Supporting Information

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SI Methods

Gene Cloning. The isolation of *A. tepidariorum* Hox gene fragments has been reported (1). The gene sequences are available under the following accession numbers: *At-lab*, FM945395.1; *At-Dfd*, FM945396.1; *At-Scr*, FM956097.1; *At-Antp*, FM956094.1; *At-Ubx*, FM956093.1; *At-abd-A*, FM956096.1. For the generation of transgenic *Drosophila* lines and synthesis of double-stranded RNA, a full-length fragment of *At-Antp* was isolated via RACE (accession no. HE608680). Based on the full sequence information, specific primers including restriction sites where designed (At-AntpFWCDS-XhoI, CcctcgagCGTGTTCTCTCT-GTGATGTGACC; At-AntpRVCDS-XbaI, CCtctagaCACTAG-GCAATATGCATTGAGG) and used for subcloning the coding sequence into the pCRII vector (Invitrogen). These fragments where then cloned into the Gal4-inducable vector pUAST for germ-line transformation. The *At-Dll* gene sequence (2, 3) and *At-dac* gene sequence (4) are available under accession no. FM876233 and FM945397.1, respectively. Fragments of the genes *At-exd-1* and *At-hth-1* were isolated by using the primers exd-fw1 (YTN AAY TGY CAY MGN ATG AAR CC), exd-bw1 (TTN CCR AAC CAR TTN SWN ACY TG), and hth-fw1 (GAY AAR GAY GCN ATH TAY GRN CAY CC), hth-bw1 (YTG RTC DAT CAT NGG YTG NAC DAT), respectively. In a nested PCR, the primers exd-fw2 (GTN YTN TGY GAR ATH AAR GAR AAR AC), exd-bw2 (GCN ARY TCY TCY TTN GCY TCY TC), and hth-fw1 and hth-bw2 (GC RTT DAT RAA CCA RTT RTT NAC YTG) were used. Accession nos. are as follows: *At-exd-1* (HE608681) and *At-hth-1* (HE608682).

Parental RNAi in *Achaearanea tepidariorum*. ***Injections for single RNAi, general procedure.*** Females were injected three times with dsRNA in water (8 μg of dsRNA in a total volume of 2 μL; i.e., total dsRNA concentration of 4 μg/μL), every second day (one day for recovery). The control animals were treated in the same way but were injected with water. The females were mated to untreated males after the last injection. After mating, the females consecutively produce several cocoons approximately every week. The first six of these cocoons of each female were collected and analyzed. Half of the embryos of each cocoon were fixed for in situ hybridization, and the other half were maintained until the larval stage. For these animals, the phenotypes were recorded. For the analysis, the offspring of all females were pooled for each consecutive cocoon (i.e., "cocoon 1" means that all offspring from the first cocoon of all females were pooled, and so on).

Controls. Two females were injected and all of them survived and produced cocoons, of which all six consecutive cocoons of the females were used in the analysis. The animals were all wild type, except for a certain percentage of animals (that differed between the cocoons) that died before hatching ("not hatched"). The results are shown in Fig. S3*A*.

At-Antp single RNAi. Four females were injected with the 1128-bp fragment (Fig. S2), and all of them survived and produced cocoons. All six consecutive cocoons of all four females were used in the analysis: no animals were excluded. The larvae were classified into wild type, moderate (animals with a small leg-like outgrowth on O1), or strong phenotype (animals with almost full walking legs on O1). Animals that died before hatching were also recorded (not hatched). The results are shown in Fig. S3*B*. Note that the strongest effect was observed in cocoon 3.

