Fig. S1. Maximum likelihood tree topology of *Sp6-9* orthologs (ln*L* = -5444.20). Numbers adjacent to nodes represent bootstrap resampling frequencies. Numbers below nodes represent posterior probabilities from Bayesian inference searches. Terminals in boldface represent newly discovered new *Sp6-9* orthologs reported in this study. Left inset: Tree topology based on all *Sp* homologs, including *Krüppel-like factor* (KLF) outgroups (ln*L* = -6504.88). Scale bars: 0.5.
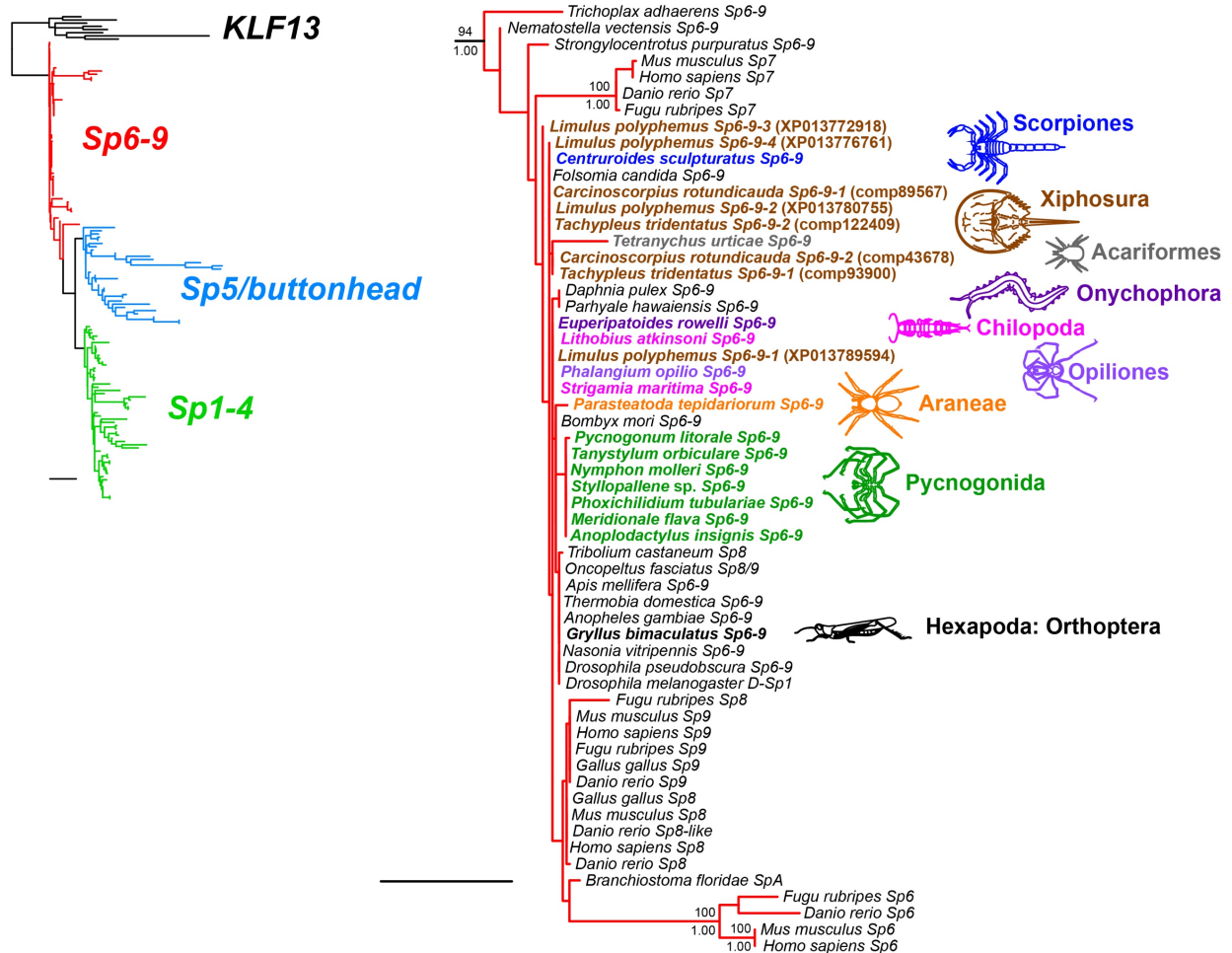
Fig. S2. Maximum likelihood tree topology of *arr/LRP* orthologs (ln*L* = -22191.04). Numbers adjacent to nodes represent bootstrap resampling frequencies.
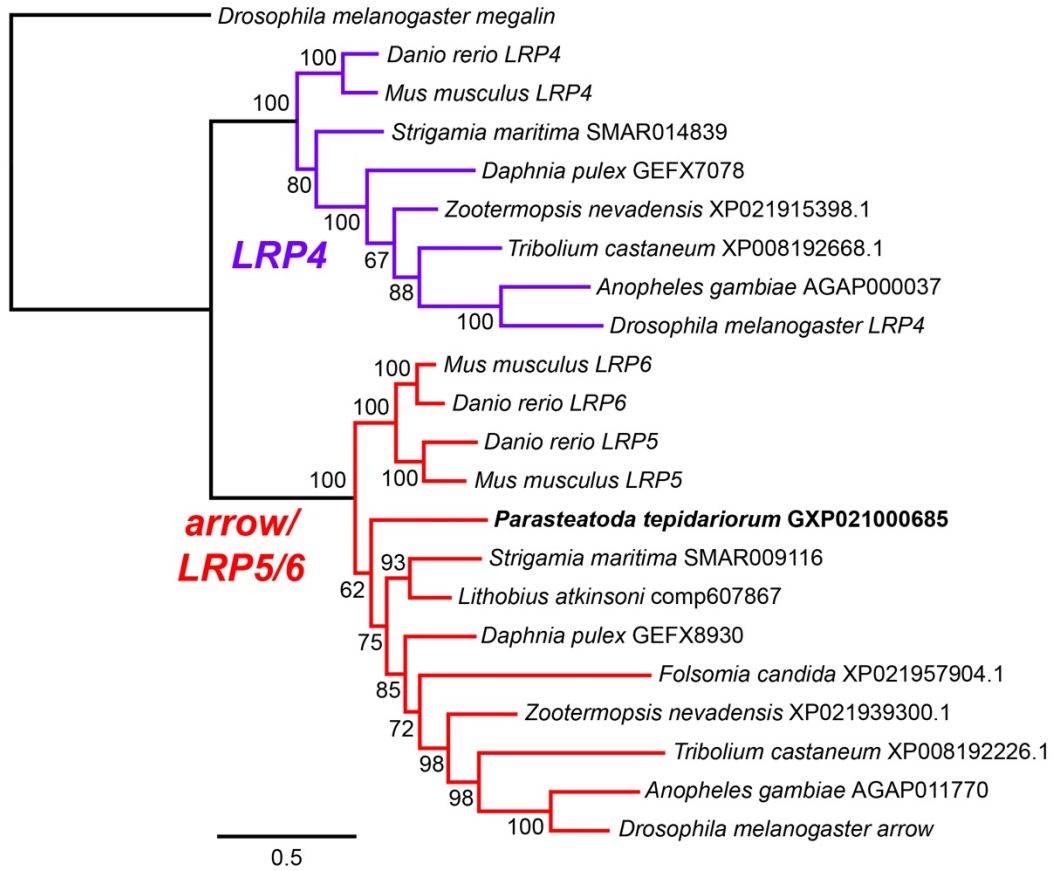
