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Report

Wnt8 Is Required for Growth-Zone Establishment and Development of Opisthosomal Segments in a Spider

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Summary

The *Wnt* genes encode secreted glycoprotein ligands that regulate many developmental processes from axis formation to tissue regeneration [1]. In bilaterians, there are at least 12 subfamilies of Wnt genes [2]. *Wnt3* and *Wnt8* are required for somitogenesis in vertebrates [3–7] and are thought to be involved in posterior specification in deuterostomes in general [8]. Although TCF and β-catenin have been implicated in the posterior patterning of some short-germ insects [9, 10], the specific Wnt ligands required for posterior specification in insects and other protostomes remained unknown. Here we investigated the function of *Wnt8* in a chelicerate, the common house spider *Achaearanea tepidariorum* [11]. Knockdown of *Wnt8* in *Achaearanea* via parental RNAi caused misregulation of *Delta, hairy, twist*, and *caudal* and resulted in failure to properly establish a posterior growth zone and truncation of the opisthosoma (abdomen). In embryos with the most severe phenotypes, the entire opisthosoma was missing. Our results suggest that in the spider, *Wnt8* is required for posterior development through the specification and maintenance of growth-zone cells. Furthermore, we propose that *Wnt8*, *caudal*, and *Delta/Notch* may be parts of an ancient genetic regulatory network that could have been required for posterior specification in the last common ancestor of protostomes and deuterostomes.

Results

Isolation and Expression of Spider *Wnt8*

To investigate the roles of *Wnt3* and *Wnt8* in chelicerates, we attempted to isolate the orthologs of these genes from *Achaearanea*. We cloned a single *Wnt8* ortholog (*At-Wnt8*) from this spider, which was confirmed by phylogenetic analysis of the full-length coding sequence (Figure S1 available online). However, despite repeated attempts, we were unable to find a *Wnt3* ortholog in *Achaearanea*. Although *Wnt3* orthologs have been found in a cnidarian and in some deuterostomes [2, 12], it is likely that this gene was lost in the lineage leading to the protostomes because no ortholog has been reported in these animals, even those with fully sequenced genomes [2, 13]. Therefore, although formally possible, we regard the presence of a *Wnt3* ortholog in *Achaearanea* as unlikely.

At-Wnt8 is first expressed in an anterior domain and at the presumptive posterior of the embryo (the center of the germ disc) just before formation of the growth zone (Figure 1A). *At-Wnt8* is then continuously expressed at the posterior end of the germband, in the ectoderm of the growth zone (Figures 1B and 1C), which is consistent with a role for *At-Wnt8* in posterior development. Expression is also observed in the brain lobes and ventral regions of mature segments (Figure 1B).

Parental RNAi Knockdown of *At-Wnt8*

To determine the function of *Wnt8* in the spider, we then performed parental RNAi in *Achaearanea* [14, 15]. *At-Wnt8*^{pRNAi} embryos displayed a range of posterior phenotypes affecting leg-bearing segments 3 and 4 (L3 and L4) and the opisthosoma. We divided these phenotypes into three classes (Figure 2 and Figure S2).

