The jointed appendages of arthropods have facilitated the spectacular diversity and success of this phylum. Key to the regulation of appendage outgrowth is the Krüppel-like factor (KLF)/specificity protein (Sp) family of zinc finger transcription factors. In the fruit fly, Drosophila melanogaster, the Sp6-9 homolog is activated by Wnt-1/wingless (wg) and establishes ventral appendage (leg) fate. Subsequently, Sp6-9 maintains expression of the axial patterning gene Distal-less (Dll), which promotes limb outgrowth. Intriguingly, in spiders, Dil has been reported to have a derived role as a segmentation gap gene, but the evolutionary origin and regulation of this function are not understood because functional investigations of the appendage-patterning regulatory network are restricted to insects. We tested the evolutionary conservation of the ancestral appendage-patterning network of arthropods with a functional approach in the spider. RNAi-mediated knockdown of the spider Sp6-9 ortholog resulted in diminution or loss of Dll expression and truncation of appendages, as well as loss of the two body segments specified by the early Dil function. In reciprocal experiments, Dil is shown not to be required for Sp6-9 expression. Knockdown of arrow (Wnt-1/1conceptor) disrupted segmentation and appendage development but did not affect the early Sp6-9 expression domain. Ectopic appendages generated in the spider “abdomen” by knockdown of the Hox gene Antennapedia-1 (Antp-1) expressed Sp6-9 comparably to wild-type walking legs. Our results support (i) the evolutionary conservation of an appendage-patterning regulatory network that includes canonical Wnt signaling, Sp6-9, and Dil and (ii) the cooption of the Sp6-9/Dll regulatory cassette in arachnid head segmentation.

Arthropoda | gene regulatory network | Wnt signaling | limb outgrowth | zinc finger

The eponymous jointed leg of Arthropoda has been closely linked to the evolutionary success of this phylum. Nearly every part of the arthropod leg has undergone extensive evolutionary modifications in different lineages, enabling adaptations to various ecological niches and environments (1–3). Arthropod legs are united by the involvement of a conserved suite of four “leg gap genes” to establish the proximodistal (PD) axis (refs. 4–10, reviewed in refs. 11 and 12) (Fig. 1A). The best-studied among them is Distal-less (Dll), the earliest marker of appendage identity, which is required for the development of the distal appendage territory across arthropods (9, 13–18), as well as being associated with body wall outgrowths in other phyla (19, 20).

Whereas the regionalization of the PD axis appears to be shared across arthropods, it is not evident whether early specification of leg identity is similarly conserved. In the fruit fly Drosophila melanogaster, two members of the Krüppel-like factor (KLF)/specificity protein (SP) transcription factor gene family were previously implicated in establishing leg fate: buttonhead (btd); orthologous to Sp5) and D-Spl (orthologous to Sp6-9); henceforth, “Dmel-Sp6-9”) (21–25). Both are upstream of Dmel-Dll and, in turn, are regulated by the activity of Wnt-1/wingless (wg) and decapentaplegic (dpp) during embryogenesis (26). Subsequently, it was shown that Dmel-btd and Dmel–Sp6-9 have two nonredundant roles in establishing leg fate and promoting leg growth (27); in null mutants of both Dmel-btd and Dmel–Sp6-9, the leg is entirely abolished and wing fate may be induced in ventral tissue. Ectopic expression of both Dmel–Sp6-9 and Dmel-btd can induce wing-to-leg transformations, but Dmel–Sp6-9 has a more important role in D. melanogaster leg fate specification, as Dmel–Sp6-9 can rescue double-null mutants, whereas Dmel-btd cannot. In later development, both Dmel–Sp6-9 and Dmel-btd are required for proper leg growth in larval stages (27, 28).

Beyond D. melanogaster, gene expression surveys of Sp6-9 have demonstrated conservation of expression domains across insects and one crustacean [all members of the same clade, Pancrustacea (29)]; taxonomically broader surveys of wg have shown broadly conserved expression patterns across Arthropoda. By contrast, functional data for this gene network are restricted to insects and one spider (ref. 30 and this study as discussed below). Sp6-9 orthologs have been functionally investigated in two insects, the beetle Tribolium castaneum (31) and the true bug Oncopeltus fasciatus (32) (Fig. 1B). In these two insects, RNAi-mediated knockdown of Sp6-9 orthologs demonstrated that Sp6-9 has a conserved role in appendage growth, but no evidence was obtained for a role of Sp6-9 in specifying leg identity or activating Dll (31, 32). Similarly, functional data for wg are restricted to insects. Consequently, the present understanding of leg-patterning mechanisms reflects an unclear evolutionary scenario regarding the network of interactions between wg, Sp6-9, and Dll, as described in D. melanogaster (Fig. 1A and B).

