Article

hunchback Functions as a Segmentation Gene in the Spider Achaearanea tepidariorum

Evelyn E. Schwager,[1,3] Matthias Pechmann,[1,4] Natàlia M. Feitosa,[1,5] Alistair P. McGregor,[1,6] and Wim G.M. Damen[1,2,*]
[1]Institute for Genetics, Evolutionary Genetics, University of Cologne, Zülpicher Straße 47, D-50674 Köln, Germany
[2]Department of Genetics, Friedrich-Schiller-University Jena, Philosophenweg 12, D-07743 Jena, Germany

Summary

Background: In insects, the gap gene hunchback (hb) is required for the formation of a set of adjacent segments through the regulation of downstream target genes of the pair rule and segment-polarity classes. In addition, hb is a major regulator of Hox genes and it has been suggested that this is the ancestral role of hb in insects or perhaps even arthropods. To date, however, hb function has been analyzed only in insects.
Results: Here we show that hb acts as a segmentation gene during anterior patterning of a noninsect arthropod, the spider Achaearanea tepidariorum. The leg-bearing segments L1, L2, and L4 are missing after downregulation of At-hb via RNAi. At-hb is required for the correct organization of target genes in this region of the embryo, suggesting that At-hb acts as a gap gene in the spider. In contrast to insects, hb does not control Hox gene expression in the spider. Furthermore, analysis of twist expression in At-hb knockdown embryos demonstrates that hb is not required for initiating the segmental organization of the mesoderm in the affected region, but only for its maintenance.
Conclusions: Our findings suggest that hb might have had a segmentation gene function in the arthropod ancestor and contradicts the suggestion that the control of Hox genes is the ancestral role of hb. Anterior spider segmentation thus utilizes a Drosophila-like genetic mode, whereas a vertebrate-like mechanism involving Wnt8 and Notch/Delta signaling is used to pattern posterior segments. These data support the hypothesis that short-germ arthropods employ two distinct mechanisms to segment their anterior and posterior body parts.

Introduction

In *Drosophila*, a regulatory cascade controls the simultaneous formation of all segments. Most arthropods, however, pattern only a few anterior segments simultaneously and generate the majority of their segments sequentially from a growth zone

*Correspondence: damen@uni-koeln.de
[3]Present address: Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Ave, Cambridge, MA 02138, USA
[4]Present address: Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie GZMB, Ernst-Caspari-Haus, Abteilung für Entwicklungsbiologie, Georg-August-Universität Göttingen, Justus-von-Liebig-Weg 11, D-37077 Göttingen, Germany
[5]Present address: Institute for Developmental Biology, Gyrhofstraße 17, D-50923 Köln, Germany
[6]Present address: Institut für Populationsgenetik, Veterinärmedizinische Universität Wien, Josef Baumann Gasse 1, A-1210 Wien, Austria

[1, 2]. It has therefore been suggested that anterior and posterior segmentation in arthropods might employ different mechanisms and that *Drosophila* segmentation is derived from such a dual system [3, 4].

In spiders, the six segments of the prosoma (cephalothorax) form almost simultaneously in the anterior embryo, before the segments of the opisthosoma (abdomen) are added in a strict anterior to posterior order (Figure S1 available online). The mechanism underlying segmentation of the spider opisthosoma involves Wnt8 and Delta-Notch signaling [4–7]. However, these pathways do not appear to play a major role in anterior segmentation and indeed are not involved in *Drosophila* segmentation.

Although we have a growing understanding of how posterior segments are patterned in spiders, little is known about the mechanisms that regulate the formation of anterior segments. In *Drosophila*, gap genes regulate downstream genes and are the key players in the transition from a nonperiodic to a periodic pattern. One of the most studied gap genes is *hunchback* (*hb*), which encodes a zinc finger transcription factor and is required for segmentation in insects [8–13].

In *Drosophila*, *hb* is active in a broad anterior domain covering the future gnathal segments and in a posterior domain corresponding to abdominal segments A7 and A8. *hb* mutants exhibit canonical gap phenotypes lacking gnathal and thoracic segments, and in addition fusion of A7 and A8 [14]. In other insects, *hb* is also involved in segmentation and knockdown causes gap phenotypes, although the exact mechanistic details can differ as the gap phenotype is obscured by homeotic transformations in *Oncopeltus*, *Tribolium*, and *Gryllus* [8–10].

In the centipede *Strigamia* [15] and the crustacean *Artemia* [16], *hb* expression is associated with nervous system and mesoderm development but not with segmentation. However, a paucity of functional data from noninsect arthropods means that it is still unclear whether *hb* evolved a role in segmentation only in insects or earlier in arthropod evolution. It has even been hypothesized that *hb* acquired its segmentation role from an ancestral Hox gene-regulating function [2]. Alternatively, *hb* may have been recruited for segmentation from an ancestral role in the nervous system [2]. In addition, the observation that *hb* is expressed in the mesoderm of various arthropods and an annelid has led to the suggestion that the ancestral role of *hb* was in mesoderm formation [11, 15–17].

Here we show that the *hb* ortholog of the spider *Achaearanea tepidariorum* is required for anterior segmentation. Knockdown of *hb* in this spider results in a gap phenotype. However, in contrast to insects, spider *hb* does not regulate Hox genes. Our data suggest that the spider employs different upstream regulatory mechanisms for anterior and posterior segmentation. In the anterior, the spider utilizes mechanisms similar to *Drosophila*, whereas posterior patterning involves Wnt8 and Notch/Delta signaling and is more similar to vertebrates.

