Hox protein mutation and macroevolution of the insect body plan

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A fascinating question in biology is how molecular changes in developmental pathways lead to macroevolutionary changes in morphology. Mutations in homeotic (Hox) genes have long been suggested as potential causes of morphological evolution[1,2], and there is abundant evidence that some changes in Hox expression patterns correlate with transitions in animal axial pattern[3]. A major morphological transition in metazoans occurred about 400 million years ago, when six-legged insects diverged from crustacean-like arthropod ancestors with multiple limbs[4–7]. In *Drosophila melanogaster* and other insects, the Ultrabithorax (Ubx) and abdominal A (AbdA, also abd-A) Hox proteins are expressed largely in the abdominal segments, where they can suppress thoracic leg development during embryogenesis[3]. In a branchiopod crustacean, Ubx/AbdA proteins are expressed in both thorax and abdomen, including the limb primordia, but do not repress limbs[8–11]. Previous studies led us to propose that gain and loss of transcriptional activation and repression functions in Hox proteins was a plausible mechanism to diversify morphology during animal evolution[12]. Here we show that naturally selected alteration of the Ubx protein is linked to the evolutionary transition to hexapod limb pattern.

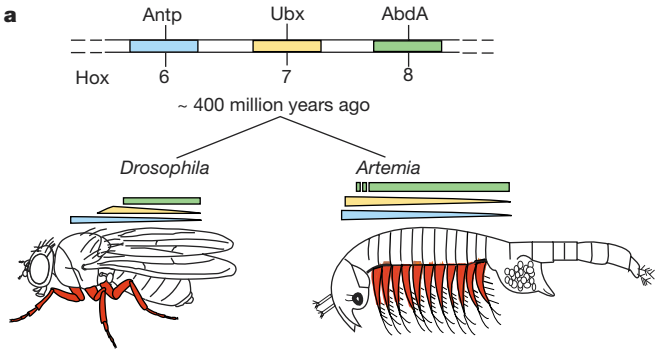
Averof and Akam[8] proposed that the hexapod body plan evolved from crustacean-like ancestors in two phases. First, mutations restricted Ubx/AbdA expression to the proto-abdominal region (Fig. 1a); second, mutations in Ubx/AbdA pathways resulted in suppression of thoracic-type limbs in the proto-abdomen. The mutations in this second 'limb suppression' phase could have occurred in *Ubx*/*AbdA* coding sequences, in regulatory or coding sequences for genes downstream of *Ubx*/*AbdA*, in regulatory or coding sequences for Hox cofactors, or in a combination of these.

In embryos of *Drosophila melanogaster*, ectopic expression of the Ubx protein in the thorax suppresses nearly all limb development; thus the cofactors required for limb repression are present in both thorax and abdomen[13,14]. This ectopic expression assay can be used to test whether a Ubx protein from crustaceans or other arthropods can repress limb development, and was recently employed to determine that the Ubx protein from an onychophoran (*Akanthokara kaputensis*, a species from a sister phylum of arthropods) does not suppress *Drosophila* embryonic limbs[15]. As there is evidence that branchiopod crustaceans and hexapod insects are sister groups[7], we chose to test the Ubx protein from the crustacean *Artemia franciscana* for a limb-suppressing function in *Drosophila* embryos.

We compared the Ubx protein sequence from *Artemia* with Ubx sequences from *Drosophila*, a hexapod mosquito (*Anopheles gambiae*) and an onychophoran (*A. kaputensis*) (Fig. 1b; see Supplementary Information for accession numbers). There are large blocks of amino-acid sequence present in *Drosophila* Ubx that are absent from *Artemia* Ubx and vice versa (Fig. 1b). Within the DNA-binding homeodomain, the *Artemia* Ubx protein has an identical sequence to the two other arthropod Ubx proteins except for a single Ala-to-Ser change (Fig. 1b). All of the arthropod and the onychophoran Ubx amino-acid sequences share six blocks of homology (shown in blue), but there are an additional six blocks of homology (shown in yellow) shared between the two hexapod Ubx sequences.

We first tested transgenic *Drosophila* lines that ectopically produced *Artemia* or *Drosophila* versions of Ubx with or without haemagglutinin antigen (HA) fused to their carboxy termini. The HA epitope was used to show protein pattern and abundance of the ectopically expressed proteins, and to distinguish them from endogenous Ubx. We found no detectable differences between the phenotypes induced by HA-tagged *Drosophila* or *Artemia* Ubx proteins and those induced by wild-type proteins, and neither *Drosophila* nor *Artemia* proteins nor their variants induced ectopic transcription of the endogenous *Ubx* or *AbdA* genes (data not shown).

