CRISPR/Cas9 Mutagenesis Reveals Versatile Roles of Hox Genes in Crustacean Limb Specification and Evolution

Graphical Abstract

Highlights

- Amphipod crustaceans display a wide array of specialized limbs
- CRISPR mutagenesis and RNAi of Hox genes generate limb transformations
- Limb identity is specified by overlapping domains of Hox expression
- abd-A expression shifts created evolutionary diversification of the crustacean body

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In Brief

Martin et al. analyze the function of six Hox genes in the crustacean amphipod Parhyale, using CRISPR/Cas9 mutagenesis and RNAi knockdown. The resulting limb transformations shed light on how each appendage is patterned and how the Hox genes have been used to create several morphological macroevolutionary transitions in the crustacean body plan.

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CRISPR/Cas9 Mutagenesis Reveals Versatile Roles of Hox Genes in Crustacean Limb Specification and Evolution

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SUMMARY

Crustaceans possess a diverse array of specialized limbs. Although shifts in Hox gene expression domains have been postulated to play a role in generating this limb diversity, little functional data have been provided to understand the precise roles of Hox genes during crustacean development. We used a combination of CRISPR/Cas9-targeted mutagenesis and RNAi knockdown to decipher the function of the six Hox genes expressed in the developing mouth and trunk of the amphipod Parhyale hawaiensis. These experimentally manipulated animals display specific and striking homeotic transformations. We found that abdominal-A (abd-A) and Abdominal-B (Abd-B) are required for proper posterior patterning, with knockout of Abd-B resulting in an animal with thoracic type legs along what would have been an abdomen, and abd-A disruption generating a simplified body plan characterized by a loss of specialization in both abdominal and thoracic appendages. In the thorax, Ubx is necessary for gill development and for repression of gnathal fate, and Antp dictates claw morphology. In the mouth, Scr and Antp confer the part-gnathal, part-thoracic hybrid identity of the maxilliped, and Scr and Dfd prevent antennal identity in posterior head segments. Our results allow us to define the role Hox genes play in specifying each appendage type in Parhyale, including the modular nature by which some appendages are patterned by Hox gene inputs. In addition, we define how changes in Hox gene expression have generated morphological differences between crustacean species. Finally, we also highlight the utility of CRISPR/Cas9-based somatic mutagenesis in emerging model organisms.

INTRODUCTION

Arthropod appendages have diversified into a remarkable repertoire of specialized morphologies. Crustaceans of the Malacostraca class, such as crabs, lobsters, shrimps, or the emerging model organism Parhyale hawaiensis, provide remarkable illustrations of this principle [1, 2], as shown by the extensive morphological and functional diversity of limbs along their antero-posterior (AP) axis (Figure 1A). This extreme specialization provides a Swiss-army knife arrangement of appendages dedicated to perception (antennae), food processing and chewing (mouthparts), prehension (claws or “chelipeds”), walking (legs or “pereopods”), and propulsion (swimmerets or “pleopods”), and at the end of the Parhyale abdomen, forked shaped appendages (uropods) are used for anchoring.

In spite of the diversity of forms they can take within a single individual, the limbs along the body axis of a crustacean or insect are serial homologs [4, 5]. Comparative anatomy and gene expression data have revealed that the proximo-distal (PD) limb axis is subdivided into two fundamental territories, a proximal protopod and a distal telopod [9–11]. In crustaceans, the protopod forms the base of this structure and is subdivided into two podomeres, the coxa and the basis (Figure 1B). The basis can be one-branched (uniramous, with an endopod) or two-branched (biramous, with both an endopod and an exite). All crustacean limb appendages are essentially variations on this common theme.