In the mildest phenotypic class (class I), we observed a slight increase in the distance between the L3 and L4 limb buds along the anterior-posterior (A-P) axis, and although the opisthosoma was narrower compared to control embryos of the same stage, segments were still evident (Figures 2A, 2B, 2E, and 2F and Figures S3A and S3C). In class II phenotypes, although elongation of the germ band was still observed, as evidenced by several segment-like structures posterior to L4, these structures were often more variable in size and fewer in number compared to wild-type opisthosomal segments (Figures 2C, 2G, and 2J). The L3 and L4 limb buds were also further apart than normal with respect to the A-P axis in class II phenotypes (Figures 2C, 2G, and 2J). In addition, the posterior part of L4 was frequently found to be narrower along the dorsoventral (D-V) axis and sometimes had completely fused limb buds (Figures 2C, 2G, and 2J). In both class I and class II phenotypes, we also observed embryos that appeared to have two or more germbands in the opisthosomal region (Figures S3H and S3J). These germbands were always narrower than normal and were often composed of varying numbers of irregular and disorganized segment-like structures (Figure S3H). In the strongest phenotypic class (class III), all opisthosomal segments were usually missing and the L3 and L4 limb buds were again further apart than normal with respect to the A-P axis (Figures 2D and 2H and Figure S3E). The L4 limb buds were also sometimes completely fused along the ventral midline in class III phenotypes (Figure S3E). In sections of germband stage *At-Wnt8*^{pRNAi} embryos, we also observed ectopic clusters of cells in a disordered pattern beneath the ectodermal cells at the posterior of the germband (Figures S3B and S3D); older embryos taken from the same cocoons (egg sacs) developed class II and class III phenotypes.

The phenotypes resulting from knockdown of *At-Wnt8* show that this gene is required for the correct development of L3/L4 and for the normal generation of the opisthosomal segments from the growth zone. The absence of *At-Wnt8* causes A-P

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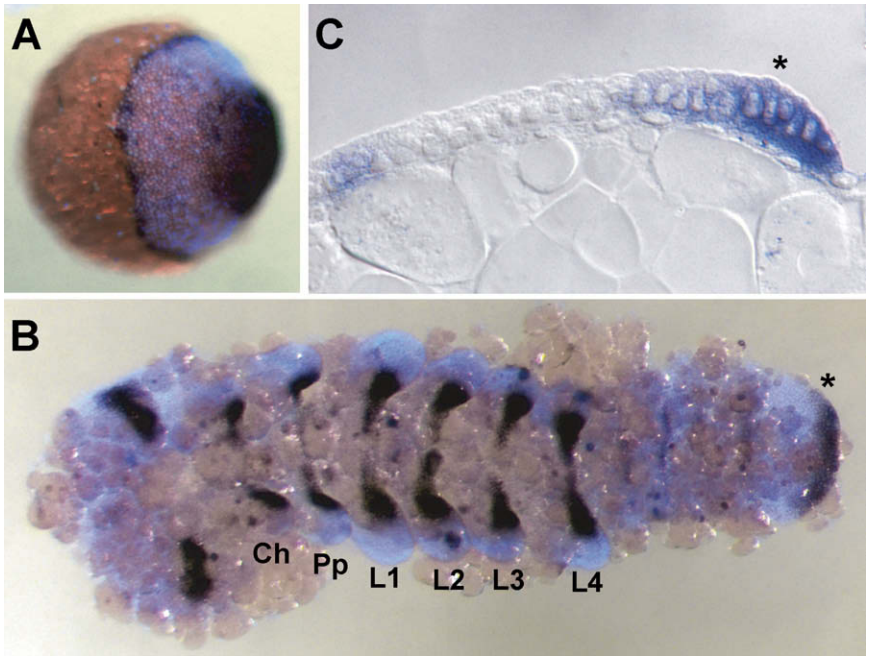

Figure 1. Expression of *Wnt8* in *Achaearanea* Embryos

(A) In situ hybridizations of *At-Wnt8* expression at stage 5. At this stage, the embryo is radially symmetrical with the future anterior to the left expressing a narrow ring of *At-Wnt8* and the posterior to the right expressing *At-Wnt8* in a solid circular domain. During stages 6 and 7, the transition is made from radial to axial symmetry.

(B) Ventral view of *At-Wnt8* expression at stage 9 in a flat-mounted embryo.

(C) Midsagittal section of a stage 8 embryo.

The growth zone is marked by an asterisk in (B) and (C). Embryos in (A) and (B) are counterstained with DAPI. The cheliceral (Ch), pedipalpal (Pp), and the four leg-bearing segments (L1–L4) are indicated in (B). All embryos are orientated with the anterior to the left.

enlargement of L3/L4 and ectopic internalization of posterior cells, and it appears that the opisthosoma is completely missing or truncated and sometimes fragmented into multiple smaller, uncoordinated, germbands.