Fig. S3. Expression of *Ptep-Sp6-9* in the spider. (*A*) In early limb bud stages, *Ptep-Sp6-9* is expressed in all prosomal limb buds, the head lobe, and as faint stripes in the opisthosoma (arrowheads). (*B*) In later stages, *Ptep-Sp6-9* expression in the head lobe becomes more prominent (arrowhead). In all but the cheliceral limb buds, expression is heterogeneous, consisting of a broad proximal ring, and a stronger distal expression domain at the terminus of the appendage. Abbreviations as in Fig. 2. All scale bars 100 μm.
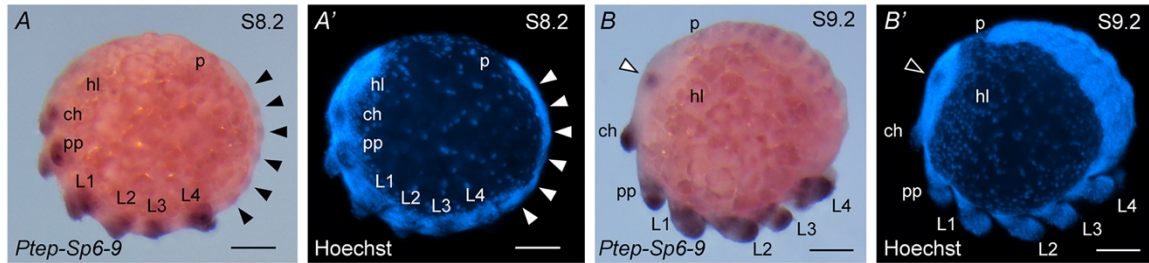
Fig. S4. Expression of *Ptep-arr* and sense probe controls. (*A*) As with insects, *Ptep-arr* is ubiquitously expressed throughout the germband. (*B*) No staining was observed in the *Ptep-arr* sense control, which was run for the same amount of time as the antisense probes. Similarly, no staining was observed in the *Sp6-9* sense