A peculiar attribute of Dil in the spider Parasteatoda tepidariorum is that it has two separate functions, the canonical leg-patterning function [phenocopies generated by embryonic RNAi (eRNAi) have distally truncated limbs (16)] and a novel head segmentation function unique to spiders [phenocopies from maternal RNAi lack the body segments corresponding to the first and second walking legs (henceforth, L1 and L2); remaining appendages develop normally.
Fig. 1. Comparative functional data on arthropod appendage fate specification and PD axis patterning. (A) Regulation of limb gap genes in the walking leg of D. melanogaster. Sp6-9 is required for leg outgrowth and specification of ventral appendage fate. (B) Summary of available gene expression and functional data for Sp6-9 orthologs in Arthropoda (gray squares: previous studies; black squares: this study). (C) Dll has two roles in spider embryogenesis: a head segmentation function and a limb patterning function. Early knockdown via maternal RNAi results in a four-legged phenotype due to the loss of the L1 and L2 segments. Late knockdown via eRNAi results in canonical truncation of appendages distal to the trochanter. cx, coxa; fe, femur; ta, tarsus; ti, tibia; tr, trochanter.

(33)] (Fig. 1C). Regulation of this early Dll function is not understood, but a recent work, completed concomitant to the present study, on Sp6-9 in the spider P. tepidariorum has shown that knockdown of Sp6-9 results in the deletion of the L1 and L2 segments, in addition to truncation of the remaining appendages (30). The description of the loss-of-function phenotype of Sp6-9 in the spider is certainly suggestive of a regulatory interaction with Dll, but neither early regulation of Dll by Sp6-9 nor its relationship with Wnt signaling has been tested.

To redress these gaps in the understanding of appendage gene network conservation, we began by assessing incidence and expression of Sp gene family members in Myriapoda and Chelicerata, the two most basally branching groups of arthropods with respect to Pancrustacea (Hexapoda + Crustacea). To test the conservation of the gene network specifying appendage development, and especially how elements of this network relate to the derived role of spider Dll as a gap segmentation gene, we performed RNAi against the wg coreceptor arrow (arr: vertebrate homologs: LRPS and LRPs), Sp6-9, and Dll in the spider P. tepidariorum. The arr homolog codes for an essential component of the canonical Wnt pathway across Bilateria (34–36) and was specifically selected herein because previous efforts to knock down wg expression directly via RNAi have exhibited variable or limited efficiency in several arthropod species (37, 38), including spiders. By contrast, severe disruption of Wnt signaling by knockdown of arr has been achieved with high penetrance in insects (34, 39, 40).

Here, we show that single-copy orthologs of arr and Sp6-9 occur in exemplars of both chelicerates and myriapods. Expression data for representatives of these two subphyla demonstrate that Sp6-9 orthologs are invariably expressed in outgrowing limbs. In strong phenocopies, down-regulation of Prep–Sp6-9 results in the abrogation of the entire appendage, as well as loss of the L1 and L2 body segments, concomitant to the loss of Prep-Dll expression. Depletion of Prep-arr disrupts both body segmentation and appendage growth, in association with depletion of Ptep–Sp6-9 expression in outgrowing legs, suggesting a conserved role for canonical Wnt signaling in segmentation and leg development across arthropods. Critically, depletion of Prep-arr does not affect the early expression domain of Prep–Sp6-9 in the presumptive L1 and L2 territory. Our results demonstrate that a conserved gene network patterns appendage development in insects and arachnids, in tandem with the cooption of an Sp6-9/Dll cassette in patterning head segments of arachnids.

Results

Single-Copy Orthologs of arr and Sp6-9 Occur in Myriapods and Arachnids. The maximum likelihood and Bayesian inference tree topologies recovered the monophyly of the Sp gene family with maximal nodal support (SI Appendix, Fig. S1). The tree topology largely corresponded to previous analyses of the Sp gene family and is consistent with basal divergence of three paralogs in the common ancestor of Metazoa: Sp1–4, Sp5, and Sp6-9. The following differences were recovered in our orthology assignment: The sequence previously identified as Trichoplax adhaerens Sp1–4 was recovered as nested within the KLF13 outgroups, and the putative T. adhaerens Sp5 ortholog was recovered as nested within the Sp1–4 cluster (both placements supported; Dataset S1).

Of the 51 Sp homologs reported herein, a single Sp6-9 ortholog was discovered for the two myriapods (the centipedes Strigamia maritima and Lithobius atkinsoni), the hemimetabolous insect Gryllus bimaculatus, four arachnids (the mite Tetranynchus urticae, the harvestman Phalangium opilio, the scorpion Centruroides sculpturatus, and the spider P. tepidariorum), seven pycnogonids (Anoplodactylus insignis, Nymphon molleri, Phoxichilidium tubulariae, Stylopallene sp., Tanystylum orbiculare, Merinodina flava, and Pycnogonum bioreale), and the onychophoran Euphorinae nowelli; two to four Sp6-9 paralogs per species were discovered in the genomes/transcriptomes of three horseshoe crabs. Single-copy Sp1–4 orthologs were discovered for the spider, the scorpion, the harvestman, both centipedes, and all seven pycnogonids; one to three Sp1–4 paralogs were discovered in the horseshoe crabs. Single-copy Sp5 orthologs were discovered for the centipede S. maritima, the horseshoe crab Limulus polyphemus, six pycnogonids, and two onychophorans. No Sp5 orthologs were found.
in the genomes of the milkweed bug *O. fasciatus*, the amphipod *Parhyale hawaiensis*, the mite, the spider, or the scorpion.