Results

Isolation of a *hunchback* Ortholog from *Achaearanea*

We recovered a full-length *hb* ortholog from the spider *Achaearanea tepidariorum*, which encodes a predicted protein

of 914 aa, containing nine zinc fingers (Figure S2): two N-terminal (NF1-2), four medial (MF1-4), one extra (ExF), and two C-terminal zinc fingers (CF1-2) (nomenclature after Patel et al. [11]). Alignment of metazoan Hb orthologs reveals that nine zinc fingers represent the ancestral structure of Hb. Insect sequences lack ExF, and although hemimetabolous insects have derived NFs, most holometabolous insects have lost these motifs (Figure S2). *At-hb* thus seems to encode a transcription factor with more ancestral features than insect Hb.

Expression of *At-hb*

At-hb is expressed maternally in developing oocytes (Figure S3A). The first detectable zygotic expression is in the blastopore region of stage 3/4 embryos (Figure 1A). At stage 4, additional expression of *At-hb* appears in a 1-cell-wide ring at the rim of the germ disc (Figures 1B and 1C). During stage 5, *At-hb*-expressing mesenchymal cells of the cumulus migrate from the center to the periphery of the germ disc whereas only a few *At-hb*-expressing cells remain in the center (Figure 1C). Then a new *At-hb* expression domain emerges as a broad stripe between the ring of expression at the rim and the central domain (bracket in Figure 1C). At stage 6, this expression becomes stronger and merges with the rim expression. At the same time, the radially symmetric germ disc transforms into an axially symmetric germband, and it is evident that the rim of the germ disc represents the future anterior of the embryo and the center the posterior [5, 18]. During this stage, the growth zone is forming, concomitant with the transformation of the now stronger posterior expression domain into a stripe and loss of *At-hb* expression in the most posterior cells (Figures 1D and 1E).

Later in stage 6, the broad anterior domain splits into two stripes, while the posterior broad stripe persists (Figure 1E). Subsequently two new narrow stripes of *At-hb* expression appear between these broader stripes (arrowheads in Figure 1F). In stage 8, the anterior broad stripe covers the head and cheliceral region, the medial stripe covers the future L1/L2 region, and the posterior domain covers the presumptive L4 region. The two narrow intercalated stripes are associated with the pedipalpal and L3 segments, respectively (Figure 1G). Sections reveal that *At-hb* is expressed in both ectodermal and mesodermal layers at this stage, with the exception of L3 where expression is exclusively mesodermal (Figures S3C and S3D). *At-hb* is expressed in every new segment that buds off from the growth zone, but not in the growth zone itself (Figure 1H; Figure S3B; and not shown). Later the *At-hb* expression is observed in the nervous system, like in other arthropods (Figure S3B) [8, 10–13, 15, 16].

At-hb pRNAi Leads to Reduced Number of Legs

To explore the function of *At-hb*, we used parental RNAi to knockdown its expression (Figure S4). First instars that hatched from *At-hb*^pRNAi^ cocoons show a reduced number of leg pairs, having only two or three pairs of legs instead of four pairs (Figures 2A–2C). Interestingly, many of these first instars actually survive the next molt and are able to walk (Movies S1 and S2).

To analyze the *At-hb*^pRNAi^ phenotype in more detail, we first examined stage 10 embryos. A phenotypic series of defects was observed (Figures 2D–2I; Figure S5), which we divided into three classes. Class I phenotypes ranged from having shorter second walking legs (L2) in mildly affected embryos (Figure 2E) to a complete reduction of L2 legs in more severe examples (not shown). Class II phenotypes lack L2 and show
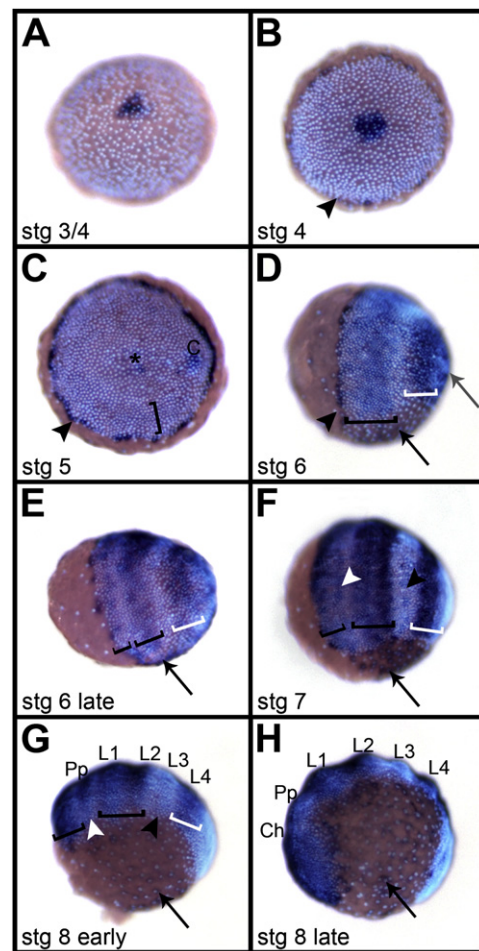

Figure 1. Expression of *At-hb* in Spider Embryos

Visualization of *At-hb* transcripts via in situ hybridization during stages 3–8 of development. Top views of the germ disc (A–C), lateral views (D–H), anterior is to the left.

(A) *At-hb* is initially expressed in the center of the germ disc.

(B) Soon thereafter it is expressed also in a ring of cells around the periphery of the germ disc (arrowhead).

(C) At stage 5, the cumulus (c) that contains *At-hb*-expressing cells is migrating to the periphery of the germ disc. The asterisk marks the future posterior of the embryo, surrounded by some *At-hb* expression. A broad expression domain arises between the outer ring (bracket) and the posterior expression.