probe controls of the spider (*C*), the harvestman (*D*), or the centipede (*E*); asterisk indicates non-specific staining in the harvestman, resulting from attached remnant of vitelline membrane. All scale bars 100 μm.
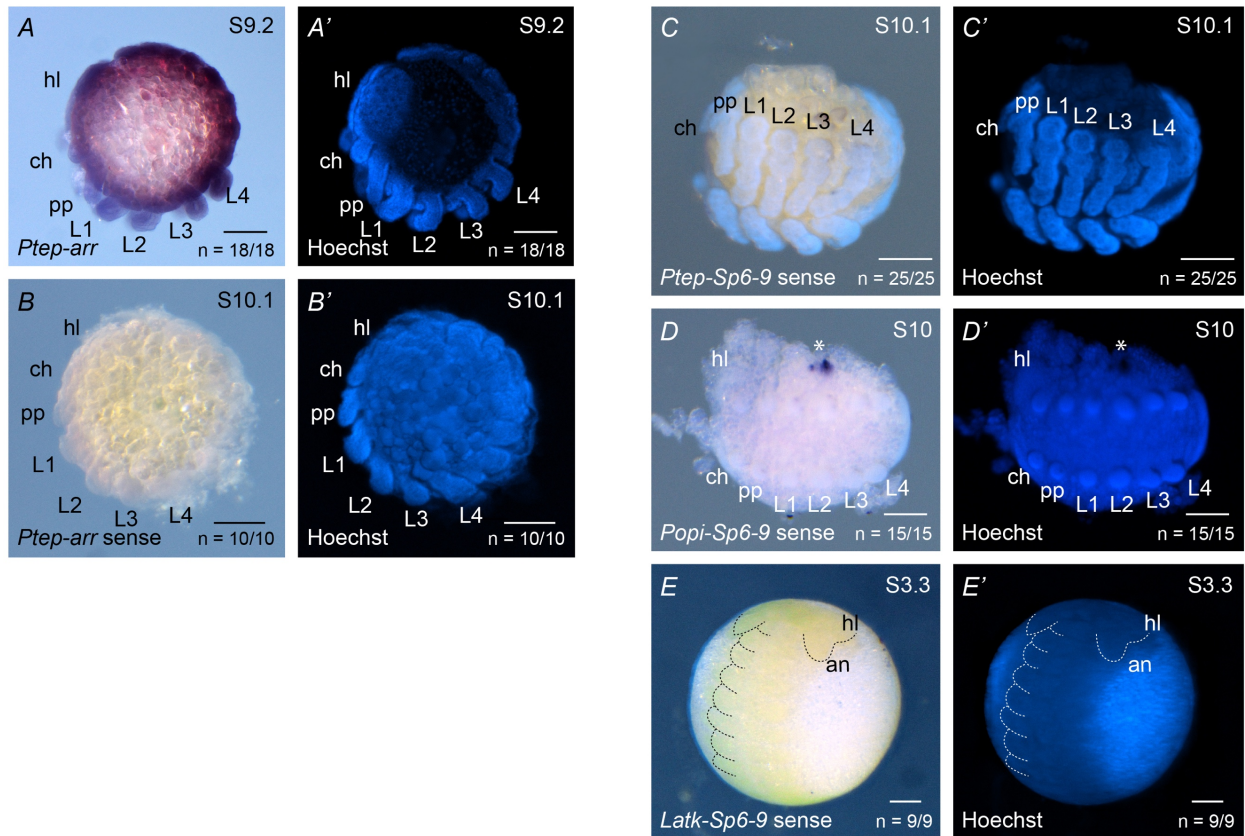
Fig. S5. Experimental design of maternal RNAi in the spider and phenotypic distribution. (*A*) *Ptep-Sp6-9* cDNA transcript indicating relative locations of two non-overlapping fragments targeted for dsRNA synthesis. (*B-E*) Phenotypic distribution of embryos from second, third, and fourth cocoons. Numbers above bars indicate sample sizes. Parenthetical integers within bars indicate number of adult females surviving injections. Colors correspond to legend at the top right. (*B*) Negative control females. (*C*) Females injected with the 