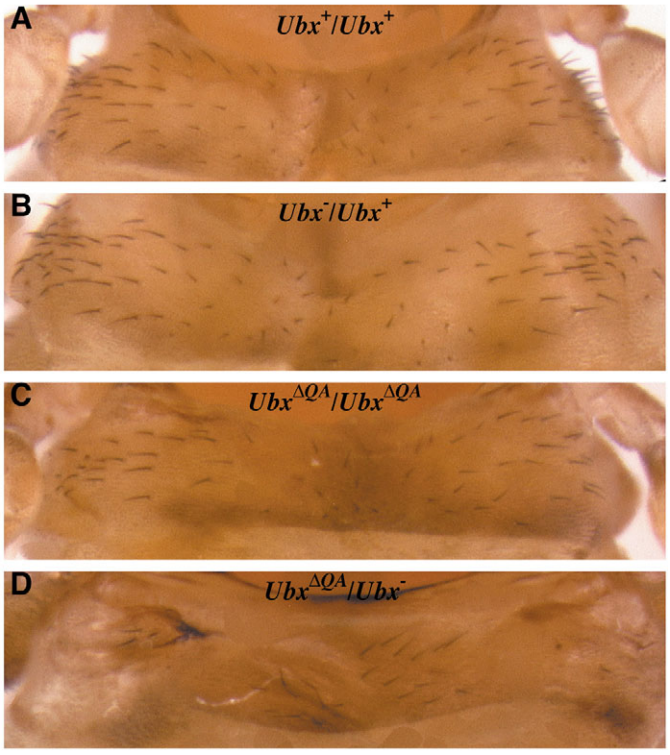


**Fig. 6.** The QA motif is preferentially required for A1 tergite formation. Deletion of the QA motif had a strong effect similar to that of removing one dose of *Ubx*. Genotypic series of A1 tergite formation as assessed by number of rows of bristles: (A) $Ubx^{+}/Ubx^{+}$ > (B) $Ubx^{-}/Ubx^{+}$ ⩾ (C) $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ > (D) $Ubx^{\Delta QA}/Ubx^{-}$. Pooling all data, $Ubx^{+}/Ubx^{+}$ A1 tergites had 5.32±0.49 rows of bristles, $Ubx^{-}/Ubx^{+}$ A1 tergites had 4.44±0.44 rows of bristles, while $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ A1 tergites had 4.26±0.49 rows of bristles (for either comparison to $Ubx^{+}/Ubx^{+}$, $P<10^{-22}$; $Ubx^{-}/Ubx^{+}>Ubx^{\Delta QA}/Ubx^{\Delta QA}$, $P=10^{-2}$; $n$=80, 120, and 180; respectively). In the *WI129* background (shown above), $Ubx^{-}/Ubx^{+}$ A1 tergites (B) had 4.68±0.39 rows of bristles, while $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ A1 tergites (C) had 4.28±0.59 rows of bristles ($P=10^{-4}$; $n$=60 and 90, respectively). In the *Oregon-R* background (not shown), $Ubx^{-}/Ubx^{+}$ A1 tergites had 4.20±0.36 rows of bristles, while $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ A1 tergites had 4.23±0.51 rows of bristles ($P$=0.7; $n$=60 and 90, respectively). Anterior is upwards.

We also found that the QA motif of Ubx was required for the full repression of non-sensory microtrichiae (trichomes) in the posterior of the T2 and T3 legs (T2p and T3p, respectively). In wild-type flies, Ubx represses trichome development on T2p, leaving an area of naked cuticle devoid of trichomes in the proximal region of T2p, while Ubx expression in T3p is sufficiently high that they are nearly completely naked (Stern, 1998). Complete loss of Ubx results in ectopic trichomes, and *Ubx* dose quantitatively determines the extent of naked cuticle (Stern, 1998). In $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ and $Ubx^{\Delta QA}/Ubx^{-}$ T2p, the area of naked cuticle without trichomes was reduced and, in some cases, nearly eliminated, whereas the phenotype of $Ubx^{-}/Ubx^{+}$ T2p was nearer to wild type (Fig. 7A-D). Similarly, we found that the QA motif was required for the repression of T3p trichomes. In $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ and $Ubx^{\Delta QA}/Ubx^{-}$ T3p, expansive regions of trichomes formed distally and ventrally, while $Ubx^{-}/Ubx^{+}$ T3p had only a few distal trichomes comparable with wild type (Fig. 7E-H). Ectopic trichomes on $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ legs were due to differences in Ubx activity and not differences in protein levels, as Ubx expression in $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ T2 and T3 leg discs was indistinguishable from wild type (data not shown).