But how do appendages diverge from this basic organization and acquire a specific morphology based on their position along the body? Hox genes play an important role in establishing segmental identity along the AP axis of arthropods and other animals by regulating the transcription of downstream target genes [12, 13]. Furthermore, subtypes of thoracic and abdominal appendages vary in number and position between crustacean species, and comparative studies suggest that spatial shifts of Hox expression have facilitated such rearrangements by modulating a combinatorial code for limb identity [12, 14–20]. For example, comparative analysis of Ubx expression across crustacean species suggested that the Hox gene Ubx plays a role in defining the transition between feeding and locomotory type appendages in the anterior part of thorax [18]. Functional work in Parhyale supported this hypothesis: RNAi-based knockdowns transformed the T2 and T3 clawed appendages into a T1 type feeding limb [21], and misexpression of Ubx resulted in ectopic locomotory thoracic appendages in the head [22]. Of note, malacostracan T1 segments deviate from the thoracic leg-like archetype as they bear a maxilliped. Although the maxilliped is part of the T1 segment, it is integrated into the mouth apparatus and shows both gnathal and thoracic features [2]. The Ubx RNAi phenotypes

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RESULTS

CRISPR/Cas9 Loss-of-Function Mutations in G₀ Embryos

RNAi-based approaches have been used for gene expression knockdown during crustacean development [21, 23, 24], and although the approach achieves moderate knockdown of mRNA levels [21], the resulting intermediate phenotypes are still useful. As an alternative, we used CRISPR/Cas9 site-directed mutagenesis and RNAi to systematically interfere with Hox function during the development of P. hawaiensis embryos. Gene knockouts of the six Hox genes expressed in the mouth and trunk generated homeotic shifts in limb features. These new results outline the combinatorial logic of a Hox code laying out the segmental identity of crustacean appendages.

(T2 and T3 to a T1 maxilliped) thus suggest that Ubx represses gnathal identity in the segments posterior to T1 in Parhyale, consistent with the observation that Ubx expression is restricted to non-maxilliped segments in every crustacean examined so far [16–18, 21].

Beyond Ubx, the potential roles of other Hox genes in determining the positional identity of crustacean limbs, and their evolutionary modification between species, remain unclear due to a lack of functional data. To fill this gap, we used CRISPR/Cas9-targeted mutagenesis and RNAi to systematically interfere with Hox function during the development of P. hawaiensis embryos. Gene knockouts of the six Hox genes expressed in the mouth and trunk generated homeotic shifts in limb features. These new results outline the combinatorial logic of a Hox code laying out the segmental identity of crustacean appendages.

Ubx Represses Mouth Features and Promotes Gill Development

As a proof of principle for generating phenotypes using CRISPR/Cas9, we first replicated the results of previous Ubx RNAi injections [21] (Figures 3A and 3B). We obtained embryos in which T2 and T3 were transformed toward T1 (the T1 appendage is a maxilliped and from now on is referred to as T1/Mxp). Importantly, T2/T3 retained a clawed morphology at their distal ends but lost the T2/T3-specific comb bristle, indicating a partial T2/T3-to-T1/Mxp homeosis, which was also what had been observed previously by RNAi. However, we also uncovered additional effects of Ubx loss-of-function in T4 and T5. As with transformed T2 and T3 appendages, T4 and T5 also acquired endites with multiple setae at their base, with the addition of a claw-type morphology.

Figure 2D shows an example where we targeted Ubx in one of two cells. In this case, the embryo shows wild-type expression of Ubx protein on one side and reduced levels on the other side. Of note for this embryo, the T2/3 limb primordia lacked one or two of the seven podomeres observed on the wild-type side, consistent with a transformation of these T2 and T3 appendages toward a T1 (maxilliped) appendage identity, similar to what was seen previously when Ubx levels were reduced by RNAi [21].

These results assaying expression after targeting suggest that CRISPR/Cas9 can be used as an efficient tool to generate somatic mutations interfering with gene function during the early embryonic development of P. hawaiensis and that the resultant mutant clones are large, most likely due to targeting soon after injection in the zygote. In this respect, it is useful to note that Parhyale embryos take 8 hr to go from the one-cell to eight-cell stage. Given this success in knocking out expression, we carried out CRISPR-based loss-of-function experiments targeting the six Hox genes expressed in the mouth and trunk [3]—Deformed (Dfd), Sex comb reduced (Scr), Antp, Ubx, abdominal-A (abd-A), and Abdominal-B (Abd-B)—injecting the one-cell stage to limit mosaicism, and examined the effect of somatic mutagenesis on limb morphology. All but one sgRNA (abd-A sgRNA#2; penetrance = 12%) generated limb-specific mutant phenotypes in hatchlings at high efficiency with a penetrance ranging between 25% and 70% (Table S1).