(D) The new broad domain (black bracket) then merges with the ring at the anterior (arrowhead), while the former posterior expression domain becomes stronger (white bracket). The most posterior cells do not express *At-hb* anymore (gray arrow).

(E) At late stage 6, the broad anterior stripe splits into two broad stripes (black brackets).

(F and G) Narrow stripes of *At-hb* expression (arrowheads) intercalate between the broad domains (white and black brackets).

(G and H) At stage 8, the first segmental grooves become visible, which allow segmental allocation of the *At-hb* expression domains: The former anterior domain covers the head and the cheliceral segment, one of the intercalated narrow stripes (white arrowhead) is in the pedipalpal segment, the broad central domain encompasses L1 and L2, the other narrow stripe (black arrowhead) is in the mesoderm of L3 (see also Figure S3C), and the posterior domain of *At-hb* expression is in L4.

At-hb is also expressed in the extraembryonic tissue, which forms when the germ disc opens dorsally (black arrow in D–H).

Abbreviations: C, cumulus; Ch, cheliceral segment; Pp, pedipalpal segment; L1–L4, walking leg segments 1–4.
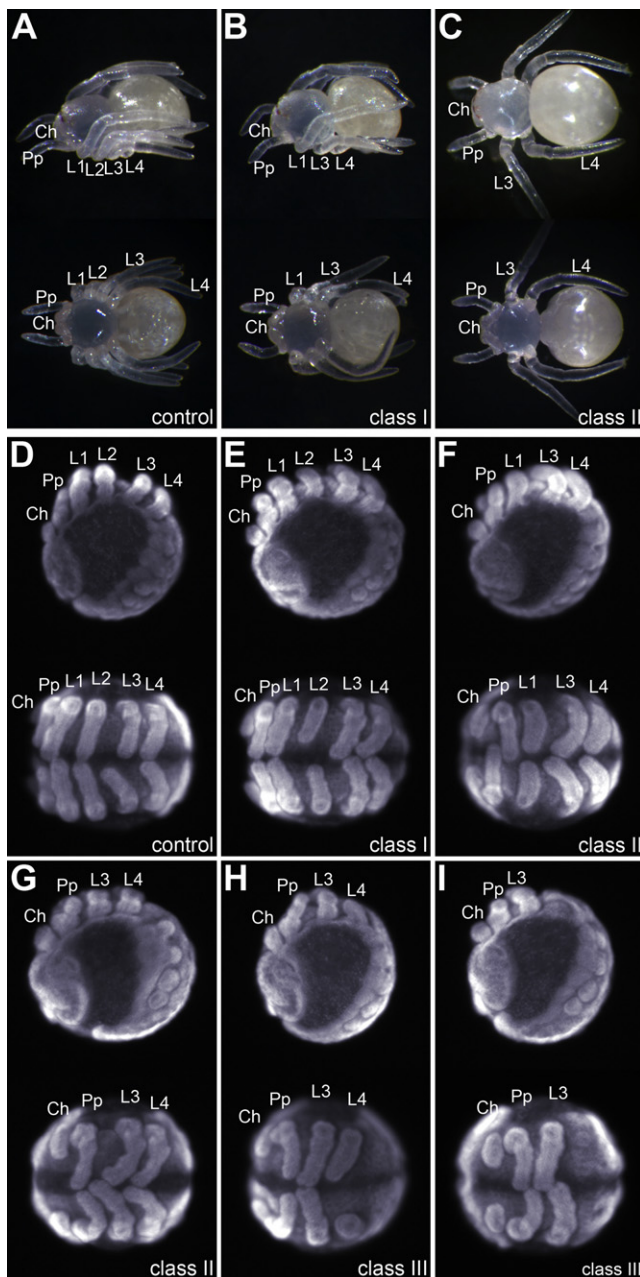
Figure 2. *At-hb* Parental RNAi Leads to Loss of Legs in the Spider *Achaearanea tepidariorum*

Bright-field images of phenotypes observed in live first instars (A–C) and epi-fluorescent images of DAPI-stained stage 10 embryos (D–I).

(A and D) Offspring of *gfp*[dsRNA]-injected control spiders. Compared to control first instars (A), class I (B) and class II (C) *At-hb*[pRNAi] first instars look normal, except they lack one and two leg pairs, respectively.

(E–I) Phenotypic series of *At-hb*[pRNAi] embryos at stage 10.

(E) The weakest class I phenotypes show only a reduction in size of the second walking leg.

(F and G) Class II phenotypes have completely lost the second walking leg (L2) and additionally show a reduction (F) or complete loss (G) of the first walking leg (L1).

(H and I) Class III phenotypes moreover show a reduction (H) or complete loss (I) of the fourth walking leg (L4).

Asymmetric phenotypes with differences in the severity of the leg reductions with regard to the left-right axis were also observed (e.g., panel H). Neither head nor opisthosomal segments were affected by *At-hb*[pRNAi] (see also Figures 5H–5L and Figures S6 and S7D–S7F). Upper pictures in

a reduction or complete loss of L1 (Figures 2F and 2G). Class III phenotypes show reduced L1 and L2 and also exhibit a reduction of L4 (Figures 2H and 2I). Severe class III phenotypes have completely lost L1, L2, and L4 legs but L3 legs are never affected (Figure 2I).

At-hb pRNAi Causes Loss of Segments and Affects the Correct Expression of *At-hairy*

To investigate whether *At-hb* pRNAi phenotypes are caused by the loss of entire segments or the appendages are merely reduced, we analyzed the expression of the segmental markers *engrailed* (*At-en*), *hedgehog* (*At-hh*), and *pby/Pax3/7* (*At-pby*).