When either *Drosophila* or *Artemia* Ubx–HA is expressed in the embryonic thorax (Fig. 2a) at levels equivalent to those of endogenous Ubx in the abdomen (see Supplementary Information), the ectopic proteins partially transformed thoracic denticle belts toward abdominal-like identities (Fig. 2b). The *Drosophila* and *Artemia* proteins were also similar in suppressing the first thoracic (T1) denticle 'beard', suppressing the formation of normal head structures, and promoting the development of abdominal denticles in



b

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AfUbx MNSYFEQN-GFYGSHP-----------------ASAPDQAYR-FPLGLN--PYSATAALANIRQTGHEQ
DmUbx MNSYFEQASGFYG-HPHQAT-GMAMGSGGHHDQTASAAAAAYRGFPLSLGMSPYANHHLQRTT----QDS
AgUbx MNSYFEQ-TGFYG-HPHQAAAGMMTTTGTHHDQTTAAAAAAYRGFPLSLGMSPYTNHHLHQTRTA--QES
AkUbx MSSFFEPANMSNTIERYQIGQGIL---------------SSAAFPQIRQSNENFYENSNTY--------

AfUbx PYDTANPAAPCKLYEHQ-------YKLPIDLQSSKEQNAYGLPSKDTNLAWLQQGQLDPTRTTDLSQGGP
DmUbx PYDASITAACNKIYGDGA----GAYKQDCLNIKADAVNGYKDIWNTGGSNGGGGGGGGGGGGGAGGTGGA
AgUbx PYDASIQAACKQIYEGSYSSKDCGTKGTSGNNGTDTSNGYKDVWNANSGATNGATTGATGSNVPAQQNSS
AkUbx -----------KLYATAPTPGQVPYKA---CYYENEQNGYKPSWSPSPSCQSSASRPAPYSPPRTSTTTN

AfUbx RYPESNAMRNVVSQGP-----------------------------------------------------
DmUbx GNANGGNAANANGQNNPAGGMPVRPSACTPDSRVGGYLDTSGGSPVSHRGGSAGGNVSVSGGNGNAGGVQ
AgUbx V-------------------PVRPSACTPDSRVGGYIDASGGSPVS-RAGSA----------------
AkUbx S-------------------------------------------------------------------

AfUbx -----------WSP--CSLSANNSNASNGSRPQTVPQGQGTPEQQHSPSQQPAAFYPWMAIAG-------
DmUbx SGVGVAGAGTAWNAN-CTIS-------------GAAAQTAAASS-LHQASNHTFYPWMAIAGKIRSDLT
AgUbx --AAAAGVPGSWNTNQCSLT-------------GSTGGQAAPSTGLHQ-SNHTFYPWMAIAG-------
AkUbx -----------WTS--CTTS-----------------------QVNPSSETTPFYPWMNVAG-------

                          Dros281Art
AfUbx -------------------------ANGL-RRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHSL
DmUbx QYGGISTDMGKRYSESLAGSLLPDWLGTNGL-RRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHAL
AgUbx ----------KRYSESLAGTLLPDWIGANGL-RRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHAL
AkUbx -------------------------ANGLQRKRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHAL

                                  Art250Dros
AfUbx CLTERQIKIWFQNRRMKLKKEIQAIKELNEQDKRITPSKLHSNCSSPTGDISDDEKDEKL
DmUbx CLTERQIKIWFQNRRMKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAVQGGHLD
AgUbx CLTERQIKIWFQNRRMKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAALHEQN
AkUbx CLTERQIKIWFQNRRMKLKKEMQTIKDLNEQEKKQRDTSLT
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Figure 1 Evolution of trunk Hox gene expression patterns and sequence comparison of arthropod Ubx proteins. **a**, The crustacean lineage (for example *Artemia franciscana*) separated from the insect lineage (for example *Drosophila melanogaster*) about 400 million years ago. Crustaceans retained multiple limbs (red) on the trunk, whereas insect limbs became reduced to three thoracic pairs. At this time in arthropod evolution, the trunk Hox genes (*Antp*, *Ubx* and *Abd-A*) had already duplicated and diverged[23]. **b**, An amino-acid sequence alignment of Ubx protein sequences from the fruit fly *Drosophila* (DmUbx), the mosquito *Anopheles gambiae* (AgUbx), the brine shrimp *Artemia franciscana* (AfUbx) and the velvet worm *Akanthokara kaputensis* (AkUbx). Sequence motifs that are shared to different extents between all of these Ubx homologues are blue; motifs shared only by the hexapods *Drosophila* and *Anopheles* are yellow. The breakpoints of two hybrid proteins shown in Fig. 3 are marked with arrowheads.