Single-copy orthologs of *arr* were discovered in genomic resources of two myriapods (*S. maritima* and *L. atkinsoni*) and one arachnid (*P. tepidariorum*). Maximal nodal support values were recovered for the placement of the spider sequence within the *arr* cluster (*SI Appendix, Fig. S2*).

**Expression of Sp6-9 in Exemplars of Chelicerates and Myriapods.**

During the formation of limb buds in spider embryogenesis, *Ptep–Sp6-9* is detected throughout all limb buds of the prosoma (the anterior tagma, which bears all six limb pairs) by stage 8 (*Fig. 2A* and *A’*). Expression is additionally observed in the head lobes and as faint stripes in the opisthosomal segments (*SI Appendix, Fig. S3A*). In later stages, expression of *Ptep–Sp6-9* becomes heterogeneous in the limb buds of the pedipalps and the walking legs, consisting of a strong distal domain and a weaker, broader proximal domain; expression in the cheliceral limb bud remains homogenous (*Fig. 2B* and *B’*). Expression is also observed as stripes in the ventral opisthosomal ectoderm of stage 9 embryos (*SI Appendix, Fig. S3B*). No expression of *Ptep–Sp6-9* was detected in the limb bud-derived organs of the opisthosoma (i.e., the primordia of the book lungs, tubular tracheae, and spinnerets; *SI Appendix, Fig. S3B*). The subdivision of expression domains in the pedipalps and walking legs in elongating appendages reflects comparable dynamics previously reported in a range of insects and a crustacean (*29*).

Similar expression patterns are observed in the prosoma of the harvestman. Specifically, heterogeneous expression of *Popi–Sp6-9* occurs in the limb buds of the pedipalps and walking legs, whereas a single distal domain with tapering proximal expression occurs in the chelicerae (*Fig. 2C* and *D*). In the centipede, *Latk–Sp6-9* is expressed in the limb buds of all head and trunk appendages, except for the mandible (*Fig. 2E*), a pattern congruent with insect and crustacean exemplars (*29*). *Latk–Sp6-9* is additionally detected in the ventral neuroectoderm, the labrum, and as complex domains in the head lobes (*Fig. 2E* and *F*).

In all three species, complementary sense probes were tested as negative controls; no staining was observed in sense controls (*SI Appendix, Fig. S4*). Taken together, these data demonstrate that expression of the *Sp6-9* ortholog is uniformly associated with developing appendages (with the exception of the mandible of insects and myriapods and the spinnerets of spiders) across all surveyed arthropods.

**Expression of Ptep-arr.** Throughout spider embryogenesis, *Ptep-arr* is weakly and ubiquitously expressed in the embryo, comparable to the *T. castaneum* ortholog of *arr* (*40*). Complementary sense probes tested as negative controls showed no staining, suggesting that the expression detected was specific (*SI Appendix, Fig. S4*).

**Ptep–Sp6-9 RNAi Results in Down-Regulation of Ptep-Dll.** To test whether *Ptep–Sp6-9* is required for maintaining *Ptep-Dll* expression, we studied the function of *Ptep–Sp6-9* using maternal RNAi (experimental design and summary are provided in *SI Appendix, Fig. S5*). In *Ptep–Sp6-9* RNAi embryos, 25% (*n = 3 of 12*) had no detectable *Ptep-Dll* expression levels using DIG-labeled probes (*Fig. 3B*) and 50% (*n = 6 of 12*) of surveyed germ disk-stage embryos had diminished (i.e., barely detectable) *Ptep-Dll* expression (stage 5; *Fig. 3C*). In later stages, 91% of surveyed limb bud-stage embryos (*n = 20 of 22*) lacked *Ptep-Dll* expression (stage 9; *Fig. 3 D–E*). In embryos exhibiting strong phenotypes (*n = 820*), the head lobes and tail bud were identifiable, but the labrum and all prosomal appendages were abolished (*Fig. 3B* and *C*). Expression of *Ptep–Sp6-9* was diminished by comparison with negative control embryos in experiments targeting two nonoverlapping fragments of *Ptep–Sp6-9* (*n = 31 of 31*), and similar phenotypic spectra were obtained from knockdown of each fragment (*SI Appendix, Figs. S5A and S6*), suggesting that the knockdown was specific and on target. In comparison to the classic *Dll* limb phenotype previously

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**Fig. 2.** Expression of *Sp6-9* orthologs in Chelicerata and Myriapoda. (*A* and *A’*) In the spider, *Ptep–Sp6-9* is expressed in all prosomal limb buds (*B* and *B’*) in later stages. *Ptep–Sp6-9* is also observed in the ventral neuroectoderm. 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. (*C* and *D*) In limb buds of the harvestman, *Popi–Sp6-9* is expressed comparable to the spider. (*E* and *F*) In the centipede, *Latk–Sp6-9* is strongly expressed in all limb buds, except for the mandibles (white arrowhead). Expression is also visible in the ventral neuroectoderm and the labrum (black arrowhead). A complex expression pattern is observed in the head lobes and developing brain. an, antenna; ch, chelicera; fp, forcipule; hl, head lobe; mx, maxilla; p, posterior terminus; pp, pedipalp; T, trunk leg. (Scale bars: 100 μm.)

