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Evolution of the interaction between *Hox* genes and a downstream target

Michael F. Palopoli* and Nipam H. Patel*†

Segmental identities along the insect body depend on the activities of *Hox* genes [1,2]. In *Drosophila melanogaster*, one well-studied *Hox* regulatory target is *Distal-less (Dll)*, which is required for the development of distal limb structures [3]. In abdominal segments, *Dll* transcription is prevented when *Hox* proteins of the Bithorax Complex (BX-C) bind to *cis*-regulatory elements upstream of the *Dll* transcription start site [4,5]. Previous evolutionary comparisons of gene expression patterns suggest that this direct repression is conserved between Diptera and Lepidoptera, but is absent in the Crustacea [6,7]. We examined gene expression patterns in three orders of hexapods, all of which develop abdominal appendages, in order to determine when the strong repressive interaction between BX-C proteins and *Dll* appeared during evolution. In each of the species examined, *Dll* expression was initiated in abdominal cells despite the presence of high levels of BX-C proteins. It appears that the strong repressive effects of BX-C proteins on *Dll* expression arose relatively late in insect evolution. We suggest that the regulatory interaction between the BX-C genes and *Dll* has evolved within the hexapods in a complex, segment-specific manner.

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Results and discussion

In *D. melanogaster*, the BX-C proteins have different effects on *Dll* expression at early and late stages of embryogenesis [5]. The early *Dll* enhancer integrates positional cues to define where the limb primordia will form: every segment provides activating signals, but only the abdominal segments provide the repressive signals of the BX-C proteins. Later, once functional levels of the *Dll* protein have accumulated in primordial cells of the thoracic limb, a

Dll-protein-dependent enhancer takes over, so that the later appearance of BX-C proteins in some of those cells does not repress *Dll* expression. This temporal difference means that, in order to assess whether a similar repressive interaction exists in another species, we must determine whether *Dll* expression is initiated in the presence or absence of BX-C proteins. To detect expression, we stained embryos using a monoclonal antibody FP6.87, raised against the BX-C proteins Ultrabithorax (Ubx) and Abdominal A (Abd-A), that works across several classes of arthropods [6,7], and a polyclonal antibody against *Dll* that works across a wide range of metazoans [7].

Co-expression of *Dll* and Ubx/Abd-A in collembolans

Collembola (springtails) are apterygote hexapods that are considered a sister group to the insects proper [8]. They are interesting in the present context because they develop three distinctive appendages on the ventral surface of the abdomen (Figure 1a). The expression patterns of Ubx/Abd-A and *Dll* in representative collembolans are shown in Figure 1.

As expected, *Dll* protein appeared in the distal tip of developing appendages, and Ubx/Abd-A proteins were expressed with spatiotemporal dynamics that parallel those in other more well-studied insects, with an early anterior boundary in the abdomen followed by expansion into portions of T3 and the posterior part of T2 (Figure 1b,f; data not shown). Each of the three abdominal appendages began development as paired primordia that eventually fused at the ventral midline; all of these primordia stained for *Dll* protein throughout development (Figure 1c). Interestingly, Ubx/Abd-A protein appeared before *Dll* protein was detectable in primordial cells of the abdominal appendage; this is shown for one of the paired A3 primordia in Figure 1d. As the abdominal appendages developed, *Dll* and Ubx/Abd-A protein distributions continued to overlap (Figure 1e). It appeared that all of the cells expressing *Dll* in segments A1–A4 also contained Ubx/Abd-A protein (Figure 1f).

These results suggest that the regulatory interaction between *Dll* and the genes in the BX-C is similar in crustaceans and collembolans, but has changed since dipterans/lepidopterans and collembolans last shared a common ancestor. Hence, although collembolans have an abdominal region that is clearly distinct from the thorax (refer to Figure 1a), and the anterior boundary of expression of the BX-C genes agrees with that observed for true insects (Figure 1f), the collembolans apparently do not share the