The small ventral patch, unusually high amount of distal trichomes present on our $Ubx^{+}/Ubx^{+}$ T3p, and the slight quantitative reductions in the size of the naked valley on our $Ubx^{+}/Ubx^{+}$ T2p relative to previously studied 'wild-type' lines (Stern, 1998) suggests that the 3rd chromosome targeted in our study contains other genetic variation affecting trichome patterning. Most, if not all, of this variation was recessive. Despite this variation, the loss of the QA motif had a strong effect on both T2p and T3p trichome patterning whether the recessive variation in our targeted chromosome was homozygous ($Ubx^{+}/Ubx^{+}$ versus $Ubx^{\Delta QA}/Ubx^{\Delta QA}$; compare Fig. 7A,E with Fig. 7C,G) or heterozygous ($Ubx^{-}/Ubx^{+}$ versus $Ubx^{\Delta QA}/Ubx^{-}$; compare Fig. 7B,F with Fig. 7D,H). Moreover, the $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ phenotype is more severe than is ever seen among numerous $Ubx^{-}$ null alleles when heterozygous (D.L.S., unpublished) (Stern, 1998). These data support the conclusion that removing the activities of the QA motif had a greater effect on the capacity of Ubx to repress leg trichomes than reducing Ubx activity by half.

## Discussion

The genetic deletion of the QA motif of Ubx produced a surprisingly subtle but highly pleiotropic homozygous phenotype. We have shown that the QA motif is partially redundant with Abd-A in A1 for limb repression, is one of several motifs within Ubx that quantitatively affect Ubx activity (Gebelein et al., 2002; Ronshaugen et al., 2002; Tour et al., 2005), and that reducing Ubx or Abd-A levels uncovers a requirement for the QA motif in limb repression. The QA