To exclude off-target effects caused by the dsRNA injections, two not overlapping *At-Antp* fragments of 474 and 516 bp (Fig. S2) were injected independently. Four females were injected with the 474-bp fragment, and four females were injected with the 516-bp fragment. All females survived the injections and produced cocoons. Because the previous analysis had shown that cocoon 3 shows the strongest effect, only cocoon 1–4 were analyzed in the off-target effect control experiments; in addition, not hatched animals were omitted. The offspring of the different females were pooled from cocoons with the same number. From each cocoon pool, 40 randomly chosen animals were analyzed for each of the two off-target effect control experiments. For comparison, 100 randomly chosen animals of each of the first four cocoon pools of the injection of the 1128-bp fragment were analyzed in the same way. These results are shown in Fig. S2 *B*–*D*. All injections led to identical phenotypes and virtually identical phenotype distributions across the consecutive cocoons, indicating that no off-target effects were present.

At-Ubx and At-abd-A single RNAi. For *At-Ubx*, we used a 925-bp subfragment of the already published fragment (accession no. FM956093) located between the primers Ubx-fwd (CGT GCT GTG ACT GAG CAT CAA CC) and Ubx-rev (GGA ACT TAG GTC CAT GTG GAT TG). For *At-abd-A*, we used the full sequence (1496 bp) of the already published fragment (accession no. FM956096). Three females were injected for RNAi with *At-Ubx*, and three females were injected for RNAi with *At-abd-A*. All females survived the injections and produced cocoons. All six consecutive cocoons of all females were used in the analysis, and no animals were excluded. The results are shown in Fig. S3 *C* and *D*. No phenotypes were observed, and the results are similar to the control injections (Fig. S3*A*). Whole-mount in situ hybridization showed that in both cases the expression level of each gene was visibly lowered compared with the controls, but not fully abolished (Fig. S4).

At-Antp/At-Ubx double RNAi. The females were injected five times every second day (one day for recovery). *At-Antp* and *At-Ubx* dsRNA were produced separately and then combined. To inject 8 μg of each gene in a total volume of 2 μL (like in the single RNAi) would have led to a total dsRNA concentration of 8 μg/μL. Because this concentration proved to be too viscous to be used with the bevelled borosilicate needles used for the injections, the total volume had to be increased. We therefore used 8 μg of each gene in a total volume of 3 μL, which was the maximum volume of liquid that could be injected without harming the animals. We also tried to inject larger volumes, but the excess liquid/hemolymph either leaked from the injection site or exited through the mouth opening during injection and spiders treated in this way did not survive.

Despite the increased number of consecutive injections, the females were mated to untreated males after injection 3. Again, the first six consecutive cocoons were collected and used for analysis.

Six females were injected, of which four survived the injection procedure. Of these injected animals, one died after producing cocoon 1, and one produced only cocoons of bad quality with only a few embryos per cocoon. These females were excluded from the analysis. The remaining two females survived, and all six of their consecutive cocoons were used for analysis. The results of these injections are shown in Fig. S3*E*. The phenotypes are classified into wild type, moderate, and strong *Antp* phenotype (see above for description) and "double phenotype" (strong *Antp* phenotype plus small rudiment on O2). Animals that died before hatching were also recorded (not hatched). The double phenotype occurred in cocoon 4 and 5. Note that the O2 rudiment never occurred alone, i.e., without the strong *Antp* phenotype, in-

dicating that both genes must be knocked down to produce this O2 appendage.

At-Antp/At-Ubx/At-abd-A triple RNAi. The females were injected five times every second day (one day for recovery). *At-Antp*, *At-Ubx*, and *At-abd-A* dsRNA were produced separately and then combined. Because the volume of 3 μL per injection could not be increased, it was not possible to inject 8 μg of each gene. Instead, each female was injected with 6 μg of each of the genes in a total volume of 3 μL (total concentration of dsRNA 6 μg/μL). Despite the increased number of consecutive injections, the females were mated to untreated males after injection 3. Again, the first six consecutive cocoons were collected and used for analysis.