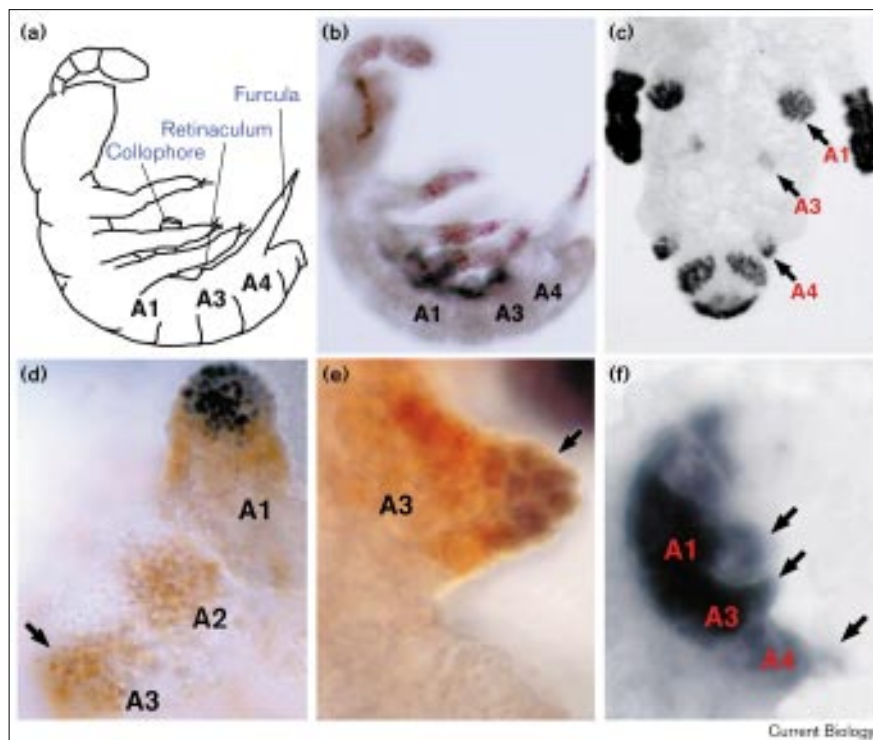
Figure 1

Antibody staining of collembolan (springtail) embryos for Dll and Ubx/Abd-A proteins.

(a) Tracing of the collembolan nymph shown in (b). Note the three pairs of thoracic legs and the abdominal appendages (labeled) on the ventral surface of segments A1, A3 and A4.

(b) Newly hatched *Folsomia candida* nymph double-labeled for Dll (red) and Ubx/Abd-A (black). As expected, Dll was expressed in the distal portions of appendages, and Ubx/Abd-A expression at this stage extended from the posterior portion of the T2 segment through most of the abdomen.

(c) Ventral view of the posterior thorax and complete abdomen of a *Xenylla grisea* embryo, at approximately 40–50% of embryogenesis, stained for Dll protein (black). Each abdominal appendage primordium stained for Dll protein (arrows). Note that Dll expression in the A3 primordium has only just initiated and is present in only a few nuclei. The staining in the very posterior of the abdomen is not addressed in the present study. (d) Abdominal hemisegments A1–A3 of an *X. grisea* embryo, at about the same stage of embryogenesis as that shown in (c), double-labeled for Dll (black) and Ubx/Abd-A (brown). Note that Ubx/Abd-A proteins are already present at high levels in the cells of the A3 primordium, but Dll expression is not apparent (arrow). (e) Lateral view of the A3 segment of a relatively old *X. grisea* embryo stained for Dll (black) and Ubx/Abd-A (brown).



Note the overlap of gene expression in the tip of the A3 appendage (arrow). (f) Lateral view of a relatively old *X. grisea* embryo stained for

Ubx/Abd-A (black). Note the persistent expression of Ubx/Abd-A in the distal portions of the abdominal appendages (arrows).

simple repressive interaction between *Ubx/abd-A* and *Dll* that is observed in dipterans and lepidopterans. We propose, instead, that the regulatory interactions between *Hox* genes and downstream targets, such as *Dll*, have evolved in a more complex, segment-specific manner. This proposition was supported by results, presented below, for two additional orders of hexapods.

Co-expression of Dll and Ubx/Abd-A in orthopterans

Grasshoppers (Orthoptera) are relatively primitive members of the Pterygota (winged insects); as such, they are phylogenetically intermediate to Collembola and the highly derived Lepidoptera/Diptera clade (see below). Morphologically, grasshoppers have lost all adult abdominal limbs; they do, however, develop embryonic pleuropodia on the first abdominal segment.