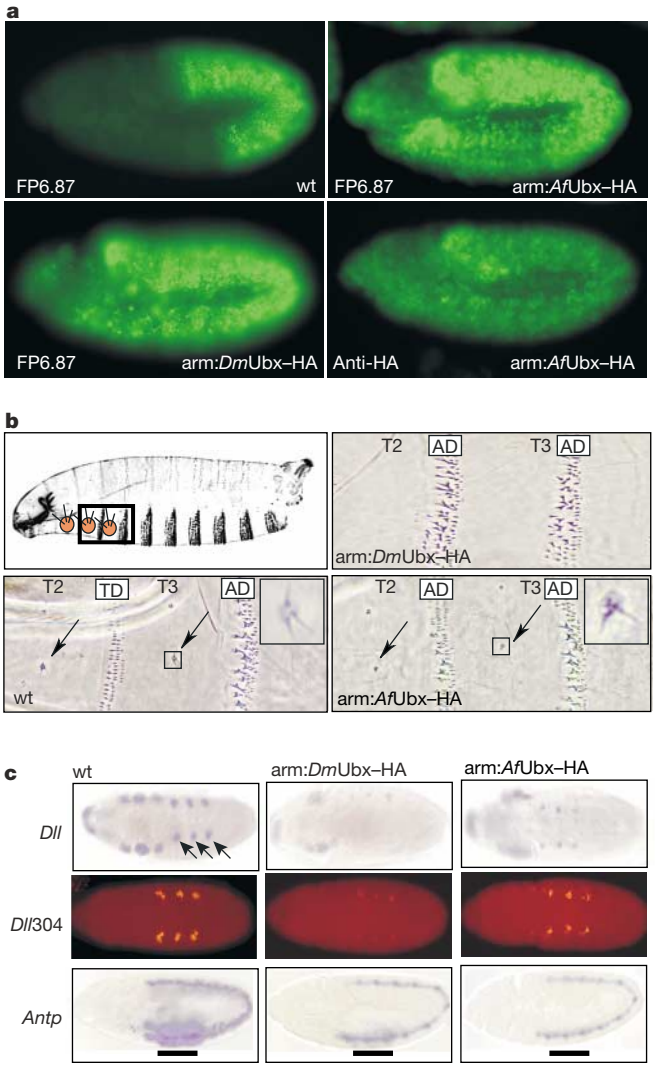

Figure 2 Comparison of the effects of ectopic *Artemia franciscana* (*Af*) Ubx and *Drosophila melanogaster* (*Dm*) Ubx proteins on *Drosophila* morphology and Ubx target genes. **a**, The two leftmost panels show *Dm*Ubx protein levels detected with the monoclonal antibody FP6.87 (ref. 24). The top left panel shows wild-type (wt) *Dm*Ubx detected in its normal domain of the posterior thorax and anterior abdomen. The lower left panel shows that equal levels of UAS–*Dm*Ubx–HA protein are produced in the thorax and portions of the head using an *arm*–GAL4 driver (arm:*Dm*Ubx–HA) under conditions described in the Methods. The upper right panel shows an embryo (arm:*Af*Ubx–HA) in which *Af*Ubx–HA protein is expressed in the thorax at levels equivalent to *Dm*Ubx–HA. In the lower right panel, an *Af*Ubx–HA embryo induced under the same conditions as in the upper right panel is stained with anti-HA monoclonal antibodies. **b**, Top left, a drawing of a *Drosophila* first-instar larva, with the positions of the thoracic limbs (Keilin's organs, KO) shown in red. Wild-type cuticles (wt) develop thoracic KO (arrows), as do cuticles from embryos in which *Af*Ubx–HA protein is ectopically expressed at the levels shown in **a**. Embryos with *Dm*Ubx–HA in the thorax (arm:*Dm*Ubx–HA) do not develop thoracic KO. *Af*Ubx–HA and *Dm*Ubx–HA are similar (with *Af*Ubx–HA slightly weaker) in their capacity to promote homeotic phenotypes such as transformation of thoracic denticle belts (TD) towards abdominal identity (AD), as well as suppression of T1 beard formation and disruption of head involution (not shown). **c**, Top row, the pattern of *Dll* transcripts in wild-type embryos and in embryos ectopically expressing either *Af*Ubx or *Dm*Ubx under the control of an *arm*–GAL4 driver. The paired patches of *Dll* transcript marking the thoracic limb primordia in wild-type embryos are marked with arrows. Middle row, the expression pattern of the thoracic-limb-specific *Dll*304–*lacZ* reporter gene in the same three genotypes. Bottom row, the expression pattern of *Antp* P1 transcripts in the same three genotypes. *Antp* P1 transcripts in the thoracic epidermis (bar) are strongly repressed by both ectopic *Af*Ubx and *Dm*Ubx proteins.

head segments (not shown). The *Drosophila* Ubx–HA protein produced stronger versions of these phenotypes than did *Artemia* Ubx–HA. However, it is clear that the *Artemia* Ubx protein produced in fly embryos is functional, and capable of ectopically inducing some aspects of abdominal identity in a manner similar to *Drosophila* Ubx.

The Ubx homologues from these two species showed striking differences in their abilities to suppress thoracic embryonic limbs (Keilin's organs): *Drosophila* Ubx–HA suppressed all of the limbs whereas *Artemia* Ubx–HA suppressed only 15% (Figs 2b and 3). *Distal-less* (*Dll*) is an important limb-promoting gene in most or all arthropods[10], and *Drosophila Dll* transcription is directly repressed by the binding of Ubx protein to an upstream enhancer called *Dll*304 (ref. 16). As expected, *Drosophila* Ubx–HA strongly repressed *Dll* transcripts and *Dll*304 reporter transcripts in embryonic limb primordia; however, *Artemia* Ubx–HA had only a modest repressive effect on *Dll* transcripts and *Dll*304 reporter levels (Fig. 2c). The inability of the *Artemia* protein to strongly repress *Dll* is not due to the absence of a general repressive function, because embryonic transcripts from the *Antennapedia* (*Antp*) P1 promoter are completely repressed by *Artemia* Ubx–HA, similar to *Drosophila* Ubx–HA (Fig. 2c).