5' *Ptep-Sp6-9* dsRNA fragment. (*D*) Females injected with the 3' *Ptep-Sp6-9* dsRNA fragment. (*E*) Combined distribution of (*C*) and (*D*).
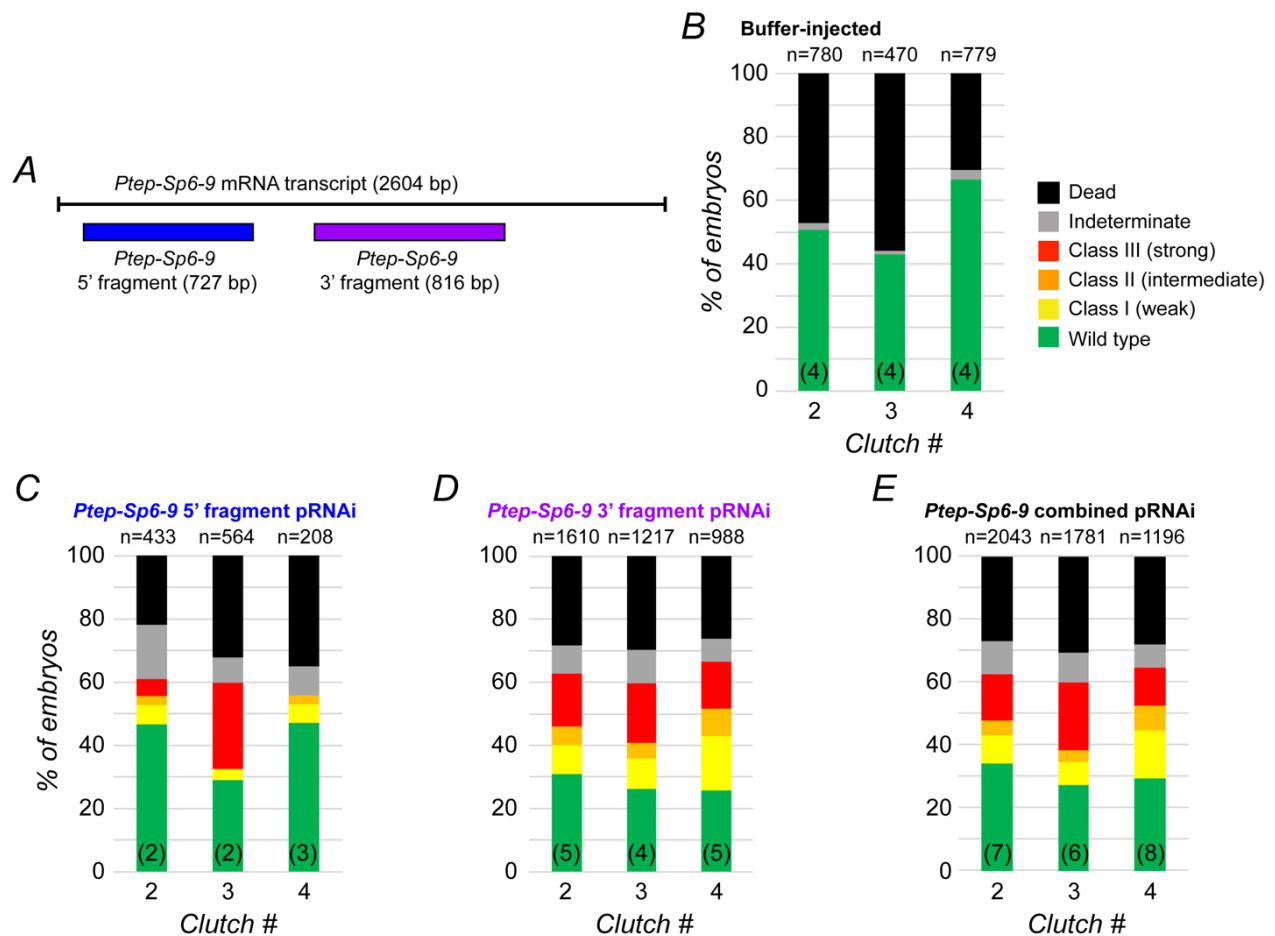
Fig. S6. Expression of *Ptep-Sp6-9* is reduced in *Ptep-Sp6-9* RNAi embryos. (*A*) *Ptep-Sp6-9* expression in a wild type embryo. (*B*) Reduction of *Ptep-Sp6-9* expression in a *Ptep-Sp6-9* RNAi embryo is correlated with appendage truncation. Abbreviations as in Fig. 2. All scale bars 100 μm.
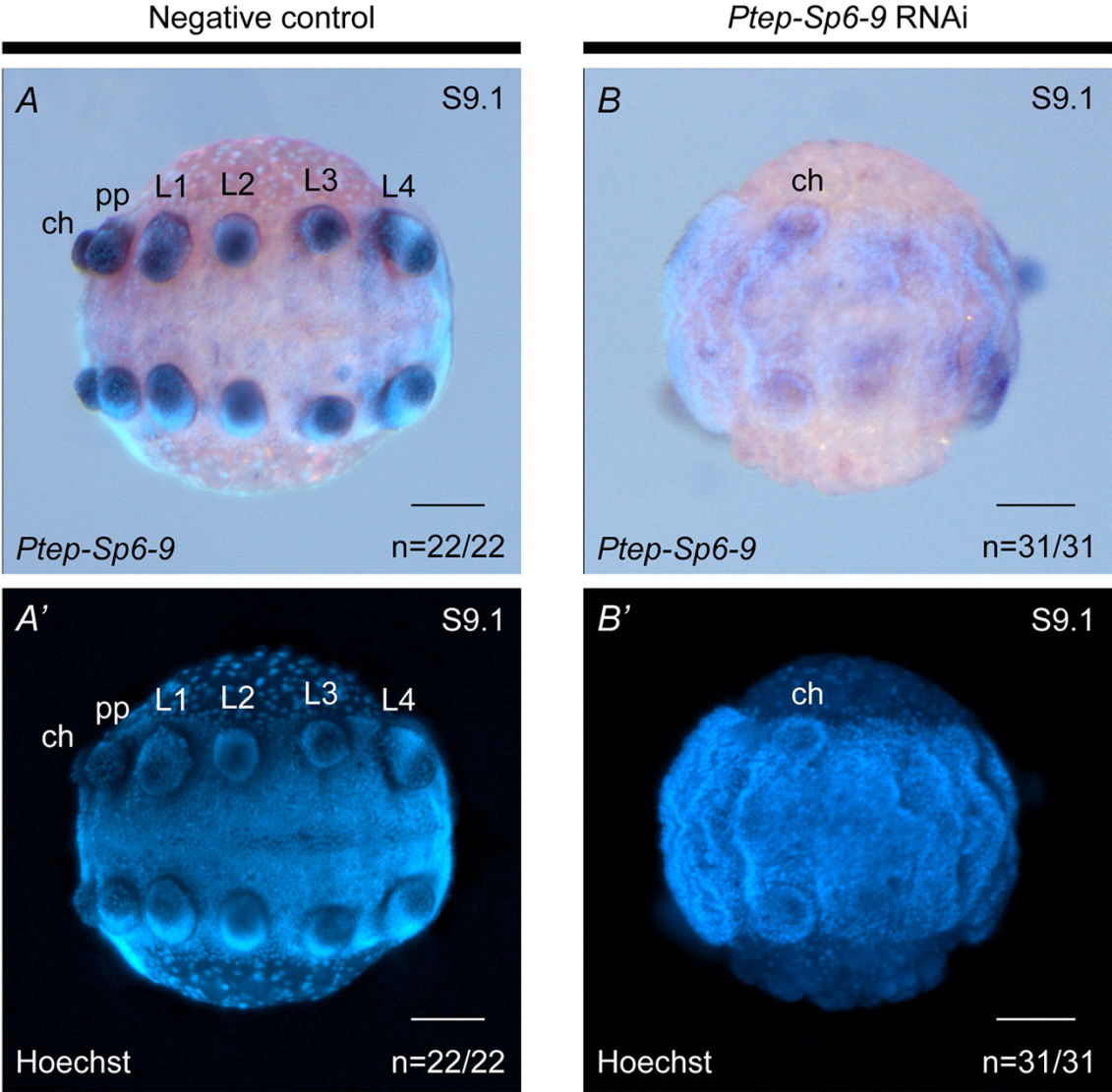
Fig. S7. Intermediate phenocopies of *Ptep-Sp6-9* RNAi reveal loss of segmentation gene expression in L1 and L2. (*A, B*) Expression of the segment polarity gene *Ptep-en-1* in negative control embryos. (*C, D*) In intermediate *Ptep-Sp6-9*-RNAi phenocopies, loss of *Ptep-en* expression is