Embryos from *At-hb*[pRNAi] cocoons do not form *At-en, At-hh*, and *At-pby* stripes in the L2 segment (class II embryos) or L1 and L2 segments (class III embryos) (Figures 3A–3D; Figure S7). Furthermore, there is no *At-pby* expression in the L4 segment of class III *At-hb*[pRNAi] embryos (Figure S7I), whereas *At-en* and *At-hh* expression in L4 is never completely eliminated (Figures 3B and 3D; Figure S7C). However, at stage 10, the L4 *At-en* stripe is distorted (Figure S7F), which must occur later during stages 9 and 10 because this affect is never observed in earlier embryos (e.g., Figure S7C). These experiments imply that not only are the appendages missing, but the entire L1 and L2 segments are not specified in *At-hb*[pRNAi] embryos. L4, in contrast, does appear to be specified (*At-en* and *At-hh* stripes form), but is then secondarily lost.

Until stage 9, the distance between the *At-en* stripes of the L1 and L3 segment in class II embryos and between the pedipalpal and L3 segment in class III embryos is larger than in control embryos (Figure 3B; Figures S7B and S7C). In control embryos, the L1 and L2 *At-en* stripes, which are missing in the RNAi embryos, are located in this region (Figure 3A; Figure S7A). At stage 10, however, this larger distance contracts and there is a general compaction of all segments. Observation of live embryos via time-lapse also demonstrates that initially tissue is present between the pedipalpal and L3 segment, but that this tissue is not patterned into segments (Figures S8 and S9).

Moreover, we observed that the expression pattern of *At-hairy* (*At-h*) is also affected by *At-hb* pRNAi. Three distinct stripes of *At-h* in L2–L4 (Figure 3E) are formed from the splitting of an initial single, broad *At-h* domain [5, 19, 20]. Intriguingly, this division of the L2/L3 stripe does not occur in *At-hb*[pRNAi] embryos and a broader *At-h* stripe remains present in the L3 segment (Figure 3F). *At-hb* thus is required for splitting the L2/L3 *At-h* stripe.

The *At-en* and *At-pby* stripes in the cheliceral, pedipalpal, and opisthosomal segments are never affected in *At-hb*[pRNAi] embryos. Likewise, the stereotypic O1 *At-pby* stripe is never affected by *At-hb* pRNAi, although the L4 *At-pby* stripe is missing (Figures S7G–S7I). The influence of *hb* is thus restricted to the central, leg-bearing segments of the spider embryo.

At-hb Does Not Appear to Be Involved in the Regulation of Hox Genes

It has been claimed that the ancestral role of *hb* was in regulating Hox gene expression in insects or even in all arthropods

each panel show lateral views except for a top view in (C), lower pictures show ventral views. Abbreviations: Ch, chelicerae; Pp, pedipalps; L1–L4, walking legs 1–4.
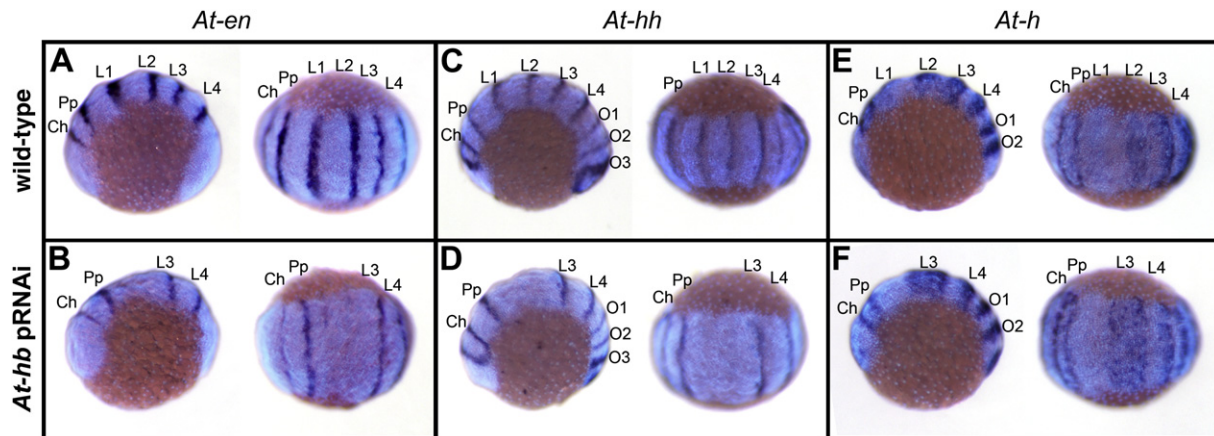
Figure 3. *At-hb* Regulates the Expression of Segmentation Genes

Expression of *At-en* (A and B), *At-hh* (C and D), and *At-h* (E and F) in stage 8 control (A, C, E) and *At-hb*^{pRNAi} class III (B, D, F) embryos. Each panel shows one embryo, viewed laterally (left) and ventrally (right).

(A and B) *At-en* expression does not appear in L1 and L2 of *At-hb*^{pRNAi} embryos. In normally developing *Achaearanea* embryos, the *At-en* stripes appear almost simultaneously, but in a stereotypic order (see Figure S1). The L2 and L3 stripes normally appear last. Because the L3 *At-en* stripe is present in the embryo shown in (B), the L2 and also L1 *At-en* stripe (which is always the first *en* stripe that appears) are missing.

(C and D) A similar situation can be found for *At-hh*, which is also expressed in the posterior part of every segment, but is not expressed in the presumptive L1 and L2 region of *At-hb*^{pRNAi} embryos.