In sum, full-length *Artemia* Ubx provides an 'abdominalizing' function in the *Drosophila* embryonic epidermis, but has little

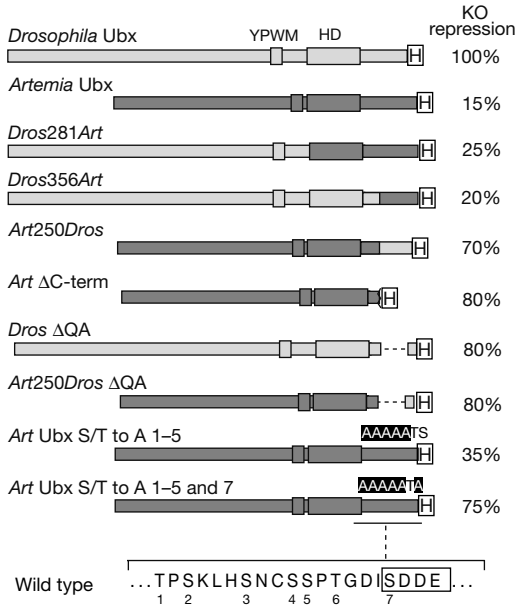

Figure 3 Repression of thoracic limbs by *Artemia*/*Drosophila* Ubx hybrid proteins. On the left are diagrams of the proteins tested in limb-repression assays. The symbols above the proteins denote the relative amounts of *Drosophila* (*Dros*) or *Artemia* (*Art*) Ubx amino-acid sequence. For example, *Art*250*Dros* has the first 250 amino acids of *Artemia* Ubx substituted for the comparable region in *Drosophila* Ubx. In *Art* ΔC-term, the 29 C-terminal amino acids of *Artemia* Ubx were deleted (see Fig. 1 or 4 for sequence). In *Dros* ΔQA and *Art*250*Dros* ΔQA, the 16 amino acids of the QA motif (highlighted in Fig. 4) were precisely deleted. The *Art*Ubx S/T to A constructs contain combinations of precise alanine substitutions in the seven *Artemia* C-terminal serine and threonine residues. These residues are numbered beneath the wild-type *Artemia* C-terminal sequence. The column immediately to the right of the proteins (KO repression) shows the percentage of larval thoracic limbs repressed (Keilin's organs, $n = 300$; rounded to the nearest 5%). This was measured in animals when the ventral thoracic concentrations of the ectopically expressed proteins were adjusted to a level that was less than 30% different to that observed for wild-type Ubx in ventral abdominal cells (see Fig. 2a and Supplementary Information). HD, homeodomain; H, haemagglutinin tag.

repressive effect on thoracic limb development in *Drosophila* embryos. Further, the limb-suppressing difference between *Drosophila* and *Artemia* Ubx is at least partly mediated by their different abilities to transcriptionally repress the *Dll* gene. Although we refer to the distinction between the two proteins as a difference in limb-repression function, we do not mean that this repression function is solely directed to limb-promoting genes.

To map the Ubx limb-repression domain(s) that *Drosophila* apparently possesses and *Artemia* lacks, we constructed a series of hybrid and mutant proteins (Fig. 3). The Ubx hybrid consisting of the amino-terminal 356 amino acids of *Drosophila* and only the C-terminal 29 residues of *Artemia* lost nearly all limb-repressing ability (~20%). Conversely, when the *Drosophila* Ubx C-terminal 26 residues replaced the C terminus of *Artemia* Ubx (*Art*250*Dros*, Fig. 3), the hybrid protein gained limb-repressing ability (70%). One interpretation of these results is that the *Drosophila* Ubx protein has a limb-repression domain in its C-terminal 26 amino acids, whereas C-terminal sequences from *Artemia* are not sufficient for limb repression. Another interpretation is that *Artemia* C-terminal sequences may regulate (inhibit) a limb-repression domain present elsewhere in both the *Artemia* and *Drosophila* Ubx proteins. This latter function would be consistent with previous studies indicating that the C terminus of *Drosophila* Ubx can be deleted with little or no effect on its embryonic limb-repression function[14,17].

To help distinguish between these possibilities, we tested an *Artemia* Ubx–HA mutant protein in which the C terminus was deleted. This mutant protein was a strong limb repressor (80%; Fig. 3). We also tested a variant of *Drosophila* Ubx and a variant of the *Art*250*Dros* hybrid in which a notable block of conserved sequence consisting of glutamines and alanines (the QA motif; Fig. 4) was deleted. Both of the QA-deleted constructs still possess potent embryonic limb-repression functions (Fig. 3). This indicates that the C terminus, and specifically the QA motif, are not required for the full repressive activities of *Drosophila* Ubx or *Artemia*/*Drosophila* Ubx hybrids, and that the C-terminal 29 amino acids of *Artemia* Ubx are inhibiting a limb-repression domain elsewhere in that protein.

In our assays, the C-terminal 45 amino acids of *Drosophila* Ubx had a largely permissive role in *Artemia*/*Drosophila* chimaeric proteins, failing to inhibit a limb-repression domain elsewhere in *Drosophila* Ubx or *Artemia* Ubx. However, some positive repression function may be encoded in the highly conserved QA motif, as the repression of Keilin's organs is reduced by about 20% when this motif is deleted. This is consistent with results from an accompanying paper[18] indicating that sequences that include the *Drosophila* Ubx C-terminal QA domain are sufficient to provide a limb-repressive function in an onychophoran/*Drosophila* hybrid protein in embryos, and are also sufficient to supply transcriptional repressive function in tissue-culture transfection assays.