observed in the L1 and L2 segments (white arrowheads in *D*). Segments anterior to L1 and posterior to L2 are not affected. Abbreviations as in Fig. 2. All scale bars 100 μm.
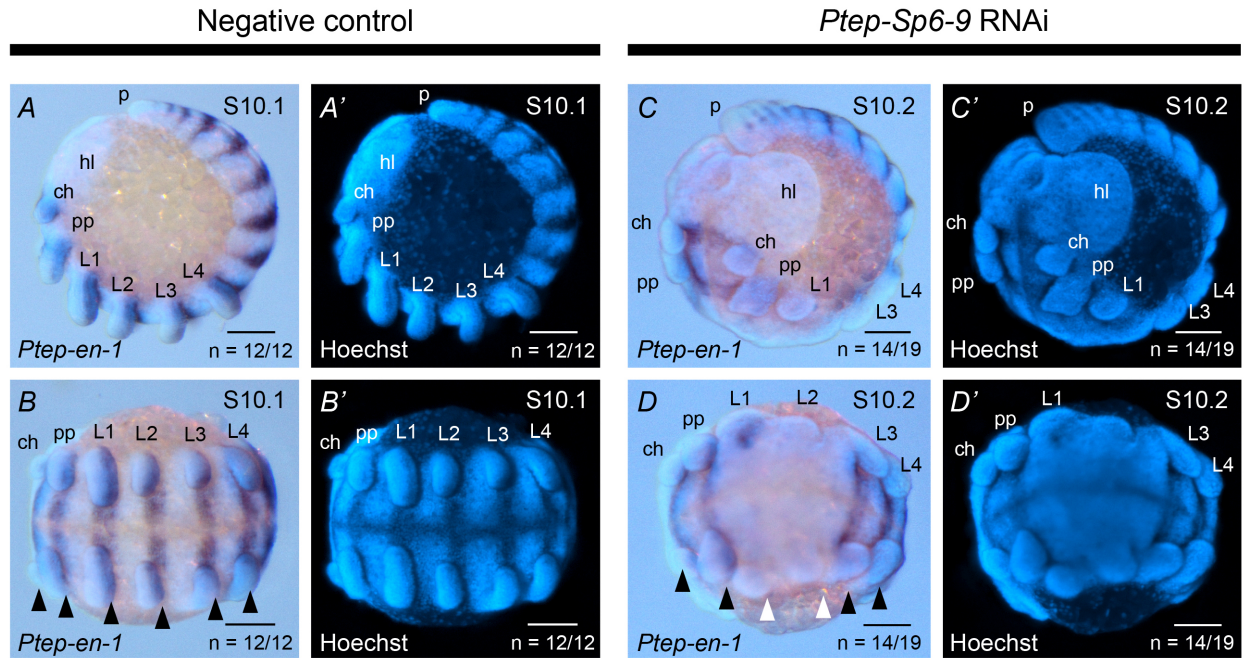
Fig. S8. In the weakest subset of *Ptep-Sp6-9* RNAi phenocopies, shortened appendages are observed. *(A)* In a wild type embryo at stage 14, the walking legs are interlaced, obscuring the ventral surface of the prosoma. *(B)* A *Ptep-Sp6-9* RNAi embryo showing walking legs of reduced length; the operculum of the prosoma is clearly visible. Note that all four walking legs are present on the animal's right side, indicating no segmentation defect, whereas fusion of adjacent appendages is observed on the left side. Arrowheads indicate the coxapophysis of the pedipalp. All scale bars 100 μm.