Posterior Expression of Developmental Genes Is Disrupted in *At-Wnt8*^{pRNAi} Embryos

We next investigated whether the *At-Wnt8*^{pRNAi} phenotypes could be explained by differences in cell division or cell death, but we found no obvious differences in the activity of these processes between *At-Wnt8*^{pRNAi} and control embryos of stages 5 to 8 (not shown). It is possible that differences in the size of the growth zone and opisthosoma are caused by fewer cells committing to a growth-zone fate. To understand the function of *At-Wnt8* in more detail, we then investigated the effect of *At-Wnt8*^{pRNAi} on the expression of other genes involved in spider development.

In *Achaearanea*, *At-Krüppel-2* (*At-Kr2*) is expressed in a stripe marking the presumptive posterior and anterior regions of L3 and L4, respectively (Figure S3F). In *At-Wnt8*^{pRNAi} embryos, this expression domain of *At-Kr2* is greatly expanded, confirming our observation that L3/L4 is larger in these embryos (Figures S3F and S3G).

Expression of *Delta* (*Dl*) arises in the posterior of *Achaearanea* embryos during stage 4 and expands during stage 5 (Figure 3A). *Dl* expression then clears from the center of the germ disc at stage 6 (Figure 3C). In *At-Wnt8*^{pRNAi} embryos, *Dl* expression arises and expands as normal in the center of the germ disc (Figure 3B). However, *Dl* expression then persists in the center of the opening germ disc, leading to an