**Figure 1. Hox Expression and the Crustacean Limb Body Plan**

(A) Summary of Hox expression in P. hawaiensis in relationship to specialized segments (after [3]). Faded color bars depict weak expression domains. Green squares indicate neuronal expression of Antp. Due to post-translational processing of Antp transcripts [3], we show here the domain for Antp protein. Red squares indicate mesodermal expression of Dfd in the median section of pleopods. The star indicates late, appendage-specific expression of Scr in T1/Mxp. The hashed bar indicates transient, weak expression of abd-A in A4.

(B) Schematic representation of the crustacean limb groundplan.
pairs of gills, normally attached at the base of the T3–T7 segments (Figures 3A and 3D). This complements the effects of gain-of-function overexpression of Ubx, which induces ectopic gills [22]. Taken together, these results suggest that Ubx is necessary for the repression of gnathal identity in the base (proximal podomeres) of T2–T5, and required for gill development in T3–T7.

We interpret the more extreme phenotype seen with CRISPR/Cas9 (with two different sgRNAs) relative to RNAi as the difference between gene knockdown and knockout. The restriction of Ubx RNAi effects to T2–T3 suggests these segments are most sensitive to the reduction in the level of Ubx expression, and it is worth noting that Ubx RNA and protein are expressed at lower levels in these two segments than in T4–T8 in wild-type animals [21]. It is important, however, to consider that CRISPR/Cas9 mutagenesis is also expected to sometimes yield similar partial knockdown effects. In some cases, this may be because the mutant alleles that are generated retain some function or because of the tissue mosaicism inherent to our somatic analysis.

**abd-A and Abd-B Organize the Specialization of Posterior Appendages**

The posterior half of *P. hawaiensis* shows three pairs of reverse walking legs in T6–T8, three pairs of swimmerets in A1–A3, and three pairs of uropods in A4–A6 (Figure 4A). This anatomical parcellation is reflected at the molecular level by the expression of abd-A in the posterior legs and swimmerets (and weakly in the uropod of A4) and by Abd-B expression, which extends from the swimmerets to the uropods (Figures 1A and S2). It follows that the partially overlapping expression domains of two Hox genes creates three Hox states that correlate with morphological differences: (1) abd-A in T6–T8, (2) abd-A plus Abd-B in A1–T3, and (3) Abd-B in A4–T6. In the appendages of A1–A3, it appears that all ectodermal cells do co-express abd-A and Abd-B during limb development (Figure S2). Here we tested the hypothesis that the posterior heteronomy of amphipods is specified by combinatorial Hox expression.

CRISPR somatic mutagenesis of abd-A validated this hypothesis and induced three notable limb modifications across its expression domain. First, posterior legs were transformed into anterior legs (T6/8-to-T4/5), as evidenced by their inverted orientation (for instance, with the dactyl pointing backward instead of forward) and by the absence of a large coxa, a characteristic of posterior legs (Figures 4A–4D). Second, the T8 segment acquired an ectopic gill, which is expected from a T8-to-T4/5 transformation (Figures 4B–4D). Third, swimmerets were transformed into uropods (A1/2/3-to-A4/6; Figures 4E–4G), and the A1–A3 abdominal body segments bearing them also transformed toward the A4/5/6 body segments in terms of size and shape, resulting in a severely contracted, narrow abdomen and an aberrant curvature of the body (Figure 4B). Interestingly, abd-A loss-of-function phenotypes can be seen as an anteriorization of T6–T8 and as a posteriorization of A1–A3. These results were replicated in CRISPR experiments that used an sgRNA targeting the second exon of abd-A (Table S1; Figure S1), thus ruling out off-target effects on limb morphology. In comparison, two independent expression knockdown experiments failed to recreate T6–T8 transformations but succeeded in replicating the effects of abd-A CRISPR in the abdomen (Figures 4H and
Amphipod thoracic legs are subdivided into three types—

Antp Functions in Claw Specification

4I). Specifically, the injection of siRNA and two transgenic lines expressing abd-A hairpin RNAs under the control of a heatshock promoter [26] all resulted in aberrant swimmerets resembling uropods (A1–A3 transformed toward A4/5/6), characterized by detached pairs of appendages, a curved basis, and a failure to develop propulsive setae. This suggests again the importance of Hox expression levels—in this case, A1–A3 are more sensitive to lowering abd-A expression than are T6–T8.