Negative control

Ptep-Sp6-9 RNAi
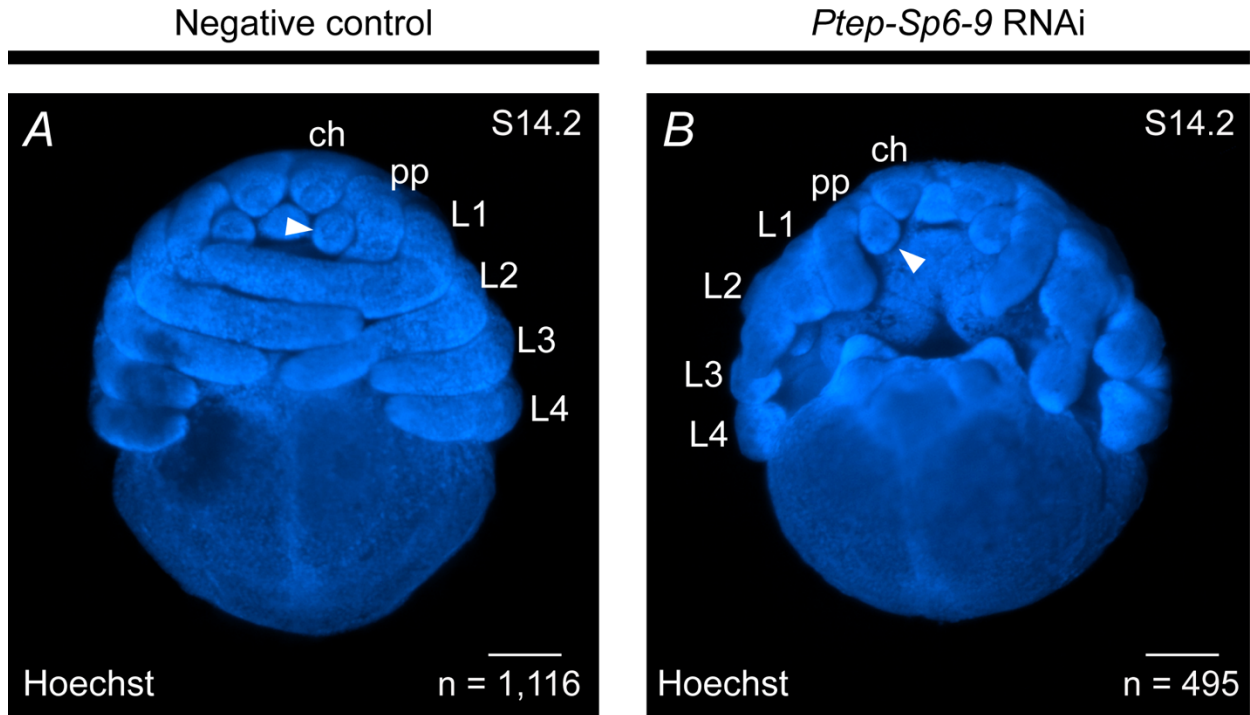
Fig. S9. Experimental design of maternal RNAi in the spider and phenotypic distribution. (*A*) *Ptep-arr* cDNA transcript indicating relative locations of fragment targeted for dsRNA synthesis. (*B*) Phenotypic distribution of embryos from second through fifth cocoons. Numbers above bars indicate sample sizes. Parenthetical integers within bars indicate number of adult females surviving injections. Colors correspond to legend at the right. For simplicity, all controls are not illustrated (data available in Table S3).