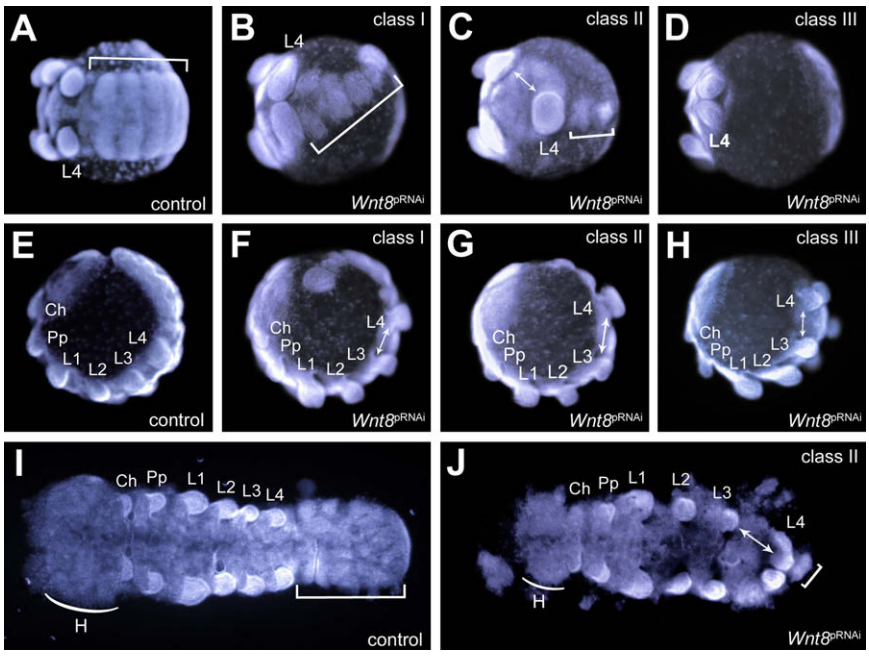

Figure 2. Embryonic Phenotypes Resulting from Parental RNAi against *At-Wnt8*

DAPI-stained stage 9 control (A, E, and I) and *At-Wnt8*^{pRNAi} (B–D, F–H, and J) *Achaearanea* embryos. Control embryos have clearly segmented opisthosomal segments and the posterior growth zone is close to the anterior edge of the head (A, E, and I). In class I *At-Wnt8*^{pRNAi} embryos, the opisthosomal segments are narrower than in control embryos (B and F). Class II *At-Wnt8*^{pRNAi} embryos show an extreme reduction of the opisthosomal segments and the appendages of L4 are also close together or fused (C, G, and J). In class III *At-Wnt8*^{pRNAi} embryos, all opisthosomal tissue is missing and the limbs of L4 are close together or fused (D and H). A larger area between the L3 and L4 limb buds was found in embryos of all three phenotypic classes (indicated by the double-headed arrows). In some *At-Wnt8*^{pRNAi} embryos, we also observed a reduction in the size of the head lobes (indicated by curved lines in [I] and [J], which is presumably related to effects on anterior *At-Wnt8* expression; however, we did not investigate this further.

(A–D) Whole-mount ventral posterior view.

(E–H) Whole-mount lateral view.

(I and J) Flat-mounted ventral view.

Square brackets indicate opisthosomal segments. Segments are labeled as described for Figure 1B.

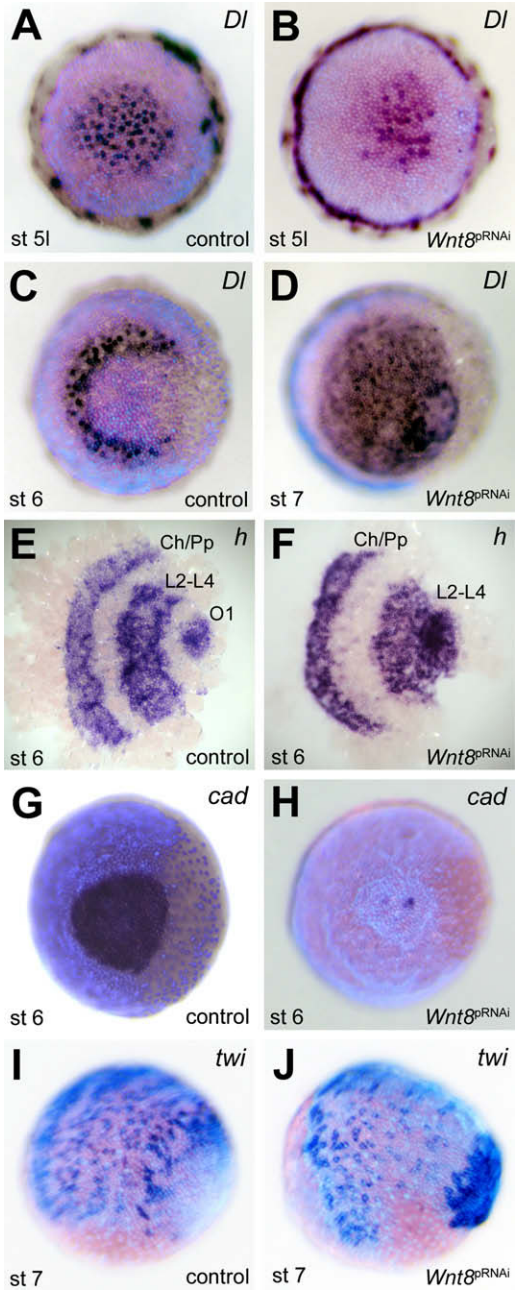

Figure 3. Gene-Expression Patterns in Control and *At-Wnt8*^{pRNAi} Embryos

Posterior *Dl* expression arises normally in *At-Wnt8*^{pRNAi} embryos (A and B) but fails to clear from the growth zone (C and D). Similarly, *h* does not clear (E and F). The *h* expression in the first opisthosomal segment (O1) appears de novo in the cleared posterior (E). *cad* expression is observed in fewer cells in *At-Wnt8*^{pRNAi} embryos (G and H). *twi* is ectopically expressed in the posterior of *At-Wnt8*^{pRNAi} embryos (I and J). Note that the transition from radial to axial symmetry is initiated at stage 6, as the embryo opens at the dorsal side and cells move toward the anterior (see [11] for a detailed description).