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**Fig. 3.** *Ptep–Sp6-9* is required for activation of *Ptep-Dll* expression. (*A* and *A’*) Expression of *Ptep-Dll* in early stages of wild-type spiders occurs as a ring corresponding to segments L1 and L2 (arrowhead). Expression of *Ptep-Dll* is lost (*B*) or diminished (*C*) (arrowhead) in *Ptep–Sp6-9* RNAi embryos. (*D* and *D’*) Expression of *Ptep-Dll* in limb bud stages of wild-type spiders occurs in all prosomal appendages and in the head lobes. (*E* and *E’*) In strong phenocopies from *Ptep–Sp6-9* RNAi, all appendages are abolished and no *Ptep-Dll* expression is detected. ch, chelicera; hl, head lobe; pp, pedipalp. (Scale bars: 100 μm.)
reported in spiders, which results in a deletion of all leg segments distal of the medial dachshund-1 (dac-1) expression domain (16, 33) (Fig. 1C). Ptep–Sp6-9 RNAi truncated limb development more proximally thanDll RNAi, as assessed by expression patterns of the medial PD axis marker dac-1 and the proximal PD axis marker extradenticle-1 (exd-1) (Fig. 4). Severe Ptep–Sp6-9 RNAi phenocopies retained only the Ptep–dac-1 expression associated with neurogenic tissues; the truncated appendages lacked Ptep–dac-1 expression altogether (Fig. 4C–D′), indicating a deletion more proximal than incurred by Dll RNAi (n = 27 of 28). Consistent with this interpretation, severe Ptep–Sp6-9 RNAi phenocopies expressed the proximal PD axis marker Ptep–exd-1 at the distal termini of truncated appendages, or not at all in a fully abrogated appendage (Fig. 4G–H′; n = 11 of 18).

The head segmentation function of Ptep-Dll specifically affects the L1 and L2 segments (33). To test whether the segmentation phenotype incurred by Ptep–Sp6-9 is distinguishable from that of Ptep-Dll, we examined the expression of the segment polarity gene Ptep–en-grailed-1 (en-1) in intermediate phenocopies (n = 256), where some truncated appendages were identifiable but a segment gap defect was still evident. In these embryos, we observed that Ptep–en-1 expression was lost in the two segments corresponding to the L1 and L2 segments (SI Appendix, Fig. S7), which is consistent with loss of Ptep-Dll expression in those segments during segmentation of the germ band (Fig. 3B and C). Moreover, the morphology of severe Ptep–Sp6-9 RNAi phenocopies in older stages shows a truncation of the prosoma incurred by the loss of two body segments, as well as truncation of all remaining appendages up to the proximal-most leg segment, the coxa (n = 826; Fig. 5). These phenotypes overlap the union of the two known Dll loss-of-function phenotypes in spiders (Fig. 1B), in addition to the more proximal truncation of the PD axis.

In the weakest subset of phenocopies, we observed shorter appendages in comparison to negative control embryos, but without any segmentation defects or appendage truncation (n = 497; SI Appendix, Fig. S8). The morphology of embryos in this phenotypic spectrum is comparable to Sp6-9 RNAi phenocopies in T. castaneum and O. fasciatus, wherein appendage segments were reduced in length and/or fused but entire appendages were not abolished (31, 32).

**Ptep-Dll Is Not Required for the Proximal Ptep–Sp6-9 Expression Domain in Appendages.** In the fruit fly D. melanogaster, Sp6-9 has been shown to be upstream of Dll. We performed both maternal and eRNAi against Ptep-Dll and assayed phenocopies for Ptep–Sp6-9 expression. Maternal knockdown recapitulated the early function of Ptep-Dll as a head segmentation gene (loss of L1 and L2 segments), with concomitant loss of the ring of Ptep–Sp6-9 expression in the germ disk, in length and/or fused but entire appendages were not abolished (31, 32).

**Fig. 4.** Ptep–Sp6-9 RNAi causes severe truncation of appendages. (A–D′) Expression of dac-1, a medial limb territory marker. As inferred from expression of Ptep–dac-1 in wild-type embryos (A–B′), strong Ptep–Sp6-9 RNAi phenocopies undergo truncations more proximally than the trochanter, resulting in the loss of the entire Ptep–dac-1 domain in all appendages (C–D′); only the expression in the ventral ectoderm is seen in strong phenocopies (D and D′). (E–H′) Expression of exd-1, a proximal limb territory marker. (E–F) Wild-type expression of Ptep–exd-1 spans the proximal-most segments and the body wall. (G and G′) Intermediate phenocopies exhibit Ptep–exd-1 in the termini of truncated appendages. (H and H′) Severe Ptep–Sp6-9 RNAi phenocopies either retain Ptep–exd-1 only at the distal remnant of truncated appendages (black arrowhead) or lose this territory entirely (white arrowhead), as, anterior spinneret; ch, chelicera; hl, head lobe; p, posterior terminus; pp, pedipalp; ps, posterior spinneret; O1, first opisthosomal segment. (Scale bars: 100 μm.)