Because the C-terminal regions of Ubx from a crustacean can exert an inhibitory effect on the limb-repressive function of proteins from the fruit fly or the brine shrimp, we surveyed Ubx C-terminal sequences from a variety of insects and other arthropods (see Supplementary Information for species names and accession numbers) for potentially informative patterns of amino-acid conservation. Notably, all of the Ubx proteins that are known or believed to lack a limb-repressive function have multiple serine and/or threonine amino acids as part of consensus phosphorylation sites in their C-terminal domains (Fig. 4). In *Artemia* Ubx, the most C-terminal

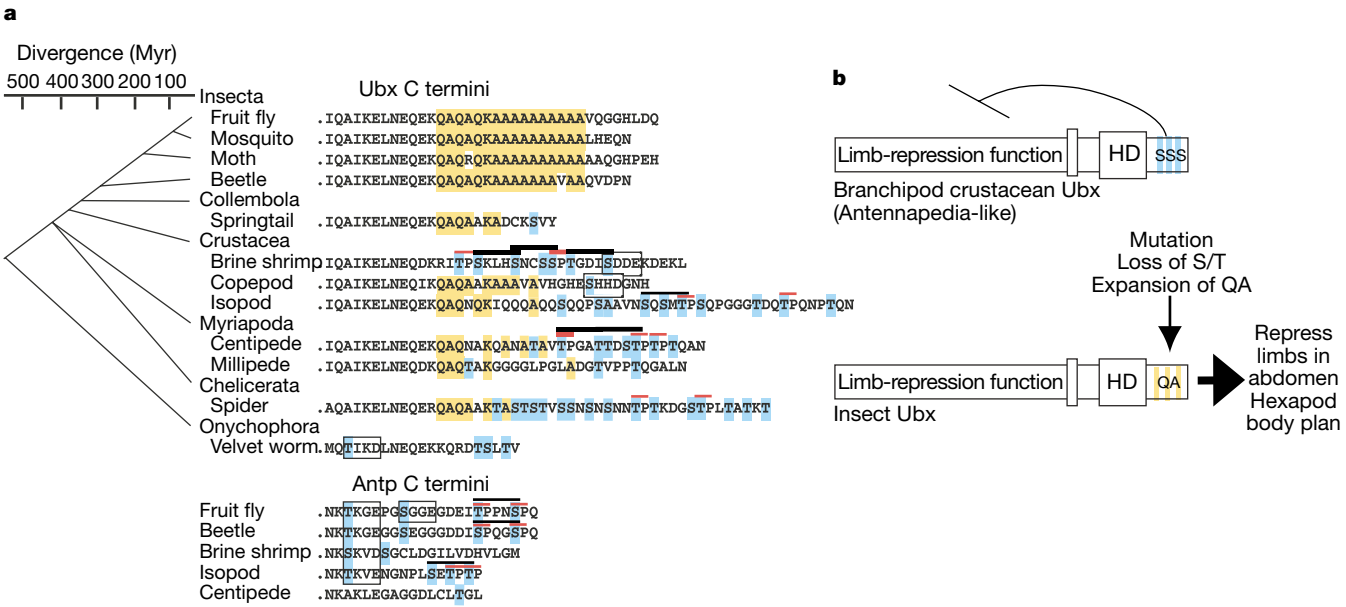

Figure 4 The evolution of Ubx and Antp protein sequence in insects and other arthropods. **a**, Comparison of Ubx and Antp C-terminal sequences. Sequences of the C termini of Ubx proteins from a variety of insects and other arthropod species are aligned on the top right. This region includes the 16-amino-acid *Drosophila* QA motif (QAQAQKAAAAAAAAAA). Matches to this sequence in the Ubx sequences of other arthropods are shown in yellow. A phylogenetic tree on the left shows the branching order of the other taxa from *Drosophila* and the approximate divergence times before present (Myr, million years ago). At the bottom, the Antp C termini from two insects and three other arthropod species are shown. The CKII consensus phosphorylation sites are boxed in both Antp and Ubx homologues. Consensus sites for GSK-3 phosphorylation are marked with black bars; S/TP motifs that are potential sites for MAP kinase phosphorylation are marked with red bars. Ser and Thr residues in these or other potential phosphorylation sites in the arthropod Antp and Ubx C termini are shown in blue. Accession numbers for the sequences shown in this figure can be found in the Supplementary Information. **b**, Model of the proposed functional change in Ubx protein in the insect and branchiopod crustacean lineages. Mutations in an ancestral form of Ubx in a crustacean/insect progenitor removed Ser/Thr phosphorylation sites and thus the inhibition of a limb-repression function located in N-terminal sequences of ancestral Ubx. This inhibitory function, of unknown mechanism, still exists in present-day branchiopod crustacean Ubx. These mutations, when assisted with an expansion of a QA-rich domain in the C terminus, generated an insect version of Ubx which had limb-repression functions that contributed to the hexapod body plan.

Ser is part of a casein kinase II (CKII) consensus phosphorylation site, which after phosphorylation would generate additional CKII and GSK-3 consensus sites[19] (Fig. 4). None of the insect Ubx proteins have Ser or Thr residues in their C-terminal domains (Fig. 4). This correlation is of great potential interest because Ser/Thr residues in the Antp Hox protein have been shown to modulate its function in embryos[20]. Replacement of Ser or Thr by Ala residues in four CKII consensus sites of Antp (including the two shown in Fig. 4) resulted in a Hox protein that was a potent repressor of limb development and *Dll* transcription[20]. One of these CKII sites, just downstream of the homeodomain, is highly conserved in Antp-like Hox proteins in mammals[21]. This, in combination with the results reported here, suggests that the inability of the Ubx proteins from *Artemia* and other multi-limbed arthropods to repress limbs might reside in Ser/Thr phosphorylation sites that inhibit a covert limb-repression domain in arthropod Ubx proteins.