A

Ptep-arr mRNA transcript (1283 bp)

Ptep-arr fragment 1 (1,169 bp)

Ptep-arr 5' fragment (380 bp)

Ptep-arr 3' fragment (578 bp)

B

Ptep-arr pRNAi combined

n=563   n=266   n=194   n=86

% of embryos

(5)   (3)   (2)   (2)

Clutch #

2   3   4   5

Dead

Indeterminate

Segmentation phenotype

Wild type

Table S1. List of gene-specific primers used for PCR amplification and cloning.

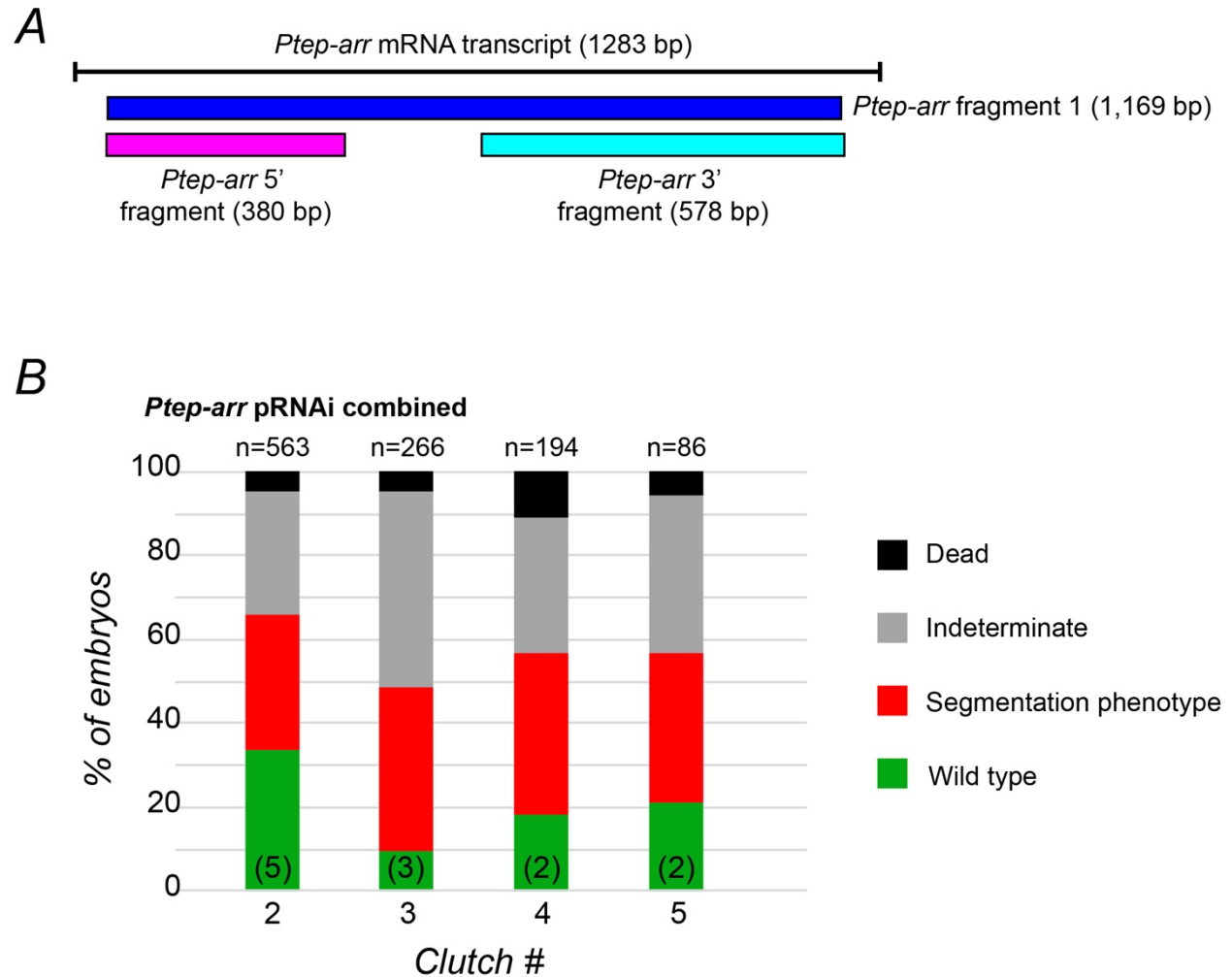| Locus/primer	Primer Sequence	Length (bp)
Ptep-Sp6-9		
Ptep_Sp6_9_5'_for	5' – TGGATCGTTTGCACGAATAA – 3'	727
Ptep_Sp6_9_5'_rev	5' – CTCAAGGTCCACTCATCGGT – 3'	
Ptep_Sp6_9_3'_for	5' – ACGCAGCCTTCATTCTCAGT – 3'	816
Ptep_Sp6_9_3'_rev	5' – CATTGCAGCAAACAAGAGGA – 3'	
Ptep-arr		
Ptep_arr_for	5' – ggccgcggTCCAGCATCTCCCCAACTGA – 3'	1169
Ptep_arr_rev	5' – cccggggcACAGACATGGGGCTAAACCG – 3'	
Ptep_arr_5'_for	5' – AATCCAGCATCTCCCCAACT – 3'	380
Ptep_arr_5'_for	5' – TTCGTTTATTGGATTGATGGAA – 3'	
Ptep_arr_3'_for	5' – GTGTGGGACAACCACAATGA – 3'	578
Ptep_arr_3'_for	5' – ATTGGACAGACATGGGGCTA – 3'	
Ptep-en-1		
Ptep_en_for	5' – CTGCTTGACATTGCCTGAAA – 3'	792
Ptep_en_rev	5' – GAATCTGCTGGCATTCCATT – 3'	
Ptep-wg		
Ptep_wg_for	5' – TTTCCGAATGCAAACATCAG – 3'	925
Ptep_wg_for	5' – ATCATGTGTGGGGTTCGACT – 3'	
Ptep-Dll		
Ptep_Dll_for	5' – ATGCCCAGGCTTACCCTATT – 3'	819
Ptep_Dll_rev	5' – TGTCCCATGAGGAGATAGGC – 3'	
Ptep-Antp-1		
Ptep_Dll_for	5' – CGACAGACTGGAAATACGACCT – 3'	694
Ptep_Dll_rev	5' – GCCTGCTTCCATCTTTGACT – 3'	
Ptep-dac-1		
Ptep_dac1_for	5' – AACCAATCCCAGCATGCTTC – 3'	724
Ptep_dac1_rev	5' – GTCGTCGTCACAGTCAACAC – 3'	
Ptep-otd-1		
Ptep_otd1_for	5' – TACACCCCCAATCCACACTT – 3'	782
Ptep_otd1_rev	5' – GGCTTGGAATCGTCCACTAA – 3'	
Ptep-lab-1		
Ptep_lab1_for	5' – CGATTCATTATAAGAGTGAGAGGC – 3'	746