Six females were injected, and only two survived the procedure and produced cocoons. All six of their consecutive cocoons were used for analysis. The results are shown in Fig. S3*F*. The phenotypes are classified into wild type, moderate, and strong *Antp* phenotype and double phenotype (this time the presence of a moderate Antp phenotype and a very small O2 rudiment has been observed as the double phenotype). Animals that died before hatching were also recorded (not hatched). The double phenotype occurred in cocoon 5 and a moderate *Antp* phenotype was observed in cocoons 5 and 6. No strong *Antp* phenotype was observed, and the number of animals with phenotype was very low. The double phenotype was moderate as well, showing only a short leg-like appendage on O1 and a very small limb bud on O2. In addition, these appendages were only on one side of the larvae (mosaic phenotype; Fig. S5). These results suggest that the reduced amount of dsRNA in the triple RNAi experiments was not sufficient to elicit strong phenotypes. In both double and triple RNAi experiments, an increased number of not hatched animals was observed. This phenomenon is probably caused by the increased number of consecutive injections that obviously harm the females and lead to problems with egg production and cocoon quality.

1. Schwager EE, Pechmann M, Feitosa NM, McGregor AP, Damen WGM (2009) hunchback functions as a segmentation gene in the spider Achaearanea tepidariorum. *Curr Biol* 19:1333–1340.
2. Pechmann M, Prpic NM (2009) Appendage patterning in the South American bird spider Acanthoscurria geniculata (Araneae: Mygalomorphae). *Dev Genes Evol* 219:189–198.
3. Pechmann M, et al. (2011) Novel function of Distal-less as a gap gene during spider segmentation. *PLoS Genet* 7:e1002342.
4. Pechmann M, McGregor AP, Schwager EE, Feitosa NM, Damen WGM (2009) Dynamic gene expression is required for anterior regionalization in a spider. *Proc Natl Acad Sci USA* 106:1468–1472.
5. Larkin MA, et al. (2007) ClustalW and ClustalX version 2.0. *Bioinformatics* 23: 2947–2948.

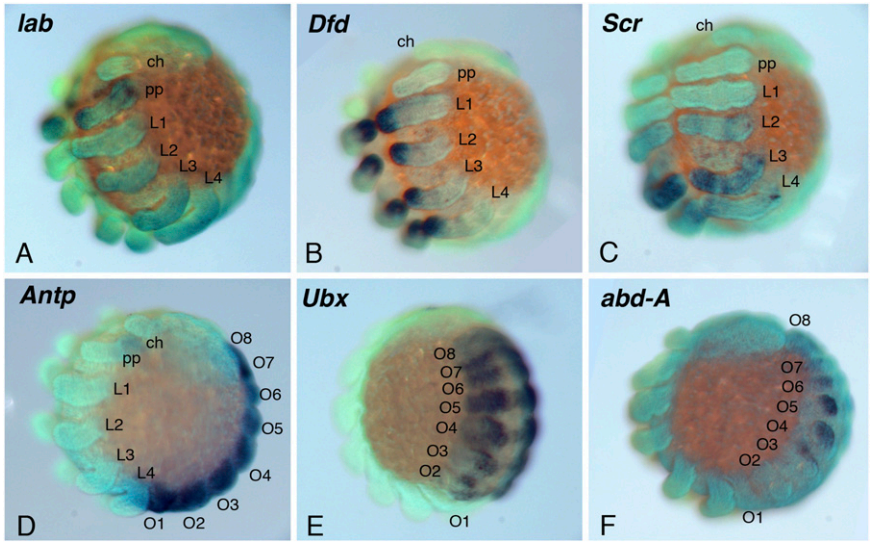

Fig. S1. Expression of the *A. tepidariorum* Hox genes *At-lab*, *At-Dfd*, *At-Scr*, *At-Antp*, *At-Ubx*, and *At-abd-A*. Embryos are shown at late stage nine of development. (*A*) *At-lab* is expressed in the pedipalps and shows weak expression in L1–L4. (*B*) *At-Dfd* is expressed from L1–L4 with strong domains of expression in the tips of the walking legs. (*C*) *At-Scr* is expressed from L2–L4, with strongest expression in L3. (*D*) *At-Antp* is expressed in the entire opisthosoma. (*E*) *At-Ubx* is expressed throughout the opisthosoma in segments O2 to O8. (*F*) *At-abd-A* expression starts at the posterior of O3 and extends to O8. ch, cheliceral segment; L1–L4, walking leg segments one to four; O1–O8, opisthosomal segments one to eight; oc, ocular segment; pp, pedipalpal segment.