In accordance with its pan-abdominal expression, Abd-B CRISPR transformed both swimmerets and uropods into walking legs (Figures 4J–4M), culminating in a densely packed array of uropods (A1–A3 transformed toward A4/5/6), characterized by modified chelipeds (Figures 5A–5D). Modified chelipeds failed to acquire normal segmentation and retained a fused ischium-merus (Figure 5C). In wild-type animals, gills are only observed in T3–T7 segments, but remarkably, T2 transformed limbs also displayed an ectopic gill indicative of a more posterior specification (T2-To-T4/5). This anatomical gain provides additional evidence for a homeotic effect of Antp on the entire appendage. As these results were obtained for two distinct sgRNAs of comparable penetrance, we have ruled out off-target effects of Antp CRISPR on limb morphology and conclude that Antp is required for the specification of chelipeds.

Complementary Effects of Antp and Scr in Maxillipeds

In contrast with the thorax and abdomen, arthropod mouthparts generally show a sequential heteronomy where all consecutive segments are distinct and differ from each other, without repetition. In the next two sections, we explore how sequential expression of Hox genes might explain the differentiation of the amphipod mouth apparatus. In addition to its effects on chelipeds, Antp CRISPR also resulted in visible defects in maxillipeds. Antp mutant jaws showed T1/Mxp-to-Mx1 transformations, as revealed by the acquisition of Mx1-specific serrated setal teeth on the basis endite and by a narrow coxal endite, topped by two long simple setae (Figures 5A–5B and 5E–5G). In contrast, wild-type T1/Mxp endites both resemble the Mx2 condition (Figure 5H). Disruption of Antp also showed graded effects of the T1/Mxp endopods, the more distal part of the limb. In the
milder forms, the T1/Mxp endopod regressed into a palp of bulging aspect, due to an abnormally narrow attachment site on the basis article (Figure 5G). These transformed limbs display three endites instead of two, due to the maintenance of the ischiium and basis endites with an ectopic and prominent, Mx1-like coxal endite. In more extreme forms, the endopod was missing from the T1/Mxp-to-Mx1 transformed limb (Figure 5F). Altogether, these results underline the dual role of Antp in maxilliped development, as it selects the identity of the proximal domain while also being required for palp growth in the distal domain.

Scr expression (Mx1-T1/Mxp) overlaps with Antp (Mx2-T3) in the mouth, and hatchlings that were injected with Scr CRISPR showed a mild to severe disorganization of the jaw due to an imperfect interlocking of the modified mouthparts. Upon closer inspection, Scr mosaic mutants revealed maxilliped-to-cheliped transformations (T1/Mxp-to-T2/3), with the distal palp acquiring both a T2/3-specific comb bristle and the morphology of a prehensile claw, characterized by an enlarged propodus and an opposing dactyl (Figures 6A–6C). In the proximal domain, T1/Mxp endites regressed upon Scr loss of function, consistent with a conversion of this appendage toward a thoracic identity.

In summary, both the CRISPR phenotypes of both Antp and Scr mutants reveal a modular, composite organization of the maxilliped, with dual effects on the proximal and distal domains. Scr functions as a determinant of the gnathal identity of the protopod, and Antp is necessary for preventing Mx1-like morphology in this limb domain. Conversely, in the T1/Mxp endopod, Scr inhibits the posterior claw-like morphology of the palp, and Antp is required for endopod presence. The antagonistic roles of these genes may thus explain the hybrid nature of maxillipeds, by conferring a combination of thoracic (presence of an endopodal extension) and gnathal (sensory endites and clawless palp) features.