To test this, we generated mutant versions of *Artemia* Ubx in which C-terminal Ser/Thr residues were mutated to Ala. In the first such mutant (*Art* Ubx S/T to A 1–5), the first five Ser and Thr residues in the C-terminus are changed to Ala. This mutant Ubx has little limb-repression function, similar to wild-type *Artemia* Ubx (Fig. 3). However, the mutation of one additional Ser in a CKII consensus site (*Art* Ubx S/T to A 1–5 and 7) results in a Ubx that strongly represses embryonic limbs (Fig. 3).

On the basis of these results, we propose that Ubx proteins in some crustacean/insect ancestors uncovered a limb-repression function by the mutation of C-terminal Ser/Thr phosphorylation sites. Together with the restriction of Ubx expression to the posterior trunk and expansion of a QA-rich domain, the loss of these sites would have contributed to the evolution of the hexapod body plan. The putative phosphorylation-mediated regulation of transcriptional repression function in arthropod Ubx proteins may occur by a similar mechanism to that recently described for the *Drosophila* Even-skipped protein[22]. In both cases, such a mechanism would provide for the mediation by signal transduction of the control of transcriptional activation and repression functions of homeobox genes.

To our knowledge, this is the first experimental evidence that links naturally selected alterations of a specific protein sequence to a major morphological transition in evolution. There are at least two major reasons why the mutation of mutiple Ser/Thr residues that inhibit a repression function might be advantageous from an evolutionary aspect. First, mutating the residues would give dominant phenotypes, eliminating the need to fix two recessive mutations in a morphologically evolving lineage. Second, the successive removal of Ser/Thr residues might quantitatively influence repression function and morphology, allowing viable micro-evolutionary steps toward "hopeful monsters"[1] with macroevolutionary alterations in body shape. □

Methods

Ectopic expression constructs

Full-length Ubx and Ubx-hybrid expression constructs were made by polymerase chain reaction (PCR) from full-length cDNAs derived from reverse transcription. PCR was used to incorporate a near-optimal translation-initiation consensus at the 5′ end. PCR was also used to incorporate codons for the haemagglutinin antigen at the 3′ end of the Ubx open reading frame. *Drosophila*/*Artemia* hybrid proteins were made by first amplifying coding fragments of *Drosophila* and *Artemia* Ubx with overlapping sequences incorporated into primers. Full-length chimaeras were then constructed by amplifying with primers that incorporated the 5′ and 3′ modifications previously described. These were blunt-end cloned into the Gal4-inducible vector pUAST. These constructs were injected into w^{1118} embryos and multiple transgenic lines were established and tested for ectopic expression and function as described in the text.

Genetics, embryonic cuticles and gene expression

Other *Drosophila* lines were obtained from the Bloomington Stock Center. These include: UAS–*Ubx1a*, *arm*–GAL4, and *arm*–GAL4; *Dll304*–*lacZ*. Male flies carrying the UAS–*Ubx* constructs were mated in cages to virgin female flies homozygous for *arm*–GAL4 on the second or third chromosome. Embryos were collected for about 12 h and aged for more than 24 h before the preparation of cleared cuticles. To establish equivalent amounts of expression of Ubx and Ubx-hybrid proteins, we varied the transformed line, the type of *arm*–GAL4 driver, and the temperature (25 or 29 °C) (also see Supplementary Information).

Antiserum staining and *in situ* hybridization

All antibody stains were performed on 3–9-hour-old embryos that were dechorionated and fixed for 20 min in 4% formaldehyde. The methods and antibodies used to detect HA, Ubx and β-galactosidase, as well as methods and probes for *in situ* hybridization can be found in the Supplementary Information.

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Competing interests statement

The authors declare that they have no competing financial interests..

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do a little dance for the other yeast, and hope that in the future, when someone says 'yeast', scientists will give equal thought to the species that was first isolated from a traditional African beer known as Pombe. ■

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Evolutionary biology

How insects lose their limbs

Mike Levine

Evolution has produced marvellous variety in the arthropods, and in their various appendages. The evolutionary processes are themselves proving highly diverse.

From the standpoint of diversity in form and sheer number, the arthropods are the most successful animals on Earth. They embrace four remarkable groups: trilobites (sadly extinct), insects, crustaceans (shrimp, lobsters, crabs and so on), and chelicerates (horseshoe crabs, spiders and scorpions). The success of the arthropods stems, in part, from their modular architecture. They are composed of a series of repeating body segments that can be modified in seemingly limitless ways. Some segments carry wings, whereas others have antennae, legs, feeding organs or specialized mating devices.