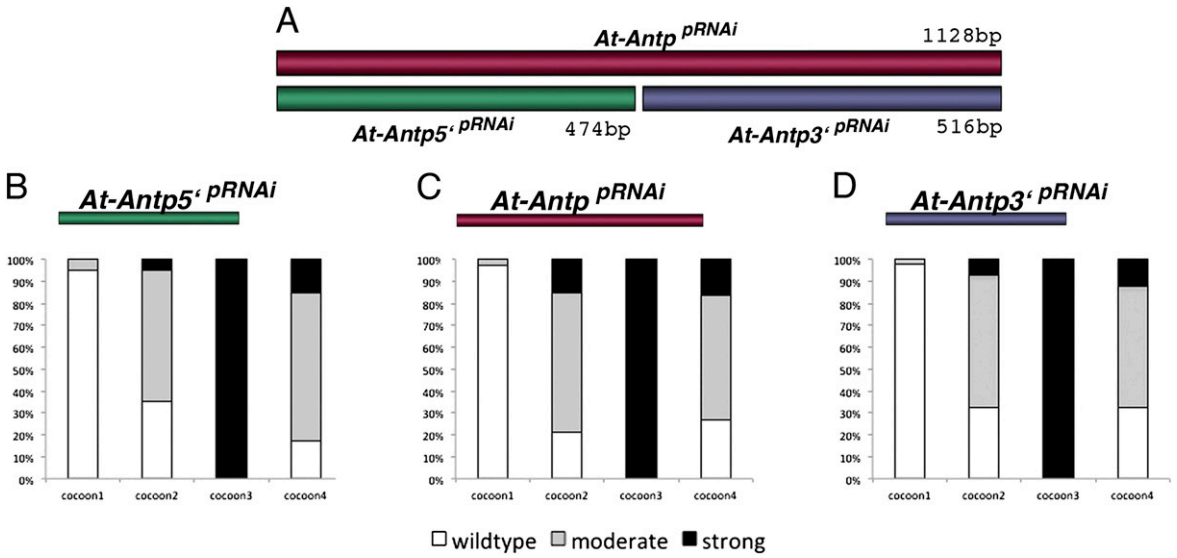

Fig. S2. Statistical analysis of *At-Antp* parental RNAi off-target effect controls. (*A*) Three different fragments of the *At-Antp* cDNA were used for synthesis of dsRNA, a 1128-bp long fragment spanning the coding sequence of the gene (highlighted in red), a 474-bp 5′ fragment (highlighted in green) and a 3′ fragment with a size of 516 bp (highlighted in blue). (*B–D*) Spiders were injected independently with each of the dsRNAs, resulting in a virtually identical phenotype distribution among the successively laid cocoons. The phenotypes have been assigned to two categories. "Moderate" refers to animals in which only a small leg-like outgrowth on the O1 segment could be seen; "strong" refers to animals in which a full walking leg (slightly smaller than the normal legs) could be observed on the O1 segment. In all injections, the strongest effect was observed in cocoon 3, slowly increasing from cocoon 1 and again decreasing in cocoon 4. (*B*) Results for the injections of the 5′ fragment (40 animals have been scored for each cocoon). (*C*) Results for the 1128-bp fragment (100 animals have been scored for each cocoon). (*D*) Results for the 3′ fragment (40 animals have been scored for each cocoon).