(E) *At-h* is expressed in segmental stripes at stage 8, whereas the L1 stripe is only mesodermal. The stripes in L2, L3, and L4 are the result of splitting of a single broad stripe.

(F) In *At-hb* pRNAi embryos, the broad *At-h* domain does not split properly and no individual L2 and L3 stripes form like in control embryos. The expression of *At-h* in L1 does not appear either.

Abbreviations: Ch, cheliceral segment; Pp, pedipalpal segment; L1–L4, walking leg segments 1–4; O1–O3, opisthosomal segments 1–3.

and that *hb* only secondarily acquired its role as a segmentation gene [2]. In several insects, *hb* prevents the activation of posterior Hox genes in anterior regions of the embryo and so knockdown of *hb* leads to ectopic anterior expression of several Hox genes resulting in homeotic transformations of gnathal and thoracic segments into abdominal identity [8–10]. To test whether the *At-hb* phenotype involves changes in Hox gene expression and homeotic transformations, we analyzed the expression of Hox gene orthologs in *At-hb*^{pRNAi} embryos. However, all Hox genes analyzed were expressed in comparable domains in both RNAi and control embryos (Figure 4; Figure S10). Moreover, the Hox gene expression patterns provide evidence that indeed only the L3 segment is present in strong class III *At-hb*^{pRNAi} embryos. The remaining leg pair in class III RNAi embryos expresses neither *At-lab* (Figure 4B) nor *At-Antp* (Figure 4H), which excludes a L1 or L2 identity, because L1 and L2 weakly express *At-lab* (Figure 4A), and excludes a L4 identity, because *At-Antp* is weakly expressed in L4 (Figure 4G).

Thus, in contrast to the role of *hb* in insects, there is no evidence for an effect of *At-hb* on Hox gene expression.

Effects of *At-hb* Knockdown on Mesoderm Formation

To determine whether *hb* has a role in mesoderm development in the spider, we investigated the expression of the mesodermal marker *At-twist* (*At-twi*) [21]. In both control and *hb*^{pRNAi} early stage 7 embryos, *At-twi*-expressing mesodermal cells are ubiquitously distributed; however, *At-twi* is more strongly expressed in the future L4 (Figures 5A and 5B). Starting at late stage 7, the *twi*-positive mesodermal cells become organized segmentally in the L1–L4 region (Figures 5C and 5E) [21]. This process is also observed in *At-hb*^{pRNAi} embryos (Figures 5D and 5F). It is surprising that mesodermal stripes of *At-twi*-expressing cells are found in the L1 and L2 segments

there (Figures 5D and 5F), because these segments are not patterned ectodermally in *At-hb*^{pRNAi} embryos. This becomes obvious in the embryos stained for both *At-en* and *At-twi*, in which expression of the mesodermal marker *twi* but not the ectodermal marker *en* is observed in the L1 and L2 segments (Figure 5J). Thus, *hb* controls initiation of ectodermal but not mesodermal layers in the leg-bearing segments of the spider. During stage 8, the mesodermal cells form broad stripes in control embryos (Figures 5E and 5G), but in *hb*^{pRNAi} embryos the L1 and L2 mesodermal stripes seem to become reduced (Figures 5F and 5H). This suggests that in the spider, *hb* is not required for the initial mesoderm segmentation, but is necessary for mesoderm maintenance in the segments affected by *At-hb* knockdown.

Discussion

hb Regulates Anterior Segmentation in a Noninsect Arthropod

In *Drosophila*, *hb* is responsible for the development of contiguous segments and therefore functions as a classic gap gene. However, data from additional insect species suggest that the ancestral role of *hb* was in regulating Hox genes rather than in segment generation in insects [2, 8–10].

We demonstrate here that the spider *hb* gene is required for the generation of segments. *At-hb* is expressed in broad domains in the presumptive L1–L2 and L4 segments before the onset of segmentation in the spider embryo, and silencing of *hb* via pRNAi leads to a gap phenotype by the deletion of L1 and L2 and the disruption of L4. This phenotype can clearly be considered a gap gene phenotype, and as such it is more similar to *Drosophila* than most of the other insect *hb* phenotypes.