Interestingly, the grasshopper (*Schistocerca americana*) embryonic pleuropodia exhibited the crustacean/collembolan expression dynamics of Dll and Ubx/Abd-A. First, Ubx/Abd-A proteins accumulated to appreciable levels, and only then did Dll protein become detectable (Figure 2a–d). Furthermore, a patch of ectodermal cells in the second abdominal segment also initiated Dll expression despite high levels of Ubx/Abd-A (Figure 2e,f); this

Dll expression in A2, however, was transient, disappearing soon after and having no obvious relationship to the morphology of a developing appendage. In contrast to the situation in A2, the cells of the developing pleuropodia in A1 continued to express high levels of both Dll and Ubx/Abd-A throughout development.

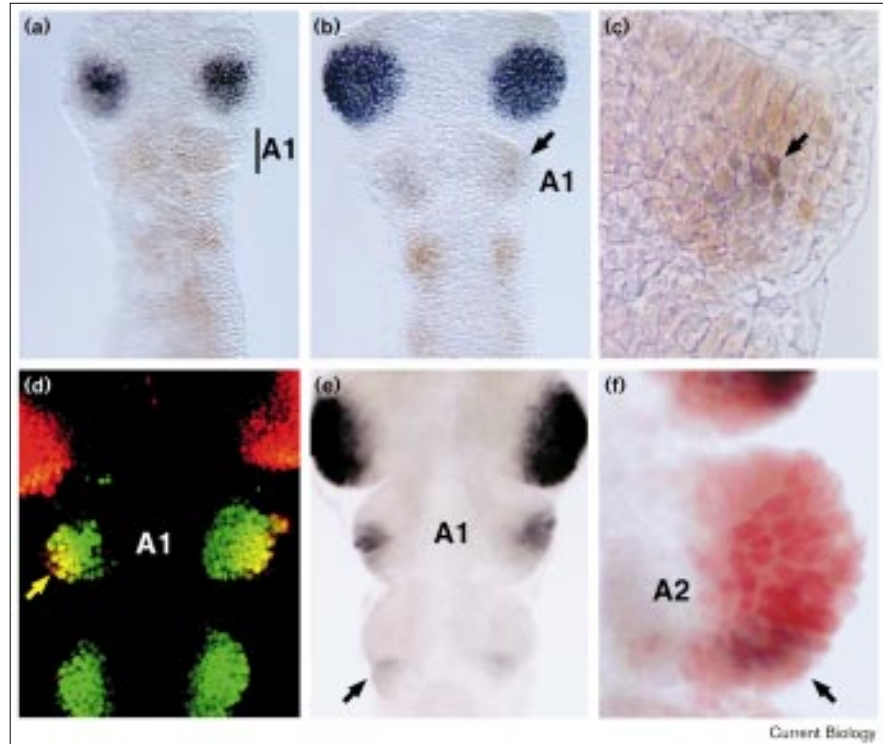
Co-expression of Dll and Ubx/Abd-A in coleopterans

Beetles (Coleoptera) fall within the Endopterygota — winged insects that undergo complete metamorphosis — but they are generally considered an outgroup to the highly derived Lepidoptera/Diptera clade ([8]; see below). Beetle embryos are interesting in the present context because they normally develop pleuropodia on A1.

In embryos of the flour beetle (*Tribolium castaneum*), both Dll and Ubx/Abd-A expression appeared to initiate at the same time in the cells of the pleuropodial primordia (Figure 3a), and early on in the growth of each primordium the nuclei near the tip continued to stain strongly for Dll and Ubx/Abd-A proteins (Figure 3b,c). Once again, these results are difficult to reconcile with the simple BX-C repression model: the presence of Ubx/Abd-A protein is expected to repress the initiation of *Dll* expression.

