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.

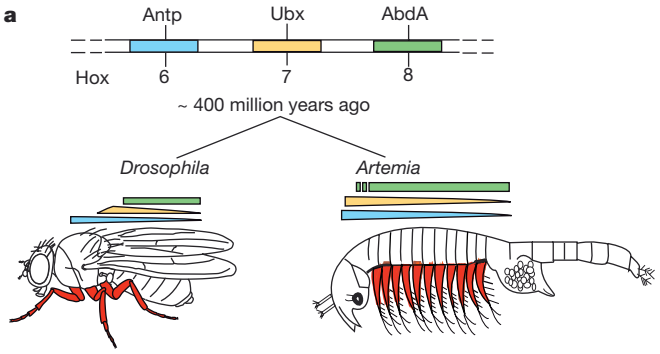
Averof and Akam[8] proposed that the hexapod body plan evolved from crustacean-like ancestors in two phases. First, mutations restricted Ubx/AbdA expression to the proto-abdominal region (Fig. 1a); second, mutations in Ubx/AbdA pathways resulted in suppression of thoracic-type limbs in the proto-abdomen. 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

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b
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.

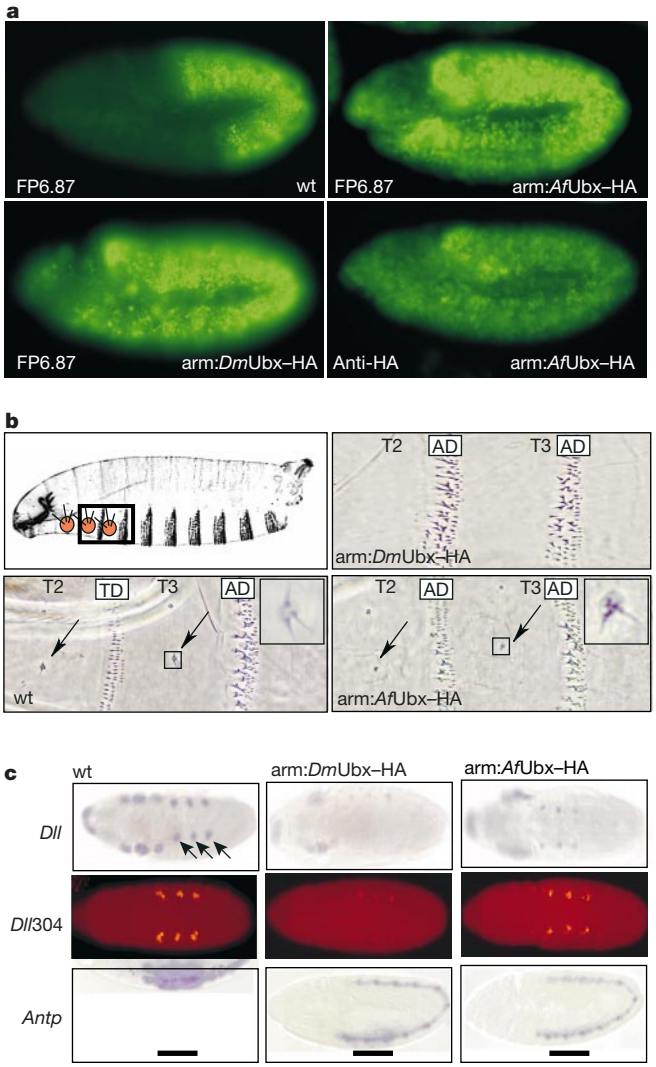

Figure 2 Comparison of the effects of ectopic *Artemia franciscana* (*Af*) Ubx and *Drosophila melanogaster* (*Dm*) Ubx proteins on *Drosophila* morphology and Ubx target genes. **a**, The two leftmost panels show *Dm*Ubx protein levels detected with the monoclonal antibody FP6.87 (ref. 24). The top 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