Conserved Functions of Scr and Dfd in Mouth Patterning

The mandible (Mn), maxillule (Mx1), and maxillae (Mx2) are consecutive mouth appendages involved in food processing along with the more posterior T1 maxilliped. Mn or Mx1 palp are common among other amphipods, but in P. hawaensis, a residual palp is apparent on Mx1 only and the three appendages thus appear to repress endopod development. CRISPR-induced mutagenesis of both Scr and Dfd revealed that these genes control different aspects of the regional identity of these segments on the PD axis.

Wild-type Mx2 have two lobes with simple setae (the coxa and basis endites). Although this appendage is thus devoid of an endopod, both RNAi- and CRISPR-based loss of function of Scr activated endopodal growth (Figures 6D and 6E). Scr CRISPR individuals showed a gradual series of Mx2 modifications, starting with the presence of an ectopic endopod and the acquisition of serrated setae characteristic of the Mx1 segment on the endites (Mx2-to-Mx1). In the most extremes cases, the endites regressed, and an antenna-like endopod protruded from the side of the mouth (Mx2-to-An). Scr RNAi resulted in less dramatic, but still striking, Mx2 phenotypes with the formation of ectopic but incomplete endopods, and the acquisition of an additional endite-bearing ischiium—a condition that exists in the maxillipeds of Parhyale and other amphipods [2]. This transformation of Mx2 to T1/Mxp (Figure 6D) is consistent with the phenotype seen when low levels of Ubx misexpression cause a reduction in the levels of Scr [22]. Scr RNAi had no effect on the Mx2 proximal domain or on Mxp, suggesting a lower expressivity than Scr CRISPR.

Like Scr in Mx2, Dfd CRISPR promoted endopodal development in Mn and Mx1 (Figures 6F–6I). In Dfd mutants, the Mx1 vestigial endopod developed into a segmented antenna, whereas the proximal domain retained an Mx1 identity (partial Mx1-to-An). Dfd CRISPR also induced the formation of an ectopic endopod on Mn, resulting in a dislocated mandible protruding from the mouth apparatus or culminating in the formation of a short and segmented antennal primordium (Mn-to-An).

Taken together, these results show that Scr/Dfd loss-of-function experiments both induce antenna-like appendages in the mouth. Similar phenotypes have been observed in homologous segments upon Scr/Dfd knockdown in hemipterans and coleopterans, suggesting an evolutionarily conserved role in the maintenance of gnathal identity between insects and crustaceans [27–31]. In the proximal domain of mouth appendages, Scr in particular patterns the setulation of Mx2/T1 endites, whereas Dfd prevents spurious Mx1 morphology in the Mx2 segment. We conclude that in addition to an ancestral function in the distal repression of antennal fate, the sequential expression of Scr and Dfd in the mouth also contributes to the heteronomy of this body region via modular effects along the limb PD axis.

DISCUSSION

Using CRISPR for Somatic Analyses of Gene Function

The recent development and apparent universality of CRISPR/Cas9 genome editing [32] allowed us to analyze the function of the six Hox genes expressed in the mouth and trunk of P. hawaensis, an emerging model organism (Figures 7A and 7B). We used zygotic injections to generate DNA lesions in the soma, without attempting stable germline transformation. Here we discuss this strategy and the extent to which it could foster discovery in analogous experimental systems.

Cost

We generated ready-to-inject samples in 2–3 days and at low cost (less than $80 per target in reagents).

Penetrance

CRISPR/Cas9 somatic loss-of-function experiments generated homeotic phenotypes at high frequency for eight out of the nine sgRNAs that were assessed (Table S1). The lower efficiency of abd-A sgRNA#2 (12% penetrance) may be explained by the fact it was the only sgRNA targeting a short second exon (Figure S1), which could be subject to splicing.