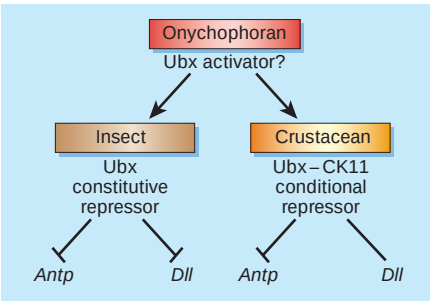

Figure 1 **Evolution through changes in Hox protein function. An interpretation of the new results[1,2] runs like this. Onychophorans, such as velvet worms, are close relatives of the arthropods, and have limbs on every segment. Here Ubx protein may function as an activator, but when onychophorans and arthropods diverged it acquired one or more repression domains, which suppressed limb development. In insects these domains mediate constitutive repression of target genes, such as *Antp* and *Dll*. During the subsequent crustacean–insect divergence, Ubx in crustaceans acquired a regulatory peptide containing potential CKII phosphorylation sites, making Ubx act as a conditional repressor. In the brineshrimp *Artemia*, for instance, Ubx represses *Antp* without influencing the expression of *Dll*. An alternative view is that the onychophoran protein contains both a repression domain and a regulatory peptide, the peptide being lost in insects but retained in crustaceans.**

Another item can be added to the list of things that are special about the arthropods: we know more about the evolutionary processes responsible for their diversification than for any other group of animals. These insights have been made possible by detailed study of the genetic mechanisms underlying the development of that most thoroughly characterized of animals — an insect, the fruitfly *Drosophila melanogaster*. After nearly a century of genetic analysis, many of the genes responsible for segmentation and limb development have been identified. Foremost among these is a class of regulatory genes, the Hox genes, which encode DNA-binding proteins and control early development. During the past ten years this information has been used in the burgeoning field of 'evo–devo', which lies at the cusp of evolutionary biology and embryology, to determine how limbs have diversified among different arthropods.

Children are taught that insects have six legs, two on each of the three thoracic (middle) segments, and this applies to every one of the more than a million species of insect. By contrast, other arthropods, such as crustaceans, have a variable number of swimming limbs. Some crustaceans have limbs on every segment in both the thorax and abdomen. Papers on pages 910 and 914 of this issue, by Galant and Carroll[1], and by Ronshaugen *et al.*[2], provide new insights into how insects have lost abdominal limbs, and so contain only six legs.

The two groups[1,2] provide evidence that suppression of abdominal limbs in insects depends on functional changes in a protein called Ultrabithorax (Ubx), which is encoded by a Hox gene. Ubx represses the expression of another gene, *Distalless* (*Dll*), which is required for limb formation, in the anterior abdomen of the *Drosophila* embryo. However, in crustaceans, such as the brine shrimp *Artemia*, all of the developing limbs have high levels of Ubx.

The other comparison to be made here is with velvet worms. These are members of the Onychophora — close relatives of the arthropods — which have limbs on all segments. In velvet worms, Ubx is expressed in at least a subset of these limbs. So Ubx expression is compatible with limb development in crustaceans and onychophorans, but is incompatible with limb development in *Drosophila* (and other insects).

The new work involved misexpression of the *Drosophila* Ubx protein in the presumptive thorax of transgenic fruitfly embryos. Limb development was suppressed because of repression of Dll. By contrast, the misexpression of onychophoran and crustacean Ubx proteins did not interfere with Dll expression and the formation of thoracic limbs. These results raised the possibility that the *Drosophila* Ubx protein is functionally distinct from Ubx in onychophorans and crustaceans. One study suggests that *Drosophila* Ubx has acquired an alanine-rich peptide that mediates the repression of gene transcription; this peptide is lacking in

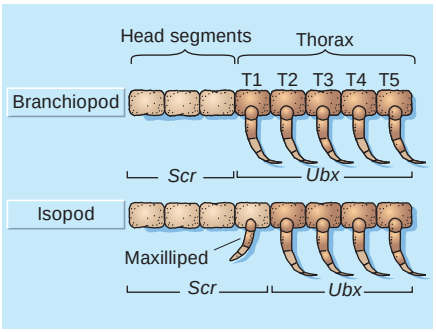

Figure 2 **Evolution through changes in Hox gene expression. In crustaceans known as branchiopods (top), the head contains feeding appendages, whereas thoracic segment T1, nearest the head, contains swimming appendages that are like those further back on the thorax (segments T2–T5). In these animals, expression of one Hox gene (*Scr*) is restricted to head segments, and *Ubx* is expressed in all thoracic segments. In other crustaceans, such as isopods (bottom), the first thoracic appendages have been modified into feeding structures called maxillipeds. This change correlates with altered patterns of Hox gene expression: *Ubx* is replaced by *Scr* expression in the first thoracic segment.**

onychophorans[1]. The other study[2] provides evidence that the crustacean Ubx contains an additional peptide that modulates the activity of the alanine-rich peptide, and possibly other repression domains, in crustacean Ubx.

Removing the regulatory peptide in the crustacean Ubx protein causes it to repress Dll in fly embryos. Conversely, modifying the fruitfly Ubx protein to include the regulatory peptide abrogates its repression activity. The peptide contains potential casein kinase (CKII) phosphorylation sites, so the crustacean Ubx protein may function as a conditional repressor: it can repress the expression of the Hox gene *Antennapedia (Antp)* in thoracic regions without altering the expression of *Dll* in the same tissues. During the divergence of the crustaceans and insects, Ubx might have evolved into a dedicated — constitutive — repressor of limb development in insects.

A scheme for the evolution of Ubx function is shown in Fig. 1. The onychophoran Ubx protein might function as an activator of appendage development. When the onychophorans and arthropods diverged, Ubx acquired an alanine-rich repression domain near its carboxy terminus. This domain mediates constitutive repression in insects. But in crustaceans the addition of the regulatory peptide causes it to function in a conditional fashion. As a result, Ubx does not suppress limb development in crustaceans. But it eliminates abdominal limbs in insects, greatly reducing the overall number of appendages compared with crustaceans.