Figure 2

Antibody staining of *S. americana* (grasshopper) embryos for Dll and Ubx/Abd-A proteins. All views are from the ventral side, and anterior is uppermost. (a) Embryo, at about 20% of embryogenesis, double-labeled for Dll (black) and Ubx/Abd-A (brown). The field of cells alongside the vertical bar is the developing A1 segment; these cells are already expressing Ubx/Abd-A at appreciable levels but no staining for Dll protein is visible. (b) Embryo, at about 22% of embryogenesis, stained as in (a). Dll expression initiated in small clusters of cells (arrow) in A1 in the middle of a field of cells already expressing Ubx/Abd-A. (c) Higher-magnification view of a portion of the embryo in (b). The Dll-expressing cells in this A1 hemisegment also contained Ubx/Abd-A (arrow). (d) Confocal micrograph of a slightly older embryo double-labeled for Dll (red) and Ubx/Abd-A (green). Overlap is yellow (arrow). The A1 nuclei that were expressing Dll also contained high levels of Ubx/Abd-A proteins. (e) Embryo, at about 26% of embryogenesis, stained for Dll (black). Interestingly, Dll was expressed in small clusters of A2 ectodermal cells (arrow). This expression was transient – older embryos invariably did not show Dll expression in A2 (data not shown). (f) Second abdominal hemisegment of an embryo, at about 26% of embryogenesis,



double-labeled for Dll (black) and Ubx/Abd-A (red). This A2 patch of Dll expression

initiated in cells that already contained high levels of Ubx/Abd-A proteins.

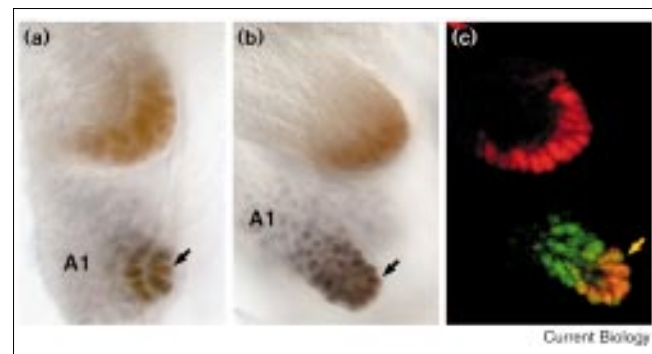
Evolutionary considerations

In all of the orders examined (Collembola, Orthoptera, and Coleoptera), Dll expression was initiated in abdominal cells despite the presence of high levels of Ubx/Abd-A proteins (Figure 4). This suggests that the strong repressive effects of these BX-C proteins on *Dll* expression, which have been well characterized in the dipteran *D. melanogaster*, arose relatively late in the evolution of insects. There are, however, alternative hypotheses that could also explain our observations.

First, high levels of another protein at the correct point in time might compete effectively with the Ubx/Abd-A proteins for binding sites in the early *Dll* enhancer, thereby overcoming any repression due to Ubx/Abd-A (see [9], for example). One attractive candidate for this role is the Antennapedia (*Antp*) protein, which is a more ‘anterior’ *Hox* gene that promotes thoracic limb development in *D. melanogaster* [10]. Interestingly, in the taxa that have been examined for *Antp* expression (Lepidoptera, Coleoptera and Orthoptera), high levels of this protein are present in those abdominal cells that initiate *Dll* expression despite an abundance of Ubx/Abd-A ([6]; our unpublished observations).

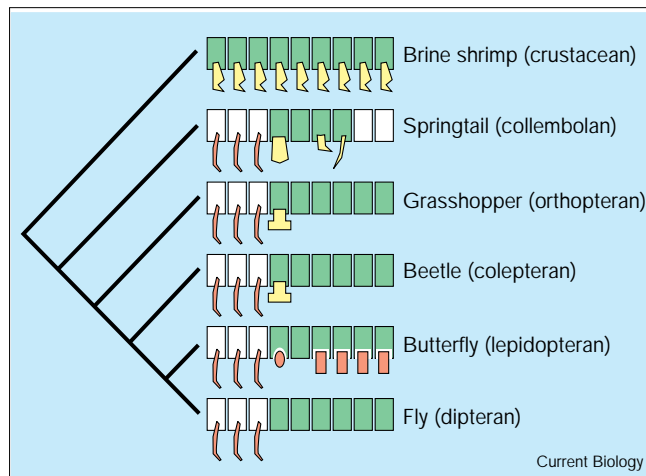
Second, it is possible that a co-factor necessary for Ubx/Abd-A repression of *Dll* is lacking in certain abdominal