Expressivity

In all our comparisons (Scr, Ubx, abd-A, and Abd-B), CRISPR showed more marked effects than siRNA injections, with an increased degree of transformation. These limitations of RNAi are most likely due to incomplete mRNA knockdown in Parhyale [21], although clearly the combination of CRISPR and RNAi data was useful in revealing the relative sensitivity of different segments to Hox gene perturbation.

Reproducibility and Target Specificity

Although it would be difficult to assess the target specificity of CRISPR in our model system, we sought to test the reproducibility of limb transformation phenotypes using non-overlapping
Figure 4. *abd-A* and *Abd-B* Pattern Functional Subdivisions in Thoracic and Abdominal Appendages

(A and B) Lateral SEM views of wild-type (A) and *abd-A* CRISPR-injected (B) Parhyale hatchlings; *abd-A* CRISPR results in leg homonomy, with anteriorization of the reverse-walking morphology in T6–T8 and ectopic gills in normally gill-less T8. Mutant abdomens curl upward due to A1–A3 posteriorization.

(C and D) Dark-field images of dissected wild-type (C) and *abd-A* mutant (D) T8, with reversed polarity in the antero-posterior (AP) axis.

(E and F) Ventral views of wild-type pleopods (A1–A3 swimmerets), characterized by a biramous morphology terminated by long setae.

(legend continued on next page)
sgRNAs (for Dfd, Scr, Antp, and abd-A). In these four cases, mutant phenotypes were equivalent regardless of the 19–20 bp nucleotides targeted. RNAi phenotypes obtained for Ubx, abd-A, and Abd-B were also consistent with the effects of CRISPR mutagenesis in these genes. These results provide independent replications and rule out off-target effects of CRISPR/Cas9 somatic mutagenesis on limb morphology.

Mosaicism
A caveat of somatic mutagenesis is linked to the random occurrence of DNA cleavage in post-zygotic stages [34]. We have seen that CRISPR injections have the potential to generate bi-allelic knockouts that spread to large sections of the injected individual by clonal inheritance (Figure 2C). For any given transformed animal, the distribution of mutant cells and their respective allelic dosage are unknown (Figure 2D). That said, the resulting mosaicism can be advantageous for several reasons. First, unilateral mutant phenotypes can be directly compared to a wild-type state within the same animal, providing an internal control. Second, for pleiotropic genes involved in several processes across development, mosaicism may increase the rate of surviving 

Figure 5. Antp Is Required for Limb Specialization in the Anterior Thorax
(A) Ventral SEM view of a unilateral mutant hatching obtained by Antp CRISPR. The mouth is dislocated due to an incomplete T1/Mxp-to-Mx1 transformation (yellow), bearing Mx1-specific setal teeth (red arrowheads). The transformed T2 limb (green) lacks a normal claw morphology and the T2/3 specific comb bristle (white arrowheads, absent; green, wild-type) and shows an ectopic gill (purple).
(B) Another example of a unilateral Antp CRISPR mutant, notably showing a T2/3-to-walking-leg transformation (green).
(C) Differential interference contrast (DIC) imaging of T2/3 limbs transformed by Antp CRISPR, with endopod podomeres false colored. Arrow, comb bristle (absent in mutants); d, dactylus; p, propodus; c, carpus; m, merus; i, ischium.
(D) Wild-type T4 and T5 forward-walking legs.
(E–H) Detailed morphology of the maxillary apparatus in wild-types (E and H) and Antp CRISPR mutants (F and G). Blue, endopods; yellow, protopods; asterisk, Mx1-basis-specific setal teeth; arrowhead, Mx1-coxa-specific endite.
(I) Unilateral Antp CRISPR mutant showing a T1/Mxp-to-Mx1 transformation with a complete ablation of the endopod.
Scale bars, 100 μm (A, B, and I).
Figure 6. Scr and Dfd Maintain Gnathal Features in Mouth Appendages
(A) Ventral SEM view of a wild-type mouth apparatus.
(B) Mouth identity defects in a unilateral Scr CRISPR mutant. Arrowhead, T2/3-specific comb bristle.
(C) Ventral view of a dissected, unilaterally transformed pair of Scr CRISPR maxillipeds. Arrow, T2/3-like claw morphology of the dactylus/propodus; arrowhead, T2/3-specific comb bristle; asterisk, regressed T1/Mxp endites.
(D) Effect of Scr RNAi on Mx2, with growth of an ectopic endopod (blue).