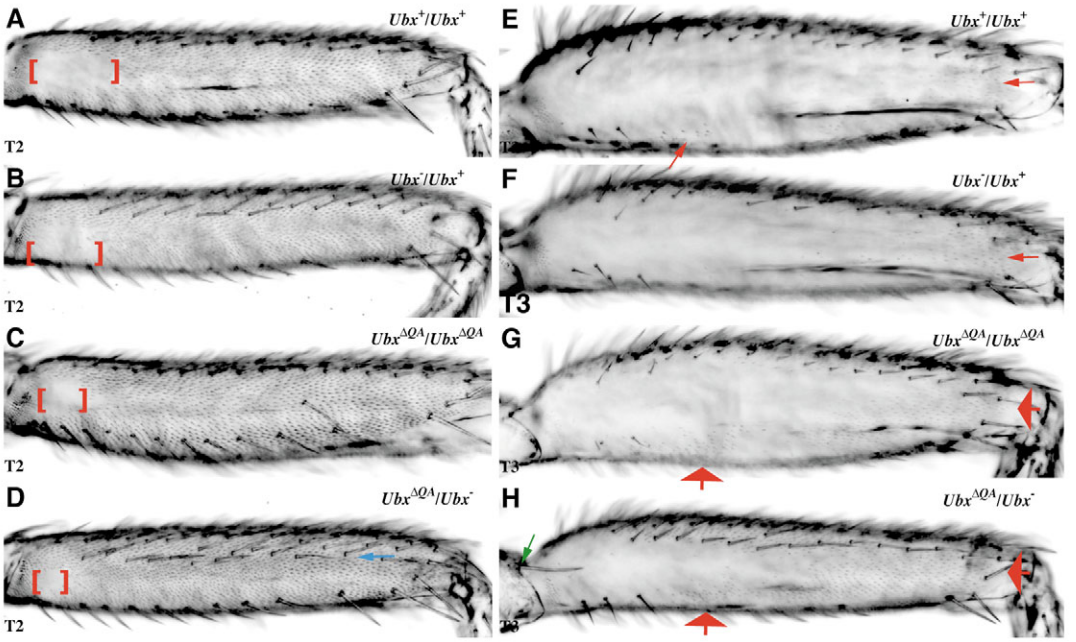


**Fig. 7.** The QA motif is preferentially required for the repression of leg trichomes. Genotypic series of T2p trichome repression (*Oregon-R* background shown): (A) $Ubx^{+}/Ubx^{+}$ > (B) $Ubx^{-}/Ubx^{+}$> (C) $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ > (D) $Ubx^{\Delta QA}/Ubx^{-}$. $Ubx^{+}/Ubx^{+}$ (A) and $Ubx^{-}/Ubx^{+}$ (B) T2p legs had similarly sized large naked valleys lacking trichomes (bracketed in red). $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ (C) and $Ubx^{\Delta QA}/Ubx^{-}$ (D) T2p naked valleys were highly reduced such that nearly the entire leg had trichomes. Genotypic series of T3p trichome repression: (E) $Ubx^{+}/Ubx^{+}$ > (F) $Ubx^{-}/Ubx^{+}$ > (G) $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ > (H) $Ubx^{\Delta QA}/Ubx^{-}$. $Ubx^{+}/Ubx^{+}$ (E) and $Ubx^{-}/Ubx^{+}$ (F) T3p legs had a similarly limited area of trichomes distally (small red arrows), although $Ubx^{+}/Ubx^{+}$ (E) also had some ventral trichomes (small red arrow) owing to a recessive allele (see text). $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ (G) and $Ubx^{\Delta QA}/Ubx^{-}$ (H) T3p legs had large ventral and distal patches of trichomes (large red arrows) that generally fused. $Ubx^{\Delta QA}/Ubx^{-}$ T2 legs (D) generally had an eighth row of bristles (cyan arrow), a phenotype of $Ubx^{-}$ clones (D.L.S., unpublished). $Ubx^{\Delta QA}/Ubx^{-}$ T3 (H) often had ectopic edge bristles (green arrows), a transformation from T3 towards T2 identity, as well as more robust dorsal and proximoventral bristles. Distal is rightwards, dorsal is upwards.

motif is preferentially required for a subset of Ubx-regulated developmental processes, a characteristic we term differential pleiotropy. The conservation of the QA motif throughout the insect lineage suggests some of its many functions are crucial for the proper patterning and fitness of insects. These findings offer a conceptual framework for understanding how pleiotropy, redundancy and selection interact to guide the evolution of selector proteins and the morphology they govern.

## Differential pleiotropy

Selector genes encode proteins that regulate many downstream target genes, often in several different tissues (Garcia-Bellido, 1975; Mann and Carroll, 2002). Therefore, coding sequence mutations are expected to be highly pleiotropic and generally deleterious, especially when the selector is expressed in several different tissues (Carroll, 2005; Carroll et al., 2005; Mann and Carroll, 2002; Stern, 2000). It is clear that regions of selector proteins such as the DNA-binding domain are likely to affect protein activity uniformly wherever the protein is expressed. However, it is uncertain to what extent peptide motifs are preferentially used in the regulation of a subset of selector targets. The $Ubx^{\Delta QA}$ allele allowed us to test genetically whether the QA motif was uniformly pleiotropic or differentially pleiotropic. The reversal of the genotypic series for $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ and $Ubx^{-}/Ubx^{+}$ (compare Fig. 2 with Figs 5-7) demonstrates a differential requirement for QA function between these tissues.

Hox selector proteins, such as Ubx, may accomplish their diverse genetic and regulatory functions by using distinct peptide motifs for the regulation of subsets of target genes. Ubx is expressed throughout development in many tissue types (White and Wilcox, 1984) and engages in both direct activation and repression of multiple target genes (Beachy et al., 1988; Capovilla et al., 1994; Galant et al., 2002; Hersh and Carroll, 2005; Krasnow et al., 1989; Vachon et al., 1992), suggesting that distinct activation and repression motifs exist. In the accompanying study, Tour et al. describe at least three motifs that quantitatively and differentially affect the expression of specific target genes when ectopically expressed, suggesting that Ubx contains several differentially pleiotropic peptide motifs that influence the expression of Ubx target genes. The YPWM motif interacts with Exd (Chang et al., 1995; Passner et al., 1999) and is differentially pleiotropic at least in part because nuclear Exd is not present in all regions where Ubx is active and required (Aspland and White, 1997). Detailed studies on the derived Hox protein Fushi Tarazu (Ftz) have also demonstrated that beetle (*Tribolium castaneum*) Ftz has distinct homeotic and segmentation functions that are differentially mediated by a YPWM motif and a nuclear receptor box or 'LXXLL' motif, respectively (Lohr and Pick, 2005; Lohr et al., 2001). By contrast, use of different peptide motifs on different targets may not be a necessary feature of selector proteins dedicated to one cell type, such as the mouse photoreceptor selector Crx (Livesey et al., 2000), or dedicated