wild-type   *At-hb* pRNAi
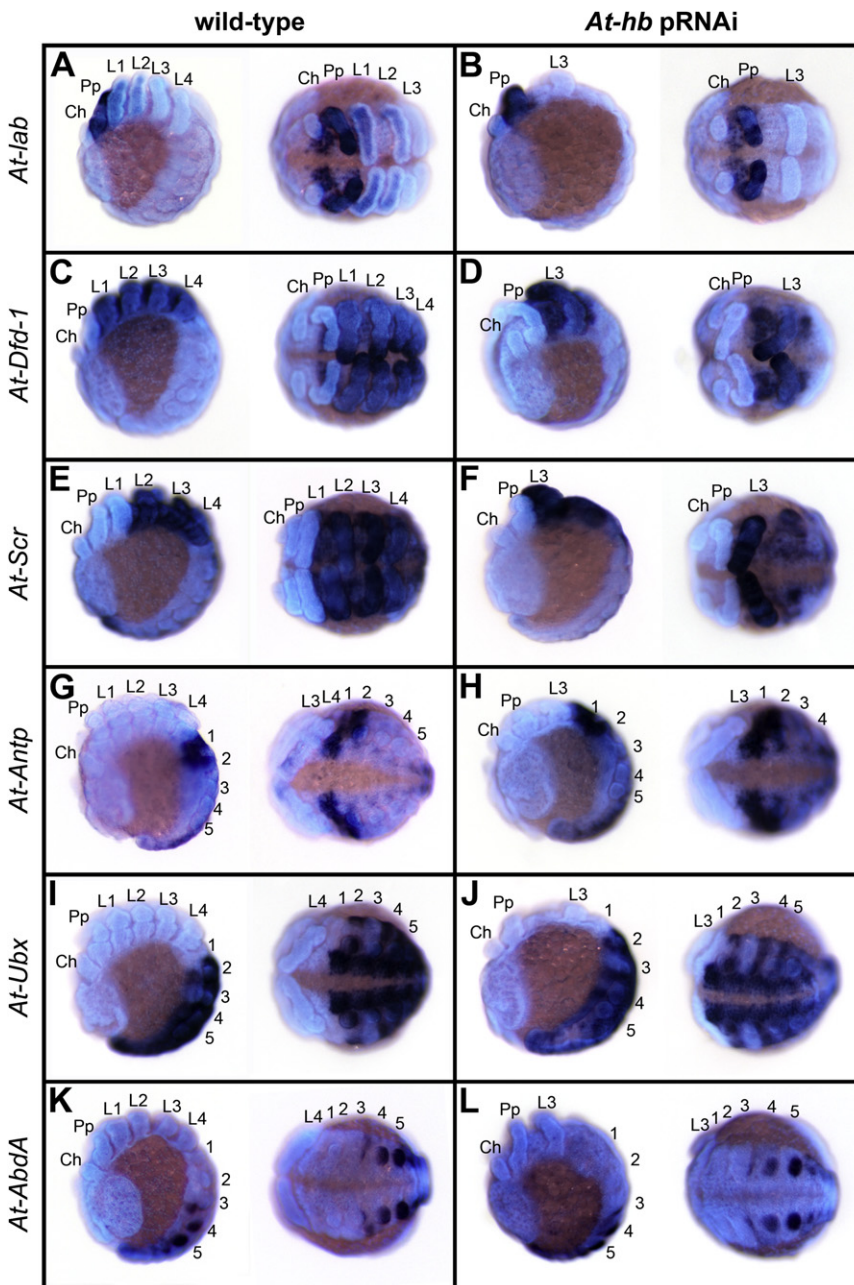
Figure 4. *At-hb* Does Not Regulate Hox Genes in the Spider

Expression of the Hox genes *At-lab* (A, B), *At-Dfd-1* (C, D), *At-Scr* (E, F), *At-Antp* (G, H), *At-Ubx* (I, J), and *At-AbdA* (K, L) in control (A, C, E, G, I, and K) and class III *At-hb*[pRNAi] (B, D, F, H, J, and L) embryos. All embryos are stage 10 embryos and viewed laterally (left) and ventrally (right). None of the Hox genes is ectopically expressed in *At-hb*[pRNAi] embryos.

(A) *At-lab* is strongly expressed in Pp and weakly expressed in L1 and L2 in WT embryos.

(B) In *At-hb*[pRNAi] embryos, *At-lab* expression is only seen in Pp. L1 and L2 are missing.

(C and D) *At-Dfd-1* is expressed in the neuroectoderm and the legs of L1–L4 in WT embryos (C), and in *At-hb*[pRNAi] embryos only in L3 and the remaining neuroectoderm of L4 (D).

(E) In control embryos, *At-Scr* is expressed in L1–L4, while the strongest expression is in L3.

(F) In *At-hb*[pRNAi] embryos, *At-Scr* is expressed only in L3, while the expression resembles that in control L3 legs. In addition, the remaining L4 ectoderm expresses *At-Scr*.

(G) *At-Antp* is expressed in two rings at the tip of the L4 legs, strongly in the posterior part of L4, in O1, and the anterior part of O2, and weakly throughout the rest of the opisthosoma.

(H) In *At-hb*[pRNAi] embryos, *At-Antp* is expressed in the same pattern, except for the missing L4 legs.

(I and J) *At-Ubx* is expressed in O2 and posterior to this segment in both WT and *At-hb*[pRNAi] embryos.

(K and L) *At-AbdA* is expressed in the posterior part of O3 and onward in both WT and *At-hb*[pRNAi] embryos.

Abbreviations: Ch, cheliceral segment; Pp, pedipalpal segment; L1–L4, walking leg segments 1–4; 1–5, opisthosomal segments 1–5.

Nonetheless, the influence of *hb* on these genes within the domain of *At-hb* expression may explain the lack of segmentation of the L1 and L2 segment area after *At-hb* RNAi and confirms that *At-hb* regulates segmentation in the spider.

In the strongest *At-hb*[pRNAi] phenotypes, only two adjacent segments (L1 and L2) are missing. In contrast, *Drosophila hb* mutant embryos lack four adjacent segments [14]. One possible explanation for this difference might be the fact that *Achaearanea* segments form in a cellular rather than syncytial environment [27], which may impede formation of short-range diffusion gradients of transcription factors like Hb.

hb Is Not Involved in Posterior Segmentation in the Spider

In insects, a second, posterior expression domain of *hb* is present in the prospective tissue of some abdominal segments and *hb* is required for segmentation of this region [10, 12, 14]. In the spider, however, *hb* is not expressed during posterior segmentation, and the opisthosoma develops normally in *At-hb*[pRNAi] embryos. In addition no comparable posterior *hb* expression has been described in other non-insect arthropods [15, 16]. This implies that this posterior

In *Drosophila,* one important function of gap genes is to regulate downstream genes in metameric patterns, which is the first sign of segmentation in the embryo. *Dm-hb* is required for the expression and positioning of particular stripes of the pair rule genes *even-skipped* (*eve*), *runt* (*run*), and *hairy* (*h*) [22–26]. In the short-germ insects *Gryllus* and *Tribolium*, *hb* also regulates stripes of pair rule gene expression, but presumably indirectly [9, 10].