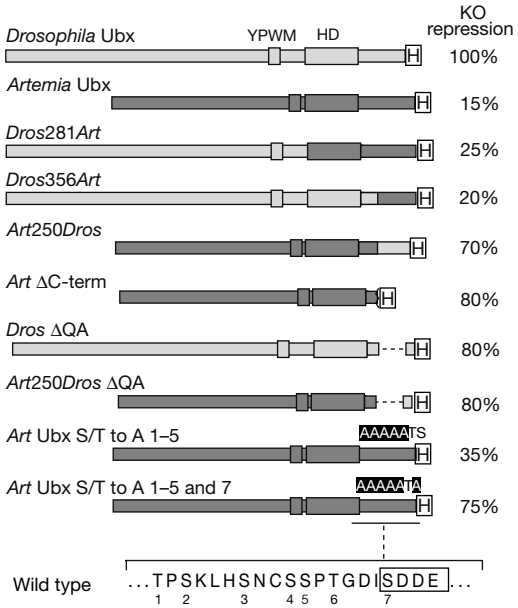

Figure 3 Repression of thoracic limbs by *Artemia*/*Drosophila* Ubx hybrid proteins. On the left are diagrams of the proteins tested in limb-repression assays. The symbols above the proteins denote the relative amounts of *Drosophila* (*Dros*) or *Artemia* (*Art*) Ubx amino-acid sequence. For example, *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

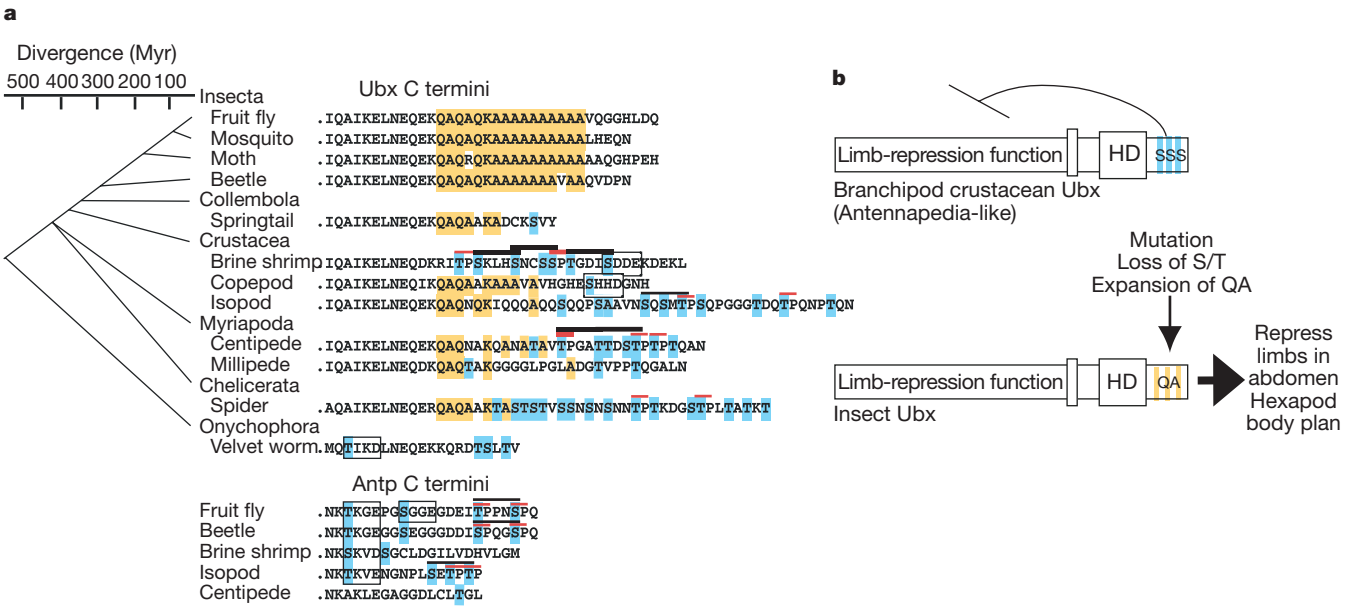

Figure 4 The evolution of Ubx and Antp protein sequence in insects and other arthropods. **a**, Comparison of Ubx and Antp C-terminal sequences. Sequences of the C termini of Ubx proteins from a variety of insects and other arthropod species are aligned on the top right. This region includes the 16-amino-acid *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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