**Fig. 5.** Severe Ptep–Sp6-9 RNAi embryos recapitulate the fusion of early and late Dll loss-of-function phenotypes. (A) In a wild-type embryo at stage 14, all six pairs of appendages are visible in the lateral view. (B) In a severe Ptep–Sp6-9 RNAi phenotype, the embryo exhibits both a head segmentation phenotype as well as truncation of all appendages distal to the coxa. While these embryos never survive to hatching, interpretations of the phenotype are shown as line drawings of hypothetical adult counterparts to convey phenotypic effects. ch, chelicera; pp, pedipalp. (Scale bars: 100 μm.)
in comparison to wild-type embryos (Fig. 6 A–B′; n = 72 of 72) The transience of this knockdown results in wild-type appendages on the remaining prosomal segments. Accordingly, Ptep-Dll parental RNAi (pRNAi) phenocopies showed typical expression of Ptep-Sp6-9 in the remaining appendages (chelicerae, pedipalps, and L3 and L4 legs), as well as elsewhere in the embryo (Fig. 6 C–D′; n = 32 of 34). As the L1 and L2 territory is deleted upon knockdown of either Ptep-Dll or Ptep-Sp6-9, we are presently unable to infer the regulatory relationship between the gene pair in early embryogenesis.

Embryonic knockdown recapitulated the canonical function of Dll as a PD axis gene; a proportion of surviving Ptep-Dll eRNAi phenocopies exhibited truncated appendages. In these phenocopies, Ptep-Sp6-9 expression is still detected in the proximal territory of the appendages, but the strongest expression domain at the distal terminus is greatly reduced upon truncation (Fig. 6 F and F′; n = 13 of 41). As with the L1 and L2 region in early embryogenesis, we cannot establish the regulatory relationship between Ptep-Dll and Ptep-Sp6-9 in the deleted region of the appendage axis.

**Ptep-arr RNAi Disrupts Anteroposterior Segmentation and Appendage Development.** To test whether canonical Wnt signaling has a conserved role in leg development, we studied the function of the Wnt-1 coreceptor Ptep-arr using maternal RNAi (the experimental design and a summary are provided in SI Appendix, Fig. S9). Loss-of-function phenocopies from Ptep-arr RNAi underwent disruption of segmentation and appendage development (n = 390), as inferred from morphology (Fig. 7) and expression assays for the segment polarity genes wg (n = 8 of 12) and en-1 (Fig. 8 A–D′; n = 7 of 7). These phenocopies were smaller than wild-type counterparts, consisting only of small, rudimentary heads in severe cases, and always lacked appendages (comparable to severe arr RNAi phenocopies of *T. castaneum* (39)). As landmarks for the anteroposterior (AP) axis, we assayed the expression of the anterior head marker orthodenticle-1 (odt-1) and the pedipalpal segment marker Hox gene labial-1 (lab-1). Strong phenocopies showed regionalization of the AP axis (expression stripes of odt-1 and lab-1 in the anterior germband), despite loss of segmentation (Fig. 8 E–H′).

To examine the regulatory relationship between Wnt signaling and Sp6-9, we assayed Ptep-Sp6-9 expression in a range of Ptep-arr RNAi phenocopies (Fig. 8 I–M′). Weak Ptep-arr RNAi phenocopies exhibited irregularly distributed and truncated limb buds showing greatly diminished Ptep-Sp6-9 expression, in addition to posterior segmentation defects (Fig. 8 K and K′). In severe Ptep-arr RNAi phenocopies lacking segments and legs, Ptep-Sp6-9 expression was never detected in ventral tissues corresponding to appendage
primordia (n = 11 of 11). Intriguingly, we did detect expression of Ptep-Sp6-9 in the presumptive L1 and L2 territory that is comparable to the broad expression band in wild-type embryos at germband stages (stage 7) in a subset of phenotypes (Fig. 8 L–M′; n = 7 of 11). We interpret this result to mean that Ptep-arr (and, by extension, Wnt activity) is required for activation of Ptep-Sp6-9 in the appendage primordia, but not necessarily head regionalization.

**Ectopic Spider Legs Induced by Hox RNAs Express Ptep-Sp6-9.** In the fruit fly, the regulation of Sp homologs and Dll in D. melanogaster is modulated by the trunk Hox gene Ultrabithorax (Ubx), with Ubx loss-of-function mutants expressing Sp genes in the ectopic appendage on the first abdominal segment (26). Notably, the identity of the anterior-most Hox gene that represses leg identity is not the same in insects (Ubx) and arachnids [Antennapedia (Antp) (41)]. To test whether convergence in Hox gene function (repression of leg development) is correlated with convergent integration of posterior Hox genes in the appendage-patterning GRN, we replicated the knockdown of Ptep-Antp-1 to generate 10-legged spider embryos (41) and assayed them for expression of Ptep-Sp6-9. The small ectopic appendages of the first opisthosomal segment in Ptep-Antp-1 RNAi embryos expressed Ptep-Sp6-9 comparable to wild-type prosomal limb buds during embryogenesis (Fig. 9; n = 73 of 73). These data are consistent with the prediction of convergent assembly of the insect and arachnid GRNs, wherein Sp homologs mediate the regulation of Dll by Hox signaling (26).