(legend continued on next page)
“escapers” by randomizing the distribution of mutant clones. Last, mosaicism can generate phenotypic series that are biologically informative. In our case, this was true for Scr CRISPR, in which intermediate (Mx2-to-Mx1) and severe homeosis (Mx2-to-An) suggested two distinct functions of this gene in the maxilla segment.

Overall, we encourage the use of CRISPR/Cas9 somatic mutagenesis for the rapid analysis of gene function in emerging model organisms with injectable eggs, complementing the already widespread use of RNAi. This should notably facilitate the systematic study of Hox gene function across a broad sample of arthropods, drawing the promise of an extended understanding of segmental and serial homolog evolution. For instance, CRISPR has been successfully carried out in the brachiopod Daphnia magna [35]. Hox mutagenesis in this species would extend existing gene expression analyses [36] and could yield important comparative insights into the macroevolution of crustaceans.

**Hox Expression Shifts and the Evolution of the Crustacean Trunk**

CRISPR mosaic mutants reveal that abd-A and Abd-B expression domains determine segmental identity in the thorax and abdomen. This combinatorial model sheds light into the evolution of abdominal appendages. In four malacostracans, Procambarus, Porcellio, Mysidium, and Mysidopsis, abd-A is expressed in the first five abdominal segments (containing pleopods), but not in the uropods of A6, the sixth and final abdominal segment [4, 16, 17] (summarized in Figure 7C). To replicate these previous results, we profiled the distribution of the Ubx/abd-A proteins using the cross-reactive FP6.87 monoclonal antibody [37, 38] in Procambarus fallax (Decapoda) and Mysidium columbae (Mysida) embryos. As previously reported, we found that the A6 segment of both decapods and mysids lacked abd-A, correlating with the presence of a pair of uropods rather than pleopods on A6 (Figures 7D–7F). In other words, abd-A-weak abdominal segments are always associated with a uropod identity. The three pleopod plus three uropod segment arrangement is unique to amphipods and may have been caused by an amphipod-specific loss of abd-A expression in A4–A5 (Figure 7H).

CRISPR somatic knockouts approximate this evolutionary scenario, as the experimental disruption of abd-A resulted in pleopod-to-uropod transformations, which validates a functional link between abd-A deployment and pleopod/uropod ratio. The posteriorization of the A1–A3 domain also suggests that abd-A works in conjunction with the overlapping expression of Abd-B in this region to establish pleopod identity. This is a new exception to the “posterior prevalence rule” [39], which would have predicted an absence of abd-A function in cells co-expressing the more posterior Hox gene Abd-B. Both comparative and functional data thus suggest that spatial shifts of abd-A deployment modulate the number of abd-A-negative uropods, explaining divergent arrangements of abdominal appendages in crustaceans.

Amphipods are also characterized by the presence of two types of legs (“amphi-poda,” gr. “different foot”), in contrast with isopods, which possess a single type of walking-leg morphology. In our amphipod model organism, abd-A mutagenesis replaced the reverse-walking legs with additional forward-walking legs, resulting in an isopod-like configuration. Accordingly, abd-A is not expressed in the legs of an isopod [16], suggesting that disruption of abd-A in the amphipod thorax effectively recapitulated the isopod state (Figure 7H). Given the central role that Hox genes play in determining arthropod segment identity, evolutionary shifts in Hox expression domains may provide a recurring strategy to generate diverse arrangements of specialized limb types [12, 40].

**Hox Functions in the Modular Evolution of Maxillipeds**

Our model for how shifts in abd-A and Antp expression have accompanied morphological evolution of the crustacean body plan is similar to the proposed role of Ubx in generating diversity in the number of crustacean maxillipeds [18]. Crustaceans exhibit anywhere from zero to three pairs of maxillipeds, and the number of maxillipeds correlates with the position of the anterior boundary of Ubx expression; in other words, appendages of the anterior thorax that lack Ubx expression become maxillipeds, whereas those that express Ubx become claws or legs [18, 21, 22].