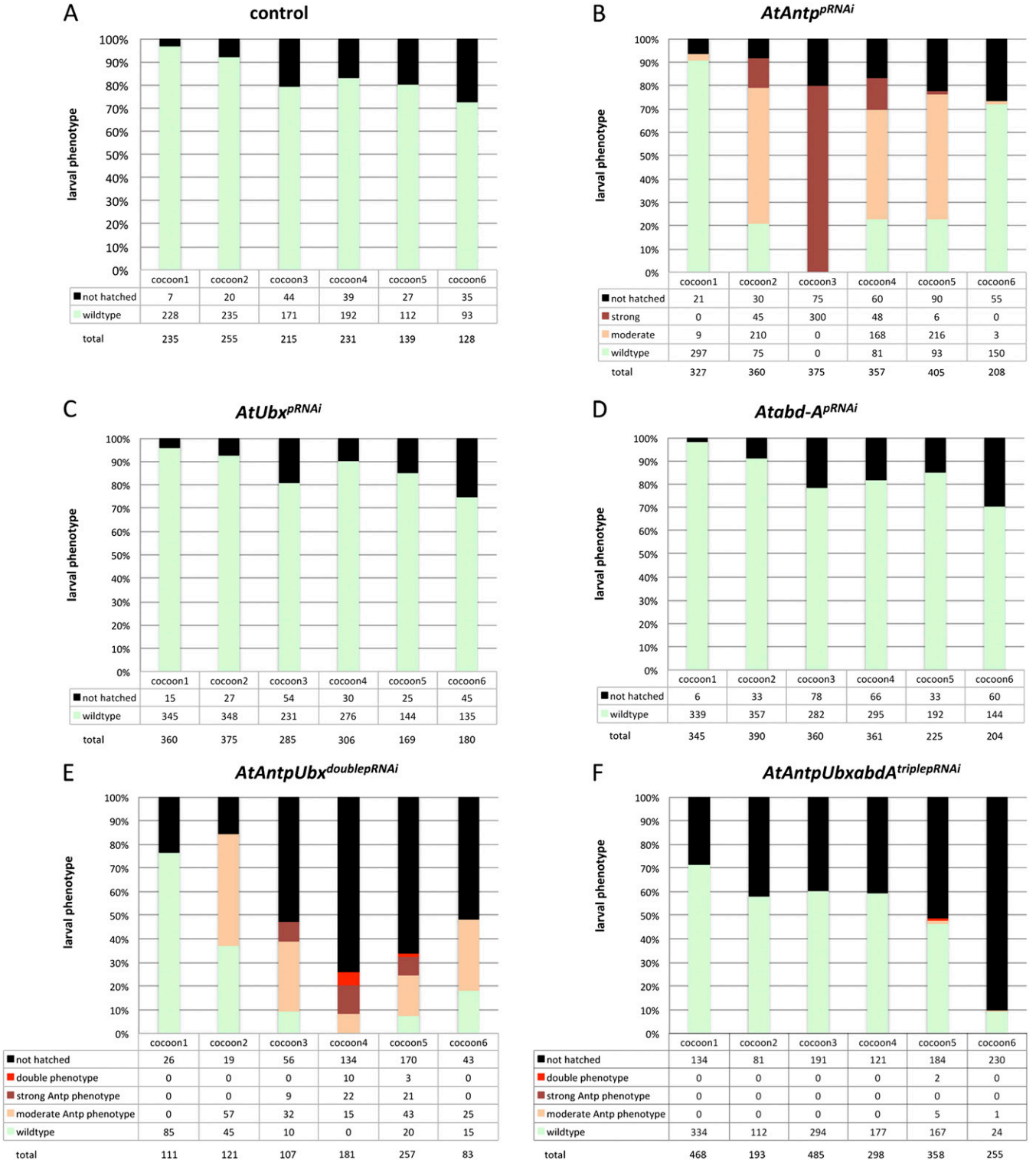

Fig. S3. Statistical analysis of control injections (*A*) and parental RNAi experiments with *At-Antp* (*B*), *At-Ubx* (*C*), *At-abd-A* (*D*), and Hox gene combinations *At-Antp*/*At-Ubx* (*E*) and *At-Antp*/*At-Ubx*/*At-abd-A* (*F*). The graphs show the combined data of several injected females, namely two females in *A*, four females in *B*, three females each in *C* and *D*, and two females each (of six injected each) in *E* and *F*. Six consecutive cocoons were analyzed for each spider in each experiment, and the data were pooled per cocoon number. Please refer to *SI Methods* for a full description of the injection procedure, analyzed animals and cocoons, and description of phenotype categories.

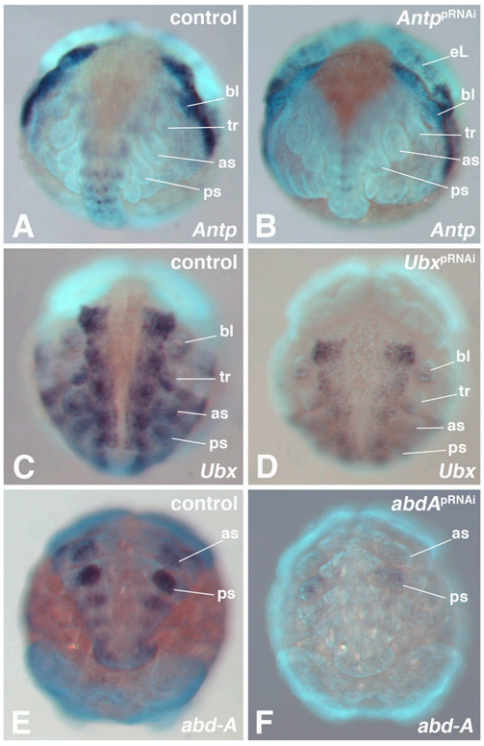

Fig. S4. Comparison of gene expression levels in control and RNAi animals examined by in situ hybridization in late stage 11 embryos. All images show ventral views of the opisthosoma. (*A* and *B*) Expression of *At-Antp* is not fully abolished in *At-Antp* RNAi