(A–D, G, and H) Posterior views of whole-mount embryos.

(C, D, G, and H) Embryos are orientated with the dorsal to the right.

(E and F) Ventral views of flattened embryos.

(I and J) Lateral views with anterior to the left and dorsal down.

enlarged domain of *Dl* expression throughout the posterior of stage 6, 7, and 8 embryos (Figure 3D). In stage 6 embryos taken from cocoons containing embryos that develop many class I and II phenotypes at stage 9, we observed some clearance of *Dl* from the posterior but less than in wild-type embryos (not shown). Similarly, expression of *hairy* (*h*), which is thought to be regulated by Delta/Notch in the spider [16], also persists in the posterior of *At-Wnt8*^{pRNAi} embryos (Figures 3E and 3F). Therefore, the lack of clearing of *Dl* and *h* seems to be associated with the expansion of L3/L4 and smaller growth zone in *At-Wnt8*^{pRNAi} embryos.

It has been proposed that the loss of posterior segments when *Notch* is knocked down in *Achaearanea* is caused by the overproduction of *twist* (*twi*)-expressing mesodermal cells at the expense of *caudal* (*cad*)-expressing ectodermal cells [14]. In *At-Wnt8*^{pRNAi} embryos, we also observed extensive ectopic *twi* expression in the posterior (Figures 3I and 3J). In *Achaearanea, cad* is expressed only after *Dl* expression has cleared from the posterior cells (Figure 3G). In *At-Wnt8*^{pRNAi} embryos, taken from the same cocoon as those with little or no clearing of *Dl* from the posterior and ectopic posterior *twi* expression, we observed only a few *cad*-expressing cells in single or multiple clusters (Figure 3H and Figures S3I and S3J) or no detectable *cad* expression. The clusters of cells expressing *cad* that have presumably assumed a growth-zone fate and older embryos with multiple unsynchronized germbands (Figures S3H–S3J) might be the result of differential RNAi knockdown of *At-Wnt8* in posterior cells. This may imply that a threshold of *At-Wnt8* activity could be required to specify growth-zone cells.

Discussion

Wnt8 Is Required for Posterior Specification in Arthropods and Vertebrates

The posterior truncation phenotypes resulting from pRNAi against *Wnt8* in the spider are at least superficially similar to those observed when *Wnt8* and/or *Wnt3* are perturbed in vertebrate embryos. Removal or blocking *Wnt8* and/or *Wnt3* in *Xenopus*, zebrafish, and mouse results in truncated embryos with only a few anterior somites and no tail bud [3–7]. Although analysis of TCF and β-catenin in *Oncopeltus* and *Gryllus*, respectively, indicated that Wnt signaling might be involved in the development of the growth zone and posterior segments in arthropods [9, 10], our data show that in fact the same ligand, Wnt8, is employed in posterior development in both vertebrates and arthropods.

In class II and III *At-Wnt8*^{pRNAi} embryos exhibiting fused L4 limb buds, it also appeared that the most ventral part of this segment is missing (Figures 2C, 2D, and 2J; Figures S3E and S4). This phenotype shows similarities to the phenotype when *short-gastrulation* is knocked down in this spider [15]. It suggests that, in addition to A-P patterning, *At-Wnt8* is involved in D-V patterning in the spider, a role *Wnt8* genes also perform in vertebrates [3, 17, 18].