**Discussion**

A Conserved Appendage-Patterning Gene Network in Insects and Arachnids. Various workers have examined the patterning of the limb PD axis across Arthropoda via a combination of gene expression surveys (e.g., refs. 4, 8, 11, 18, and 42–45) and functional studies (e.g., refs. 6, 7, 9, 15, 16, 18, and 46–48). By comparison with the regionalization of the PD axis, evolution of the GRN underlying specification of arthropod leg fate is poorly understood from a functional standpoint (Fig. 1A and B). As examples, dpp and wg both play a role in establishing positional information along the PD axis, and dpp also patterns dorsal fate in ventral appendages of D. melanogaster. However, dpp expression in other surveyed arthropods is not comparable to the patterns described in D. melanogaster; thus, dpp may not serve the same role in appendage development across Arthropoda (11, 49, 50). In spiders, functional interrogation via maternal RNAi has demonstrated a role for Ptep-dpp in AP axial patterning during early embryogenesis, but its role in appendage patterning has not been explored, likely due to the severity of Ptep-dpp RNAi defects (51). Similarly, no comparative functional data exist for wg orthologs outside of winged insects, and these vary widely in penetrance and phenotypic spectrum (37, 38, 49, 52). As an example, in the cricket Gryllus bimaculatus, Gbm-wg cRNAi resulted in only transient and
early diminution of Gbim-wg expression, followed by wild-type expression by onset of limb bud stages, and corresponding wild-type morphology of hatchlings from all injected embryos (38).

To test for evolutionary conservation of leg-patterning mechanisms across arthropods, we identified the regulatory subnetwork formed by Wnt signaling, Sp homologs, and Dll as a key target for functional comparison, and focused on arachnids for reasons of phylogenetic significance and limited representation of functional data. Our results constitute an instance of systemic disruption of Wnt-1/Wg signaling in a noninsect arthropod (Fig. 8 B and B’), which we achieved by targeting the downstream coreceptor arr.

In contrast to previous efforts to knock down wg, RNAi against arr results in a highly comparable phenotypic spectrum in insects and arachnids, wherein both segmentation and appendage development are disrupted and leg-patterning genes are not activated in the developing appendages. These results support a common requirement for Wnt activity for leg patterning in the common ancestor of insects and arachnids.

A discrepancy in function of Sp6-9 has previously been observed across the three available insect data points (Fig. 1B). In two cases (the true bug O. fasciatus and the beetle T. castaneum), Sp6-9 orthologs were linked to allometric growth by RNAi has previously been observed. Knockdown experiments is that allometric growth studies, verification of Sp6-9/Dll ortholog expression with developing appendages in previously undersampled parts of arthropod phylogeny (Myriapoda and Chelicerata) supports our inference of conserved Sp6-9 dynamics in the arthropod common ancestor (Fig. 10).

We note that the phenotypic spectrum observed in limbs of Ptep–Sp6-9 RNAi embryos spans the loss-of-function phenotypes in D. melanogaster, as well as the range of outgrowth phenotypes reported in D. melanogaster, T. castaneum, and O. fasciatus knockdown experiments. Therefore, a possible explanation for the discrepancy in Sp6-9 knockdown experiments is that allometric growth phenotypes observed in T. castaneum and O. fasciatus reflect incomplete penetrance of Sp6-9 RNAi and, in turn, incomplete knockdown of Dll expression in these developing appendages. This hypothesis could be tested in the future via CRISPR-Cas9-mediated mutagenesis in T. castaneum and O. fasciatus.

Cooption of the Sp6-9/Dll Module in Head Segmentation of Arachnids.

Among metazoans, Dll and Sp transcription factors play critical roles in the development of several tissues. Previous phylogenetic inferences have supported the presence of at least three Sp gene family members in the common ancestor of Metazoa (29, 34). Subsequent divergences of the nine paralogs present in vertebrates (Sp1 to Sp9) are likely attributable to twofold whole-genome duplication in the vertebrate common ancestor (55). Within the Sp6-9 orthogroup, at least two vertebrate paralogs, Sp8 and Sp9, regulate Fgf8 expression and outgrowth of the apical ectodermal ridge in the mouse, chick, and zebrafish (56, 57). Homopomorphic mutants of Sp8 lose expression of various appendage markers, including Dlx [vertebrate Dll ortholog (55)]. While fewer comparative data are available among spiralian, in the planarian Schmidtea mediterranea, RNAi against Smel-Dlx or Smed–Sp6-9, followed by excision of the head, resulted in the inability to regenerate eyes as well as other tissues (58). Recently, it was shown that regenerating appendages in the annelid Platynereis dumerilii express Pdm–Sp6-9, Pdm-Dll, and orthologs of other limb-patterning genes (59). In some cases, the spatial relationships of
these annelid genes are comparable to those in developing arthropod appendages, but their functions remain unknown. Thus, a Sp6-9/Dll regulatory cassette has been reported in various roles and lineages across Bilateria (27, 60).