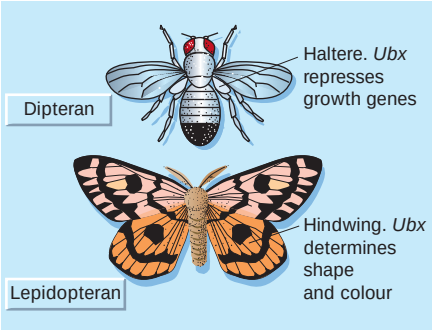

Figure 3 **Evolution through changes in Hox target genes. Among the insects, dipterans (such as *Drosophila*, top) have rudimentary wings, called halteres, in place of hindwings. Ubx represses growth in the halteres, suppressing wing development. In contrast, lepidopterans (such as moths, bottom) have well-developed hindwings. Ubx does not suppress growth in lepidopteran hindwings, and it has been proposed that the *cis*-regulatory sequences associated with these genes lack binding sites for the Ubx repressor. In butterflies, Ubx primarily regulates genes that determine characteristics of the hind- and forewings, such as those involved in determining shape and colour.**

The work of Galant and Carroll[1], and Ronshaugen *et al.*[2], is a striking demonstration of the importance of protein evolution in the diversification of arthropod limbs. The analysis[2] of the crustacean Ubx protein provides a particularly rigorous standard for future evo–devo studies, in that these authors identified the exact amino-acid substitutions that are responsible for the suppression of insect limbs.

However, there are other sides to the story. For instance, changes in gene expression, rather than changes in protein function, have been implicated in the conversion of swimming limbs into feeding appendages in certain crustaceans[3,4] (Fig. 2). In this example, the shift in the *Ubx* pattern is accompanied by a change in the expression of another Hox gene, *Sex combs reduced* (*Scr*).

Another example comes from the evolutionary conversion of hindwings into rudimentary wings (halteres) in the insect group, the Diptera, that includes *Drosophila*[5,6]. This process centres on '*cis*-regulatory sequences', which are stretches of DNA adjacent to a gene that influence its expression. In *Drosophila*, the production of halteres may have depended on the gradual acquisition of binding sites for Ubx protein in the *cis*-regulatory DNAs of different 'growth genes', such as *wingless* and *decapentaplegic*. As discussed above, Ubx functions as a dedicated repressor in insects. Although it is expressed in the hindwings of butterflies, it does not suppress their growth, possibly because there are no Ubx-binding sites in the *cis*-regulatory DNAs of the butterfly growth genes[5,6].

In summary, evo–devo studies provide evidence for three distinct mechanisms of limb evolution in arthropods. First, there are changes in Hox gene expression patterns (Fig. 2). Second, a given Hox protein can regulate different target genes in different insects, owing to the evolution of Hox-protein-binding sites in the *cis*-regulatory DNAs of the target genes (Fig. 3). Third, as exemplified in the new studies[1,2], Hox proteins can evolve new activities (Fig. 1). Once again we are reminded that evolution is opportunistic and uses every trick in the book to generate "endless forms most beautiful and most wonderful"[7]. ■

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Daedalus

The cliff of stability

Large atomic nuclei, containing many protons and neutrons, tend to be unstable. But stable nuclei do exist, and can be seen as an 'island of stability' on a graph of proton number against neutron number. As the number of protons increases, the number of neutrons required for stability increases too. Daedalus now points out that neutron stars are stable, even though they have no protons but enormous numbers of neutrons. So the graph should have a 'cliff' of stable neutron-rich nuclei along the neutron axis, rising out of the sea of instability. DREADCO physicists are now looking for such a cliff.

X-ray spectroscopy irradiates an atom with an energetic photon that ejects an electron from a low energy level. A higher electron then 'falls' into the vacancy. At some frequency the electron should emit all of its energy and fall not just into a lower orbit, but right into the nucleus. This nuclear transformation would create a new element, with one more neutron and one less proton than the original.

The process would absorb or emit large amounts of energy, and would have to be conducted slowly. But hydrogen and its two isotopes deuterium and tritium — which have one and two neutrons, respectively, in addition to hydrogen's single proton — should become pure neutrons if their electrons drop into the nucleus. A single neutron is unstable; how many must come together for them to be stable?

'Nuclear matter' would be so dense it would be hard to handle. But, says Daedalus, a heavy element such as gold could have most of its electrons dropped into the nucleus, and still keep some in orbit to balance the nuclear protons. The resulting large atomic nucleus would be stabilized by its excess of neutrons, although it might slowly acquire orbiting electrons by beta-capture. These orbiting electrons would make it a low-atomic-number element, such as hydrogen. Their vast orbital space would give it a high but controllable density, around a hundred times that of water. This would be 'super-heavy' hydrogen, although you could do the same for helium or lithium, for example.

Daedalus anticipates new chemistry. 'Superheavy hydrogen' should give dense types of water and hydrocarbons, probably incompatible with life. A nucleus of hundreds of neutrons stabilized by a few protons could be taken up the periodic table by a beam of protons until it approached the elusive 'island of stability' from below. And dense anti-tank shells would not need depleted uranium. David Jones