Wnt8 May Establish and Maintain Growth-Zone Cells in Spider Embryos

There is evidence that Wnt signaling acts upstream of Delta/Notch in vertebrate somitogenesis [19–21]. Although the expression of *Wnt3a* and *Wnt8* is not cyclical during somitogenesis in vertebrates, some downstream components of Wnt signaling, such as *Axin2,* are cyclically expressed in mice [20–22] and possibly are integral to the Delta/Notch-dependent segmentation clock [20]. However, recent experiments have shown that *Axin2* and components of the Delta/Notch pathway continue to oscillate in the presence of stabilized

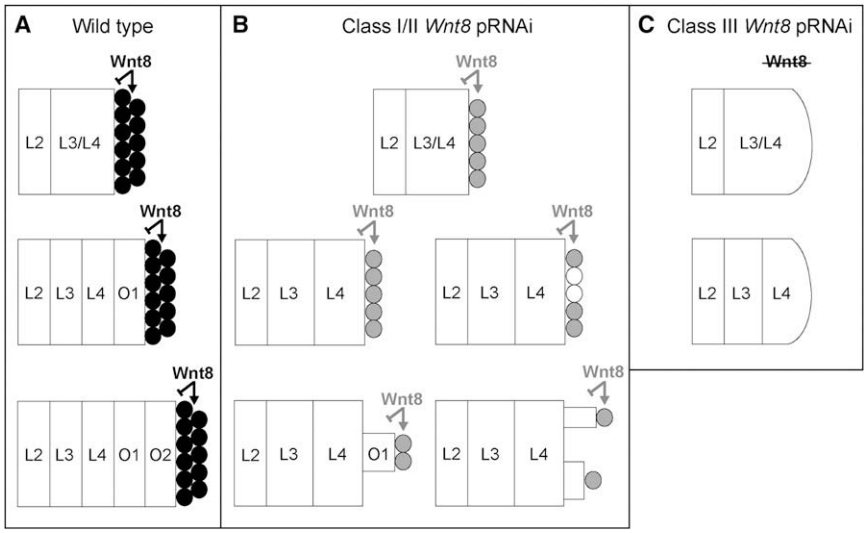

Figure 4. Proposed Model of the Role of *Wnt8* in the Growth Zone of *Achaearanea* Embryos

(A) In wild-type embryos, the establishment and maintenance of growth-zone cells (circles) depends on Wnt8 activity (represented by black filling), possibly through preservation of these cells in an undifferentiated state (arrow) and/or repressing factors that promote segmentation (blunt arrow).

(B) Reduced Wnt8 activity (represented by gray or white-filled circles) in class I and II *At-Wnt8*^{pRNAi} embryos results in a depletion of growth-zone cells in favor of L3/L4 and internalization. This manifests as narrower segments and posteriorly truncated embryos or isolated clusters of cells that generate independent irregular opisthosomal germbands.

(C) When Wnt8 activity is low or absent, as is presumed to be the case in class III *At-Wnt8*^{pRNAi} embryos, the growth zone is not established because all posterior cells become part of L3/L4, or are internalized, resulting in embryos with no opisthosoma.

Prosomal regions and differentiated segments are represented by rectangles. Internalization of cells and reduction of L4 along the D-V axis are not illustrated. L2, L3, L4, O1, and O2 are leg-bearing segments 2, 3, and 4 and opisthosomal segments 1 and 2, respectively.

β-catenin, which suggests that in mice, Wnt signaling may be permissive for the segmentation clock rather than instructive [23, 24]. Similarly, in zebrafish it is thought that *Wnt8* may act to maintain a precursor population of stem cells in the PSM and tailbud rather than directly regulate the segmentation clock [5]. We propose that the same ligand, Wnt8, could play a similar permissive role for segmentation in the growth zone of the spider by establishing and possibly maintaining a pool of cells that develop into the opisthosomal segments. When *At-Wnt8* activity is reduced, cells are ectopically used in L3/L4 or internalized, depleting the putative growth-zone pool (Figures 2 and 4 and Figure S3). This depletion manifests as a smaller opisthosoma, separated clusters of cells that give rise to separate irregular germbands, or even no opisthosoma (Figure 4).