to either activation or repression, such as the posterior compartment selector Engrailed (En) (Courey and Jia, 2001; John et al., 1995; Smith and Jaynes, 1996).

### Redundancy with Abd-A

The QA motif is not strictly necessary for limb repression in A1 at any stage of development because of the additive roles played by other peptide motifs in Ubx and because it is partially redundant with the Hox protein Abd-A (Figs 3, 4). We observed extensive limb derepression in A1 in embryos and adults when both the QA motif was absent and when the *Ubx* and *abd-A* doses were reduced but not when either was manipulated singly. The partial redundancy of the Ubx and Abd-A in limb repression is mechanistically explained by their direct repression of the *Dll* limb primordia enhancer through the same binding site (Gebelein et al., 2004; Vachon et al., 1992). The absence of ectopic limb primordia or limbs on the more posterior abdominal segments of $Ubx^{\Delta QA}/Ubx^{-}$ *abd-A*$^{-}$ flies suggests that the higher level and broader expression of Abd-A (Karch et al., 1990) are sufficient to repress limb formation in more posterior segments (A2-A7).

### Differential pleiotropy and redundancy may facilitate selector protein evolution

Compared with the relatively rapid turnover of *cis*-regulatory elements, the evolution of selector protein function appears to be a rare occurrence, owing, at least in part, to the pleiotropic consequences of mutations in protein coding regions (Carroll, 2005; Mann and Carroll, 2002). By contrast, many *cis*-regulatory elements have a modular architecture and mutations in these elements can more easily adjust the expression of a single gene in a single tissue. Analogously, the differential pleiotropy we observed for the QA motif may provide a degree of modularity to some selector proteins. If natural selection can quantitatively alter a specific trait by modifying selector protein sequence and accrue minimal pleiotropic fitness trade-offs in other tissues, this route might be taken if the fitness gains are great compared with any offsets, if genetic suppressors arise, or if it is the most readily available path.

Redundancy may further limit the number of functions subject to intense purifying selection. For example, if a selector protein performs *n* functions but *n–1* are redundant with the function of other selectors, natural selection may be free to modulate the *n*th function through coding changes with limited effects on the other traits. The two most extreme cases of the evolution of Hox protein function have involved Hox genes that were co-opted for other regulatory functions (Alonso et al., 2001; Lohr and Pick, 2005; Lohr et al., 2001; Stauber et al., 2002). The ancestral Hox3 and Ftz expression domains both overlapped with multiple Hox proteins (Hughes and Kaufman, 2002), suggesting they were at least partially redundant with neighboring Hox genes during their co-option. We propose that the rare instances of the evolution of selector protein function tend to be facilitated when a combination of redundancy and the differential pleiotropy of peptide motifs alleviates the constraints on selector protein evolution.

### The power of purifying selection

There is an intuitive but misleading contradiction between the $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ phenotype and the macroevolutionary time-span over which the motif has been conserved. The QA motif has been conserved in all insects, but the phenotype we observed in $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ *D. melanogaster* affected traits that vary between insects, not between insects and other arthropods. This suggests that, as Ubx has acquired different genetic targets in different insect lineages (Tomoyasu et al., 2005; Weatherbee et al., 1999), so has the QA motif. We have shown that some of the phenotypic effects of deleting the QA motif are mitigated by the contributions of other motifs, redundancy with Abd-A, and differential pleiotropy. Yet, we have also argued that these same forces could facilitate the evolution of selector function under the right combination of circumstances. Why, then, is the QA motif still present in all insect orders studied?