embryos (*B*), but visibly lower than in control embryos (*A*). Note the well-developed ectopic leg (eL) on O1, indicating that the level of interference with *At-Antp* expression is sufficient to derepress leg development in O1. (*C* and *D*) Expression of *At-Ubx* is not fully abolished in *At-Ubx* RNAi embryos (*D*), but visibly lower than in control embryos (*C*). (*E* and *F*) Expression of *At-abd-A* is almost fully abolished in *At-abd-A* RNAi embryos (*F*), compared with control embryos (*E*). as, anterior spinneret bud; bl, book lung bud; ps, posterior spinneret bud; tr, trachea bud.

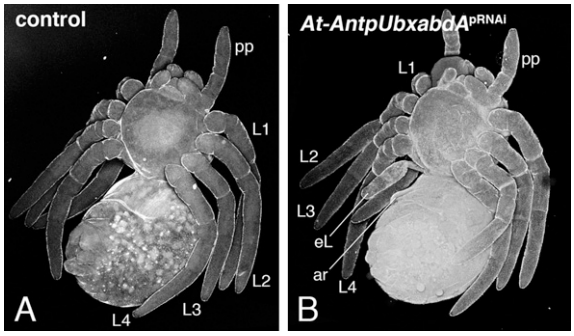

Fig. S5. Moderate mosaic phenotypes result from triple RNAi with *At-Antp*, *At-Ubx*, and *At-abd-A*. (*A*) Wild-type larva (ventral view). (*B*) Triple RNAi larva (ventral view). A short ectopic leg is present on O1 and a very small rudimentary bud is visible on O2, but the effect is restricted to one body half of the larva.: ar, appendage rudiment; eL, ectopic leg; L1–L4, walking legs one to four; pp, pedipalps.