Ptep_lab1_rev	5' – GCACTGTCTTAAGCAGTTACATTC – 3'	
Popi-Sp6-9		
Popi_Sp6_9_T7_for	5' – ggccgcggCCCTTCATTCGCAAGTCAAT – 3'	775
Popi_Sp6_9_ T7_rev	5' – cccggggcTCCAGGTGGTCCAGGTACTC – 3'	
Latk-Sp6-9		
Latk_Sp6_9_for	5' – CTTCTTACTCCCGTTCCCGT – 3'	708
Latk_Sp6_9_rev	5' – ACCGTCAACTCCTTGCCTAA – 3'	
Cscu-Dll		
Cscu_Dll_for	5' – TCCCATTCGGTCGACTTATC – 3'	696
Cscu_Dll_rev	5' – GCAGGATTCATGTCCCAAGA – 3'	
Cscu-Sp6-9		
Cscu_Sp6_9_for	5' – ggccgcggGCTCTTGCTGGCTAGCTTGT – 3'	917
Cscu_Sp6_9_rev	5' – cccggggcGATGGCATACGGGAGAAAGA – 3'	

Table S2. Tabulation of raw phenotypic distribution data in *Ptep-Sp6-9-RNAi* experiments.

5' *Ptep-Sp6-9* RNAi	N	Wild type	Class I	Class II	Class III	Indeterminate	Dead
cocoon 2	433	202	26	13	23	74	95
cocoon 3	564	164	19	0	154	45	182
cocoon 4	208	98	12	6	0	19	73
Total	1205	464	57	19	177	138	350

3' Ptep-Sp6-9 RNAi	N	Wild type	Class I	Class II	Class III	Indeterminate	Dead
cocoon 2	1610	496	151	92	271	144	456
cocoon 3	1217	320	118	58	230	131	360
cocoon 4	988	255	169	86	148	71	259
Total	3815	1071	438	236	649	346	1075

Negative control	N	Wild type	Class I	Class II	Class III	Indeterminate	Dead
cocoon 2	780	396	0	0	0	17	367
cocoon 3	470	202	0	0	0	5	263
cocoon 4	779	518	0	0	0	25	236
Total	2029	1116	0	0	0	47	866

Table S3. Tabulation of raw phenotypic distribution data in *Ptep-arr*-RNAi experiments.

Ptep-arr RNAi	N	Wild type	Segmentation phenotype	Indeterminate	Dead
cocoon 2	563	190	181	166	26
cocoon 3	266	26	103	125	12
cocoon 4	194	35	75	63	21
cocoon 5	86	18	31	32	5
Total	1109	269	390	386	64

Negative control	N	Wild type	Segmentation phenotype	Indeterminate	Dead
cocoon 2	303	215	0	73	15
cocoon 3	448	356	0	71	21
cocoon 4	297	227	0	55	15
cocoon 5	497	409	0	49	39
Total	1545	1207	0	248	90