Given the recently described novel role of Ptep-Dll as a gap segmentation gene in spiders, our experiments with Ptep–Sp6-9 are poised to address whether this phenomenon constitutes a new case of cooption of an Sp/Dll cassette. In our Ptep–Sp6-9 RNAi experiments, the union of both the Ptep-Dll maternal RNAi (head segmentation) phenotype and the eRNAi (distal limb truncation) phenotype in the strong Ptep–Sp6-9 RNAi phenocopies (Figs. 1C and 5) was associated with the diminution or complete loss of Ptep-Dll expression in the relevant stages of embryogenesis (Fig. 3). These data support a model of activation and maintenance of Ptep-Dll by Ptep–Sp6-9 for both the head segmentation and leg-patterning functions of Dll. Furthermore, we observed that the head segmentation domain of Ptep–Sp6-9 was not lost in severe Ptep-arr RNAi phenocopies, which bear part of a regionalized AP axis but never develop segments or appendages in later developmental stages. This result suggests the independence of the Ptep–Sp6-9/Dll gap gene function from Wnt signaling, and is hypothesized herein to be due to cooption of an ancient Sp/Dll gene cassette.

**Loss of Sp5 is Characteristic of Arachnids.** Why would the Sp6-9/Dll cassette be recruited for this function in an arachnid? One possibility may be that Sp6-9 fulfills the role of the Sp5/btd ortholog in insects. In *D. melanogaster*, btd is one of the classically known gap segmentation genes, and expression surveys of btd orthologs support evolutionary conservation of this head segmentation role, at least in the common ancestor of Mandibulata (29, 61). The outstanding question then is whether Sp5 could also retain this function in Chelicerata.

By comparison with Sp6-9, Sp5 lacks the same breadth of functional data points, obviating clear polarization of gene function on a tree topology even within Pancrustacea. As an alternative approach to inferring evolution of Sp5 function, we mapped the evolutionary losses of Sp5 across Panarthropoda (with the assumption that loss of critical Sp5 functions was rescued through their cooption by other genes). Our survey of recently sequenced genomes and developmental transcriptomes of various Panarthropoda pinpoints the evolutionary loss of Sp5 in the common ancestor of the four arachnid lineages that we surveyed (spider, scorpion, mite, and harvestman). The presence of an Sp5 ortholog in Onychophora, one centipede, one horseshoe crab, and six sea spider species supports the inference that Sp5 was present in the common ancestor of panarthropods and also of chelicerates, and that a shared loss of Sp5 likely occurred in the common ancestor of arachnids. The absence of Sp5 in the genomes of *O. fasciatus* and *P. hawaiiensis* is interpreted to constitute independent loss events (Fig. 10).

If we interpret the shared absence of arachnid Sp5 to mean that an Sp6-9/Dll cassette could have replaced the role of Sp5 in the common ancestor of arachnids, then we should expect to find evidence for gap gene-like expression for Sp6-9 and Dll in other arachnids as well. To test this prediction, we surveyed expression of Sp6-9 and Dll in early embryogenesis of the scorpion *C. sculpturatus*, following our previous approach to the study of this species (62) (harvestman embryos proved too fragile to examine at equivalent stages). Consistent with our prediction, we discovered that before the germblast stage, Cscu–Sp6-9 and Cscu–Dll are expressed as a
ring around the blastopore, which subsequently splits at one end, precisely as in spiders (Fig. 10 B and D).

While functional tools do not exist for scorpions, this datum accords with the interpretation that the cooption of the Sp6-Dll cassette into head segmentation occurred before the divergence of spiders from other arachnids. Future tests of this evolutionary scenario should emphasize expression surveys of Sp5 orthologs in Xiphosura and Pycnogonida to establish gap gene-like expression patterns in tandem with knockdown experiments of Sp6-9 in mites and harvestmen.

Methods
Bioinformatics and Phylogenetic Analysis. Orthologs of Sp gene family members were identified in genomes of S. maritima (63), C. sculpturatus (64), T. paeoniarius (65), L. palustris (65), and E. rovelli (66) and from transcriptomes of P. opilio (67), L. atkinsoni (this study), Carcinocarcinus rotundicauda (65), Tachypleus tridentatus (65), Peripatopsis capensis (67), and seven pycnogonids. In addition, the genomes of O. fasciatus (68) and P. hawaiensis (69) were examined for the incidence of an Sp5 ortholog, which had not been found previously (29, 30, 32). For all searches, D. melangeaster Dsp1 (National Center for Biotechnology Information [NCBI] accession no. ABW09374.2), P. hawaiensis Sp6-9 (NCBI accession no. CBH08881.1), and D. melangeaster arr (NCBI accession no. NP254737.2) were initially used as peptide sequence queries in BLAST searches, and hits with an e-value \(< 10^{-5}\) were retained. All putative orthologs were verified using reciprocal BLAST searches.