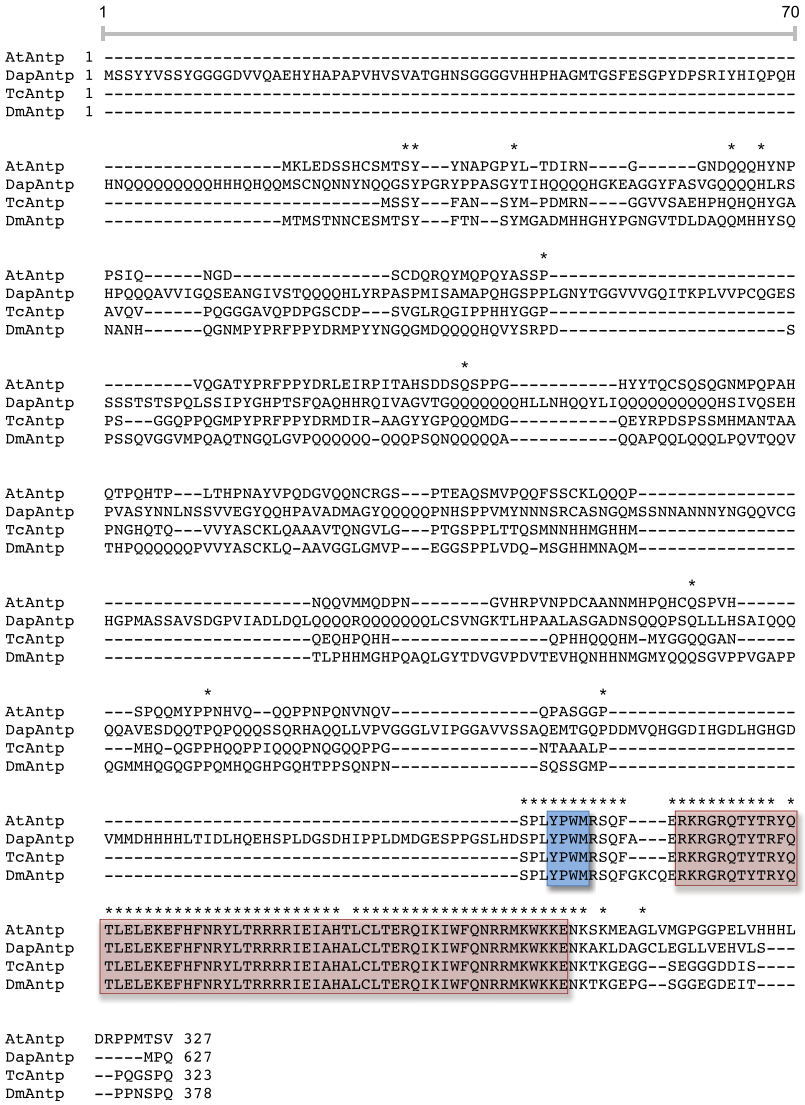

Fig. S6. (Continued)