Figure 3



Antibody staining of *T. castaneum* (flour beetle) embryos for Ubx/Abd-A and Dll proteins. Anterior is uppermost. (a,b) Embryos were double-labeled for Dll (brown) and Ubx/Abd-A (black). (c) Confocal micrograph; the same reagents as in (a,b) but depicted in red (Dll) and green (Ubx/Abd-A). (a) Lateral view of T3–A1 segments of an embryo soon after thoracic limb buds became prominent. Apparently, both Dll and Ubx/Abd-A proteins accumulated simultaneously in the developing pleuropodial cells (arrow), as no embryos were observed to stain for just one or the other protein alone. Slightly older embryos are shown in (b,c). Distal pleuropodial cells (arrow) expressed both Dll and Ubx/Abd-A proteins. In (c), the overlap of Ubx/Abd-A and Dll expression domains is shown in yellow (arrow).

Figure 4



Phylogenetic distribution of different patterns of Ubx/Abd-A and Dll expression and the associated appendage morphologies. Phylogenetic relationships of five orders of hexapods are depicted, with the brine shrimp (*Artemia*; a branchiopod crustacean) included as an outgroup. The morphologies of the appendages along the trunks of these arthropods are illustrated along with the patterns of Dll and Ubx/Abd-A expression. For the trunk segment ectodermal tissue, green represents strong staining early for Ubx/Abd-A, whereas white represents lack of strong staining early for Ubx/Abd-A. For the developing limbs, red represents the initiation of Dll expression in the absence of Ubx/Abd-A proteins regardless of whether there is overlap later on in development; yellow represents the initiation and maintenance of Dll expression despite the presence of Ubx/Abd-A protein. It is clear that the simple BX-C repression model described for *D. melanogaster* cannot be generalized to all hexapods. We propose instead that the interaction between the *Hox* genes and downstream targets, such as the *Dll* gene, has evolved in a complex, segment-specific manner within the hexapods.

cells at the relevant stage(s) in these other species. From work in *D. melanogaster*, one promising candidate for this role is the Extradenticle protein — spatiotemporal modulation of cytoplasmic versus nuclear localization of this protein is thought to have a profound effect on the binding specificities of Hox proteins in a cell and, hence, on the segmental functions of the *Hox* genes [11].

Third, although Ubx and Abd-A proteins both function as effective repressors of *Dll* expression in *D. melanogaster*, it is possible that Abd-A evolved this function first. As there is apparently a complete lack of overlap between Dll and Ubx/Abd-A protein expression in the pleuropodia that develop on the A1 segment of the butterfly *Precis coenia* (data not shown), and as the anterior half of this segment apparently expresses Ubx but not *abd-A* in this species [6], we think it likely that the repressive interaction between Ubx and *Dll* was present in the common ancestor of dipterans and lepidopterans. In the flour beetle, however, genetic studies have demonstrated that the *Ubx* ortholog functions to prevent the development of leg-like appendages on A1 in place of the pleuropodia, whereas the

beetle *abd-A* ortholog functions to repress the development of ectopic pleuropodia in abdominal segments A2–A8 [12,13]. These results indicate that the beetle *Ubx* ortholog does not repress *Dll* expression but modifies the morphology of the resulting appendage instead, suggesting that the strong repressive interaction between Ubx and *Dll* evolved in the dipteran/lepidopteran lineage since they last shared a common ancestor with coleopterans. This scenario is also consistent with our results for grasshopper A1. The grasshopper Ubx protein is apparently not a repressor of *Dll* expression. Even more interesting, the transient expression of Dll in grasshopper A2 is apparently initiated in the presence of high levels of Abd-A protein [14], suggesting that the *abd-A* gene is not as effective a repressor of *Dll* expression in orthopterans as it is in dipterans.

One way to distinguish between these hypotheses would be to manipulate somatic gene expression, for example of the *Antp*, *Ubx* and *abd-A* genes, directly in each of these organisms. Experiments are currently underway to develop an expression system that would make such experiments feasible.

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