Sequences previously compiled by Schaeper et al. (29) were downloaded from the GenBank database, and new putative orthologs were added for all genes. Multiple sequence alignments were conducted de novo using MUSCLE (73). Outgroup sequences used to root the tree consisted of KLF-9/13 orthologs of Nemastoma vectensis (XP_001624390.1), T. castaneum (EE293873.1), Danio rerio (NP_001070240.1), and Mus musculus (NP_067341.2). For the Sp5 gene, we inferred phylogenies both with and without the KLF-9/13 outgroups and both with and without masking of ambiguously aligned sites using GBlocks v.091b (71) with parameters as specified in our previous work (64); all alignments are constructed anew, using LRP4 orthologs and a megalin sequence as outgroups. Due to the paucity of ambiguously aligned sites, the LRP alignment was not treated with GBlocks (Dataset S4).

Phylogenetic reconstruction of amino acid alignments consisted of maximum likelihood analysis with RAxML v.8.0 (72) under the LG + 1 model, with 250 independent starts and 250 bootstrap resampling replicates, and with Bayesian inference analysis with MrBAYES v3.2 under a mixed model of evolution. Convergence was independently assessed using average split frequency and with Tracer v.1.6. As a conservative treatment, 10\(^{6}\) generations (20%) were discarded as burn-in.

Cloning of Orthologs and Probe Synthesis. Fragments of Ptep–Sp6-9 were amplified using standard PCR protocols and cloned using a TOPO TA Cloning Kit using One Shot Top1 chemically competent Escherichia coli (Invitrogen) following the manufacturer’s protocol, and their PCR product identities were verified via sequencing with M13 universal primers. All gene-specific primers sequences are provided in SI Appendix, Table S1.

Embryo Collection, Fixation, and in Situ Hybridization. Animals were maintained, and embryos fixed and assayed for gene expression, following established or minimally modified protocols, as detailed previously (62, 74). PCRs for generating riboprobe templates, synthesis of digoxigenin-labeled probes, and preservation of embryos all followed our recently detailed procedures (74). Probes were used at a concentration of 30–50 ng/μl. Sense probes were always developed for the same duration as the antisense probes to ensure a completion of staining lasted 0.5–6 h at room temperature. Images were taken using a Nikon SMZ25 fluorescence stereomicroscope mounted with a DS-F2i digital color camera driven by Nikon Elements software.

Double-Stranded RNA Synthesis and RNAi in P. tepidiarium. Double-stranded RNA (dsRNA) was synthesized following the manufacturer’s protocol using a MEGAscript T7 kit (Ambion/Life Technologies) from amplified PCR product. The remnant of dsRNA was checked, and the concentration was adjusted as described previously (74).

Maternal RNAi was performed in virgin spider females (sisters from the same clutch) with injections every other day along the lateral surface of the opisthosoma, for a total of four injections. The dsRNA was injected at a concentration of 2.5 μg/μl, and 5 μg of dsRNA was delivered at each injection (total of 20 μg). Females were fed the first day after the final injection and mated within 24 h of the first injection (Ptep-dll O. fasciatus RNAi) or 24 h after the first injection (Ptep-Dll-arr RNAi) or filial O. fasciatus (Ptep–Sp6-9 RNAi) injection. Each set of pRNAi experiments was accompanied by a set of negative controls, which were injected with an identical volume of 1× Tribolium injection buffer. To rule out off-target effects, dsRNA was synthesized for injection as two nonoverlapping Ptep–Sp6-9 fragments of similar size (727 bp and 816 bp), with each injected into five females. Phenotypes were scored by severity, as described above; raw counts are reported in SI Appendix, Tables S2 and S3. Development was followed until stage 14, and embryos were periodically fixed and scored. Efficiency of knockdown was verified using in situ hybridization. An identical procedure was used to perform maternal RNAi against Ptep–Antp–1, Ptep-dll, and Ptep-arr (SI Appendix, Fig. S9).

eRNAi against Ptep-Dll followed the original report of this procedure (33), with an 819-bp fragment. The first clutch of a newly mated female was obtained and divided into four sets of 100. One-quarter of the embryos were injected under halocarbon-700 oil with 1× Tribolium injection buffer, and remaining 300 embryos were injected with Ptep-Dll-dsRNA; both solutions were mixed with a 1:20 dilution of rhodamine dextran for visualization. Embryos were reared for 4 d, and a subset of surviving embryos was assayed for Ptep–Sp6-9 expression.

ACKNOWLEDGMENTS. Comments from the editors and two anonymous reviewers greatly improved the final manuscript, and experiments presented. Sea spider egg clutches were collected by Georg Brennes for sequencing of developmental transcriptomes. Carlos Santibañez López assisted with scorpion gene expression assays. Access to the onychophoran draft genome was kindly provided by Georg Mayer and Stephen Richards. Edits from Jesús A. Ballesteros, Guilherme Gamiett, Gonzalo Giribet, Carlos E. Santibañez López, and Andrew Z. Ontano were incorporated into the manuscript. This material is based on work supported by the National Science Foundation under Grant IOS-1552610.