Wnt8 May Be Part of an Ancient Regulatory Network

It was previously shown that Delta/Notch signaling is also involved in posterior development in the spiders *Cupiennius* [16, 25] and *Achaearanea* [14]. Our new results reveal that in the spider, *Wnt8* is required for the clearing of *Dl* and *h* expression in the posterior and that this is necessary for repression of *twi*, activation of *cad*, and establishment of the growth zone.

The involvement of *Wnt8*, Delta/Notch signaling, and *cad* in the posterior development of other arthropods has also been directly demonstrated by functional analysis or inferred from expression patterns [13, 26–28], and in vertebrates, *Wnt3a* and *Wnt8* probably act upstream of Delta/Notch and *cad* during somitogenesis [4, 19–21]. Taken together, this suggests that a regulatory genetic network for posterior specification including *Wnt8*, Delta/Notch signaling, and *cad* could have been present in the last common ancestor of protostomes and deuterostomes, but has subsequently been modified in some lineages. For example, in *Drosophila*, Delta/Notch signaling is not involved in segmentation [29], and although the *Drosophila Wnt8* ortholog, *WntD*, is required for D-V patterning, it is not involved in posterior development [30]. Segments arise almost simultaneously in *Drosophila*, rather than sequentially from a growth zone, so this may suggest that the role of *Wnt8* in posterior development was not required for this mode of development and therefore was lost during the evolution of these insects.

Conclusions

Our results suggest that *Wnt8* regulates formation of the posterior growth zone and then maintains a pool of undifferentiated cells in this tissue required for development of the opisthosoma. Wnt signaling thus regulates the establishment and maintenance of an undifferentiated pool of posterior cells in both vertebrates and spiders and in fact the same Wnt ligand, Wnt8, is used in both phyla. Therefore, *Wnt8* could be part of an ancient genetic regulatory network, also including *Dl*, *Notch, h*, and *cad,* that was used for posterior specification in the last common ancestor of deuterostomes and protostomes.

Experimental Procedures

Achaearanea adults and embryos were obtained from our laboratory culture at the University of Cologne. *At-Wnt8* and *At-Kr2* were cloned with degenerate PCR from embryonic cDNA via the following primers: wnt8F1 TGGGAYMGNTGGAAYTGYCC, wnt8F2 TGGGGNGGNTGYWSNGA, wnt8R1 NAYNCCRTGRCAYTTRCA, wnt8R2 RTCNSWRCANCCNCCCCA, Kr2 KrF1 GGNTAYAARCAYGTNYTNCA, KrF2 CARAAYCAYGARMG NACNCA, and KrR GCYTTNARYTGRTTNSWRTC. The sequences of the full-length *At-Wnt8* coding region and partial *At-Kr-2* transcript were obtained with RACE PCR (Clontech). Embryos were staged according to Akiyama-Oda and Oda [31]. Embryos were fixed and in situ hybridizations performed with DIG (Roche)-labeled probes as previously described with minor modifications [31, 32]. 6 μm cross-sections were made from embryos from whole-mount in situ hybridization experiments mounted in durcupan (Sigma). Cell division and cell death were assayed in control and *At-Wnt8* RNAi embryos with phosphohistone H3 and Caspase 3 antibodies, respectively [33]. Parental RNAi in *Achaearanea* was carried out as described previously [14, 15] by injecting dsRNA synthesized from a single 800 bp fragment of the *At-Wnt8* coding region or two nonoverlapping fragments of 393 and 323 bp, respectively, into adult female spiders. Control spiders were injected with dsGFP (Figure S2).

Accession Numbers

The sequences of the full-length *At-Wnt8* coding region and partial *At-Kr-2* transcript were deposited in GenBank with accession numbers FJ013049 and FJ013048, respectively.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/20/1619/DC1/.

Acknowledgments

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