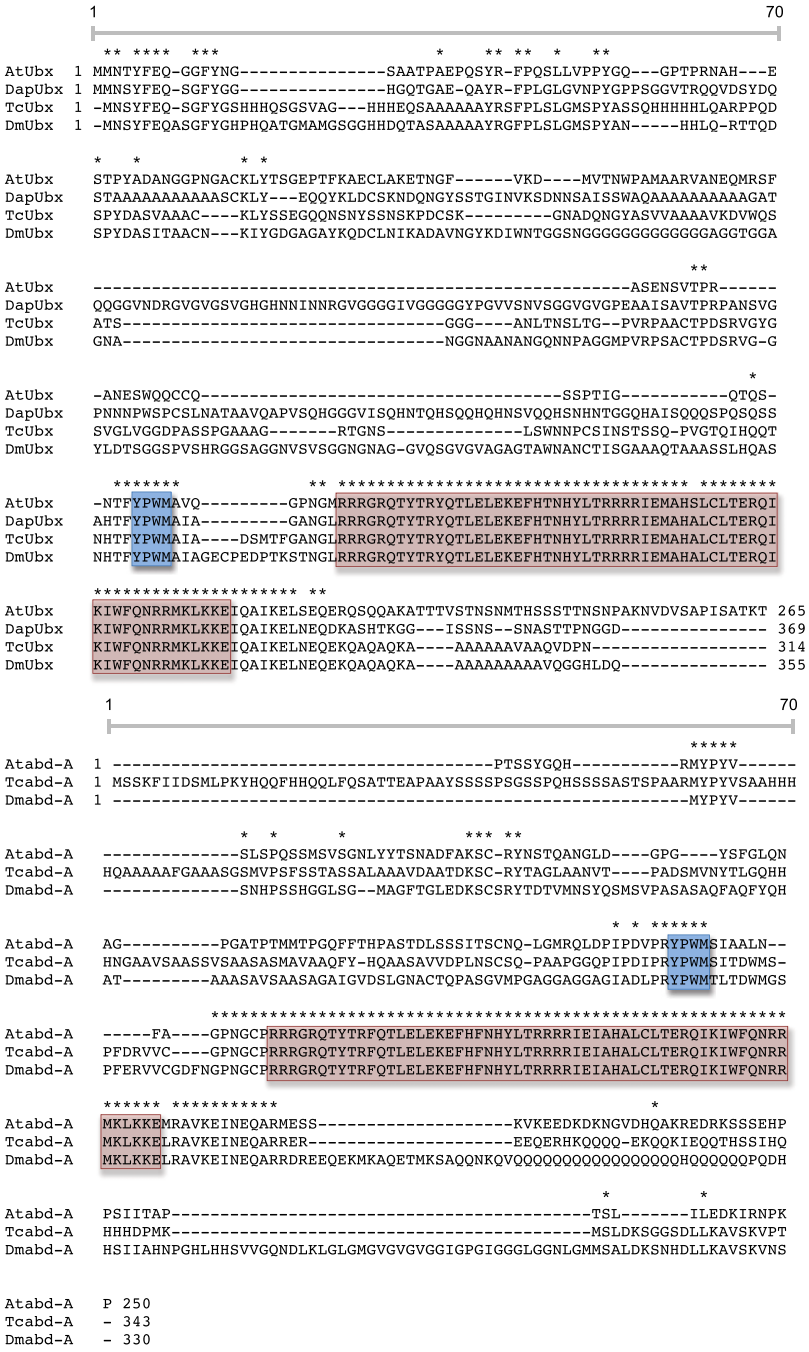

Fig. S6. Alignments of arthropod Antennapedia, Ultrabithorax, and abdominal-A proteins. The alignment has been produced with ClustalW (5). The hexapeptide is highlighted in blue, and the homeodomain is highlighted in red. Asterisks indicate identical amino acids. Dashes denote gaps that were introduced to improve the alignment. The total number of amino acid residues in a protein is given at the C-terminal end of each protein. Only complete protein sequences (where available) were used for the alignments from the following arthropod species: *Achaearanea tepidariorum* (At), *Daphnia magna* (Dap), *Tribolium castaneum* (Tc), and *Drosophila melanogaster* (Dm). Accession nos.: At-Antp, HE608680; At-Ubx, CAX11340.1; At-abd-A, CAX11343.1; Dap-Antp, BAE96991.1; Dap-Ubx, BAE96992.1; Tc-Antp, EEZ99250.1; Tc-Ubx, NP_001034497.1; Tc-abd-A, EEZ99248.1; Dm-Antp, NP_996167.1; Dm-Ubx, AAS65158.1; Tc-abd-A, ACZ94928.1.