In the spider *Achaearanea*, *hb* is required for correct formation of the stripes of the *hairy* gene. The L2/L3 *hairy* stripe does not split in *At-hb*[pRNAi] embryos and no separate L2 stripe forms. *At-hb* is thus required for division of this initial broad stripe. Although the effects on *At-h*, as well as on the segment polarity gene orthologs *At-en*, *At-hh*, and *At-pby*, are within the expression domain of *At-hb*, it is unclear whether *hb* directly or indirectly regulates these target genes.
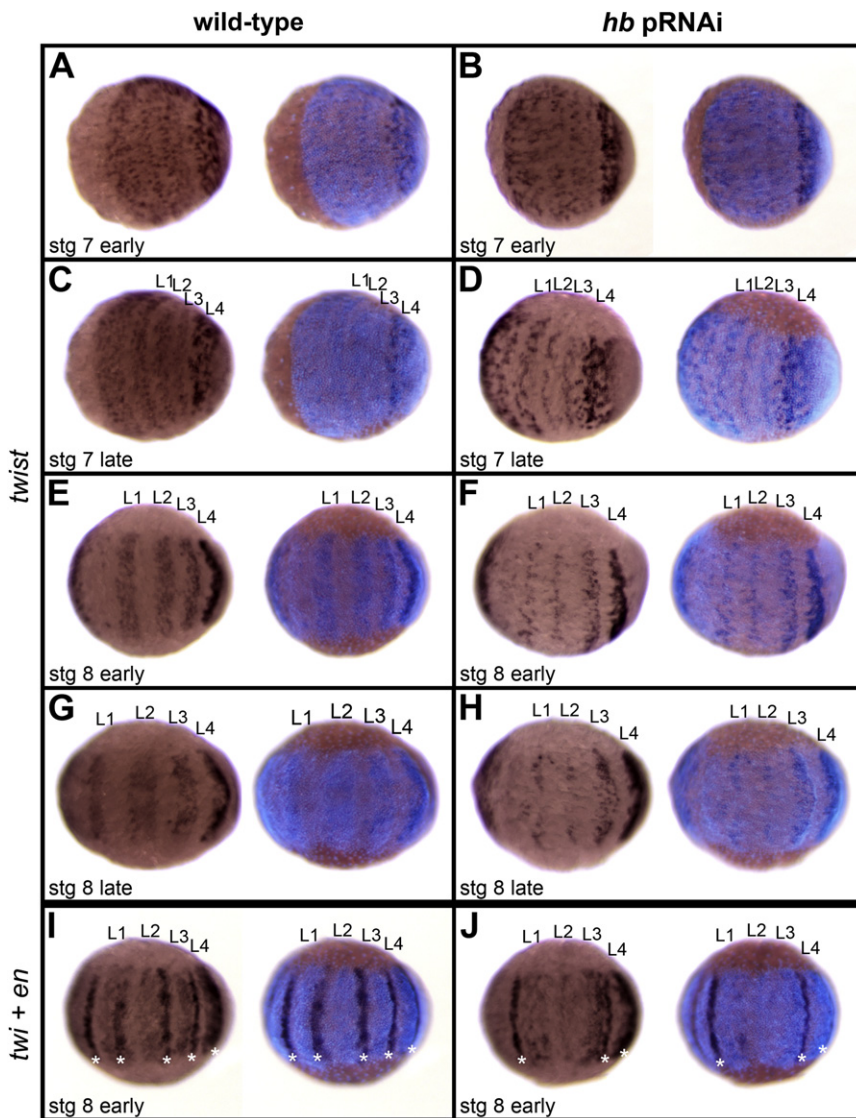
wild-type   *hb* pRNAi

Figure 5. Initiation of Mesodermal *At-twi* Expression Does Not Require *At-hb*

Expression of *At-twi* in stage 7 and 8 wild-type (A, C, E, G) and *At-hb*[pRNAi] (B, D, F, H) embryos. The early stage 8 embryos in (I) and (J) are hybridized with *At-twi* and *At-en* probes (single color double in situ hybridization). Initially, *At-twi*-expressing mesodermal cells are ubiquitously distributed (A, B), but then become organized segmentally in the L1–L4 region (C–F). In stage 8 embryos, the mesodermal cells form broad stripes (E, G), but in *hb*[pRNAi] embryos the L1 and L2 stripes appear to be reduced (F, H). *At-hb* pRNAi does not affect sorting of *At-twi*-expressing cells into stripes, even in segments that are not segmented ectodermally (I, J). All embryos are shown with bright-field pictures only (left) and a bright-field picture with merged DAPI fluorescent staining (right). Mesodermal *At-twi* stripes are labeled with corresponding segments L1–L4, and the ectodermal *At-en* stripes in (I) and (J) are marked with asterisks. All *At-hb*[pRNAi] embryos were taken from cocoons that exhibited class III phenotypes in more than a third of embryos at later stages.