Sudden variation in all of the traits governed by the pleiotropic QA motif would probably not be tolerated in a natural, competitive environment. Even though $Ubx^{\Delta QA}/Ubx^{\Delta QA}$ flies are viable and fertile and have a modest phenotype from a developmental perspective, natural selection acts on genetic variation that has a selection coefficient as small as the inverse of twice the effective population size (Li, 1997; Wright, 1931). For insects, which are likely to have effective population sizes of $10^5$ to $10^6$ (Lynch and Conery, 2003), the difference between the production of an average of one fewer offspring out of a million literally makes the difference between variation that is tolerated and that which is selected against. Therefore, despite a turnover of targets and traits governed, pleiotropic peptide motifs that subtly modulate selector protein function can experience consistent purifying selection that preserves them across vast periods of time.

We thank K. G. Golic and G. Boekhoff-Falk for reagents; K. Vaccaro for *P* element injections; D. K. Young and the Department of Entomology for access to their Syncroscopy Auto-Montage Pro setup; J. P. Gruber and S. W. Paddock for imaging assistance; T. M. Williams, B. M. Hersh and J. H. Yoder for critical reading of the manuscript; and Carroll Lab members for helpful discussion. C.T.H. is a predoctoral fellow of the Howard Hughes Medical Institute. D.L.S. is funded by a grant from the National Institutes of Health and by a David & Lucile Packard Foundation Fellowship. S.B.C. is an investigator of the Howard Hughes Medical Institute.

### Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/23/5261/DC1

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Table S1. Sequences of *Ubx* reduction alleles

| Reduction line | A30 | B1 | C4 | E7 | A31 | B2 | C3 | |
|---|---|---|---|---|---|---|---|---|
| Ubx allele | ΔQA | ΔQA | ΔQA | ΔQA | + | + | + | |
| Position (bp) | | | | | | | | Notes |
| -24 | del | del | del | del | del | del | del | |
| -20 | T | T | T | T | T | T | T | |
| 155 | A | C | A | A | A | C | A | |
| 500 | C | T | C | C | C | T | C | |
| 532 | A | C | A | A | A | C | A | |
| 749 | C | T | C | C | C | T | C | |
| 754 | G | T | G | G | G | T | G | |
| 787 | C | G | C | C | C | G | C | |
| 819 | G | C | G | G | G | C | G | |
| 966 | A | G | A | A | A | G | A | |
| 1002 | CA | GT | CA | CA | CA | GT | CA | |
| 1007 | G | A | G | G | G | A | G | |
| 1231 | G | A | G | G | G | A | G | |
| 1382 | CA | GG | CA | CA | CA | GG | CA | |
| 3256 | A | A | A | A | G | A | A | |
| 3362 | T | T | T | T | C | T | T | |
| 3483 | A | A | A | A | T | A | A | |
| 3548 | C | C | C | C | A | C | C | |
| 3886 | G | G | G | G | A | G | G | |
| 5318 | G | G | G | G | A | A | A | |
| 5769 | A | A | A | A | T | T | T | |
| 6036 | T | T | T | T | C | C | C | Introduced stop codon |
| 6042 | T | T | T | T | C | C | C | Introduced stop codon |
| 6048 | T | T | T | T | C | C | C | Introduced stop codon and *Avr* II |
| 6051 | G | G | G | G | A | A | A | Introduced *Avr* II |
| 9146 | T | T | T | C | T | C | T | |
| 9625 | A | A | A | T | A | A | A | |
| 9786 | A | A | A | del | A | A | A | |
| 9825 | G | G | G | A | G | G | G | |
| 9941 | T | T | T | A | T | T | T | |
| 9977 | GT | GT | GT | AA | GT | GT | GT | |
| | | | | | | | | |
| Minimum | | | | | | | | |
| crossovers | 1 | 1 | 1 | 3 | 1 | 3 | 3 | |

Nucleotide positions are with respect to the 10,875 bp *Aat* II/*Xma* I (bp 1/bp 10875) fragment.

All other nucleotides match the sequence of the BX-C from DS03126, including several on each side not included in the targeting construct.

All polymorphisms, except the introduced four, are non-coding and not expected to impact phenotype.

Red, sequence from pTV2 targeting construct used.

Blue, sequence from targeted endogenous Ubx locus.