Although Ubx represses gnathal identity the thorax, it does not explain the “chimeric”—both gnathal and thoracic—identity of the maxilliped [41], a composite identity that relies on the selector activities of more anterior Hox genes. Indeed, we found that interfering with Hox gene functions triggered modular effects on feeding appendages, with endite-bearing articles requiring Scr, the maxilliped endopod requiring Antp, and either Scr or Dfd repressing antenna-like endopods in maxillae (Mx1) and maxillules (Mx2). The compartmented functions of these consecutive genes may contribute to the robust establishment of differentiated morphologies in adjacent mouth segments, and they also shed light on the composite nature of maxillipeds. Indeed, Scr and Antp show dual functions that are complementary in each section of T1/Mxp. In the proximal section, Scr is necessary for the growth of endites while Antp provides positional identity. The Mxp distal domain shows a reverse pattern, with a requirement of Antp for palp growth, while Scr provides positional identity. The ability of Hox genes to perform different functions along the PD axis has been linked to the Hox co-factors Homothorax (Hth) and Extradenticle (Exd) in insects [31, 42–51]. Because Exd and Hth expression mark proximal limb domains in the crustacean limb [6, 7, 10, 11], Exd/Hth/Hox protein interactions could explain the differential effects of genes such as Antp, Scr, and Dfd in the proximal versus distal domains of the Parhyale feeding segments. Combinations of Hox genes and proximal co-factors may thus form a molecular canvas for
Figure 7. The Control of Crustacean Limb Identity by Hox Genes

(A and B) Summary of all known Hox loss-of-function phenotypes in *P. hawaiensis*. Arrows indicate the directionality of the homeosis (red, anteriorization; green, posteriorization); dotted lines indicate gills.

(C) AP shifts in *abd-A* expression recapitulate the evolution of limb-type subdivision in both thoracic legs and abdominal appendages (this study) [16, 17, 26]. Notice that *abd-A* loss of function triggers homeotic shift of opposite directions on each side of its expression domain in *P. hawaiensis* (B). F legs, forward-walking legs; R legs, reverse-walking legs.

(legend continued on next page)
transformation vector, and germline transformation was carried out as pMinos
intron, was then placed downstream of the abd-A fragment in opposite orientations on either side of the Parhyale Scr tool (Thermo Fischer Scientific) in non-conserved coding regions of the RNA Interference for injection visualization.

abd-A expression) and continuing until hatching. Two independent transgenic shocks beginning around stage 14 (i.e., just before the onset of endogenous Embryo injection followed a published protocol [57]. For CRISPR somatic Injections and Imaging and DIC optics. For SEM, hatchlings were fixed for 2 hr in 3.7% formaldehyde, and appendages were removed individually, mounted in 70% glycerol, and visualized with dark-field hatchlings were fixed for 2 hr in 3.7% formaldehyde, dehydrated via an ethanol series prior to critical point drying, and examined on a Hitachi TM-1000. M. columbiae specimens used for SEM were obtained from a stock of adults that had been fixed and stored in MeOH. SEM images were false-colored using the “Darken” and “Soft Light” layer functions of Adobe Photoshop.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL INFORMATION includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.11.021.

AUTHOR CONTRIBUTIONS


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REFERENCES


DIAGRAMS

(D) Ventral SEM view of the abdominal appendages of a decapod crayfish hitchling, with only the A6 segment bearing a uropod. Asterisk, A1 appears to be limbless in hitchlings, but males develop a gonopod (modified pleopod involved in reproduction) at the juvenile stages.
(E) F6.87 staining of a crayfish embryonic abdomen (red); staining is absent from A6 and uniform in A2–A5 limb primordial.
(F and G) F6.87 staining of the embryonic abdomen of a mysid shrimp at successive stages (red); staining is absent from the uropod-bearing A6 segment and uniform in A1–A5 pleopod primordia.
(H) Modification of abdominal limb distribution in Amphipoda (tree topology after [33]).