New Insights into Mesoderm Development and Segmentation in the Spider

One outstanding question concerning the origin and evolution of segmentation is the role of the mesoderm in this process. Although it has been proposed that mesoderm and ectoderm in the spider are patterned in parallel [21], we demonstrate here that, at least in the leg-bearing segments, the mesoderm becomes segmentally organized first: *twi*-positive cells are arranged into stripes before segmentation becomes evident in the ectoderm. Similarly, in *Artemia,* mesodermal cells are segmentally arranged before ectodermal segmentation becomes obvious [16].

function of *hb* evolved only in the lineage leading to the insects.

hb Does Not Regulate Hox Gene Expression in the Spider

hb also plays a major role in regulating Hox genes in insects [2]. For example in *Drosophila*, Hb represses anterior expression of *Antp* and *Ubx*, which is obvious from particular *hb* mutants that show homeotic transformations superimposed on the deletion phenotype [14, 28–31]. Additionally, in most other insects, a segment deletion phenotype is complemented or sometimes superimposed by homeotic transformations caused by ectopic expression of Hox genes [8–10, 12, 32]. Marquez-Souza and colleagues even suggest that the conserved function of *hb* and other gap genes in insects is restricted to Hox gene regulation [9].

We did not observe any ectopic Hox gene expression in *At-hb*[pRNAi] embryos. *At-hb* thus does not appear to regulate Hox genes in the spider. Consequently, *hb* has either lost its ability to control Hox genes in the spider or else this regulation is an evolutionary innovation of *hb* in the lineage to the insects. To further address this evolutionary problem, functional data from crustaceans and myriapods are needed.

Moreover, we demonstrated that ectodermal and mesodermal segmentation in *Achaearanea* are at least initially two independent processes. *At-hb* pRNAi does not affect the initial segmental arrangement of the *twi*-positive mesodermal cells. However, after their initial independence, the mesoderm appears to disintegrate in the absence of the segmented ectoderm in segments affected by *At-hb* pRNAi. However, it is not yet known which mechanisms initially pattern the mesoderm in spiders and how the initially scattered *twi*-positive cells become arranged into stripes.

Spiders Use Both Vertebrate- and *Drosophila*-like Modes of Segmentation

There is a clear distinction in the regulation of segmentation between the anterior prosoma and the posterior opisthosoma in the spider. Our previous work showed that opisthosomal segments are sequentially generated from a posterior growth zone and that their specification depends on Wnt8 [5] and Notch-Delta [4, 6, 7] signaling. Both pathways are also used by vertebrates to sequentially pattern their somites [33]. The present study demonstrates that in contrast, the prosomal segments are specified almost simultaneously from a pre-existing

field of cells and thus patterned via a mechanism more reminiscent of *Drosophila* segmentation.

The use of distinct upstream genetic pathways for segmentation of the prosoma and the opisthosoma in the spider is most obvious from the RNAi experiments. No segmentation defects were observed in the opisthosomal segments of *At-hb* (this paper) or *At-otd* [20] RNAi embryos, whereas in *At-Wnt8* RNAi embryos all prosomal segments form, but the opisthosoma was missing [5]. The spider thus utilizes a "*Drosophila*"-like approach to pattern its anterior segments and a vertebrate-like way to pattern its posterior segments. The spider data provide the first functional evidence for the hypothesis that short-germ arthropods may employ two separate mechanisms to segment the anterior and the posterior [2–4, 34].

This hypothesis has important implications for our understanding of the evolution of segmentation in arthropods. We propose that the last common ancestor of arthropods utilized different mechanisms for anterior and posterior segmentation. The anterior segments were presumably patterned simultaneously via a mechanism depending on *hb* and *otd* and reminiscent of what is seen in the spider prosoma and in *Drosophila*. The ancestral posterior patterning mechanism likely employed both Wnt8 and Notch-Delta signaling, because involvement of these pathways in posterior development has recently also been shown for other arthropods [2–4, 34]. We postulate that simultaneous specification of all segments as seen in long-germ insects, like *Drosophila,* might be due to an expansion of the anterior specification mechanism to the posterior during the course of evolution [1, 34].

Experimental Procedures

Animal Culture
Embryos and adults of *Achaearanea tepidariorum* were obtained from our culture in Cologne [5].

Gene Cloning
Fragments of *At-hb*, *At-pby*, *At-Antp*, *At-Ubx* were recovered by degenerate PCR with the following primers: *At-hb*: hbfw1 AARCAYCAYYTNGARTAYCA, hbfw2 AAYCAYTTYGGNWSNAARCC, hbbw RTGRCARTAYTTNGTNGCRTA; *At-pby* [35]; *At-Antp* and *At-Ubx* [36]. Additional sequence for these transcripts and for published fragments of *At-Scr*, *At-Hox3*, and *At-AbdA* [19] was obtained by 5′ and/or 3′ RACE PCR with the Marathon RACE kit (Clontech). Sequences of *At-Dfd-1* (FM945396), *At-en* (AB125741), *At-h* (AB125743), *At-hh* (AB125742), *At-lab* (FM945395), *At-otd-1* (AB096074), *At-Pax6* (FM945394), *At-Six3* (FM945393), and *At-twi* (AB167807) were available from GenBank [6, 18, 20, 37].

In Situ Hybridizations
Achaearanea embryos were fixed as described in [18], and whole-mount in situ hybridizations were carried out with minor modifications to [38]. Embryos were sectioned as described in [5].

Parental RNAi
Parental RNAi in *Achaearanea* was performed as described previously [6, 37]. dsRNA was generated from PCR fragments of a 1168 bp 5′ fragment corresponding to nt 1–1168 of the *At-hb* sequence and a 583 bp 3′ fragment corresponding to nt 1192–1775 of the *At-hb* sequence as well as *gfp* with the Ambion T7 Megascript Kit without additional annealing of the RNA. Spiders were injected 5 times every 2–3 days with 3 μg dsRNA per injection.

Movies
Movies of second instar spiders were taken on a Leica MZ16FA with Leica Application Suite version 2.8.1. Movies were assembled with iMovie and Quicktime software.

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