Novel Function of Distal-less as a Gap Gene during Spider Segmentation

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Abstract

Despite many aspects of the regulation of segmentation being conserved among arthropods, the evolution of novel gene functions has played an important role in the evolution of developmental regulation and the emergence of new segmental structures. Moreover, the study of such novel gene functions can be informative with respect to the patterns and direction of evolutionary changes in developmental programs. The homeobox gene Distal-less (Dll) is known for its conserved function in appendage development in metazoa. In arthropods, Dll is required for the specification of distal appendage structures. Here we describe a novel and unexpected role of Dll in the spider Achaearanea tepidariorum. We detect At-Dll transcripts not only in the appendages, but unexpectedly also in an anterior domain during early development, prior to the specification of the limb primordia. A similar early Dll domain is present in the distantly related spider Pholcus phalangioides. In A. tepidariorum this early At-Dll expression is required for head segmentation. RNA interference results in spiders that lack either the first or the first and the second walking leg segments. The early At-Dll expression is also required for the activation of the segment polarity genes engrailed and hedgehog in this region. Our work identifies the Distal-less gene as a novel factor in anterior spider segmentation with a gap gene-like function. This novel role of Dll is interesting because Dll expression is reduced in this region in crustaceans and the homologous insect segment, the mandible segment, does not express Dll and does not require this gene for patterning. We therefore discuss the possible implications of our results for understanding the evolution and diversification of the mandible segment.

Introduction

The genetic regulation of head segmentation in Drosophila melanogaster is one of the best-studied examples of how tissues become specified and patterned during early embryonic development [1,2]. Moreover, some features of the Drosophila segmentation gene cascade are evolutionarily conserved among other arthropods [3]. However, although the arthropod head itself is morphologically an ancient and evolutionary conserved structure, composed of a protocerebral (or ocular) region followed by a series of five homologous segments [4,5], there is increasing evidence that the upstream mechanisms regulating head patterning vary among arthropods [3,6].

The formation of the head in Drosophila melanogaster begins with a maternal and early zygotic gradient system involving the proteins encoded by the bicoid (bcd), hunchback (hb), and orthodenticle (otd) genes [2,3,7–9]. These gradients provide a coordinate system for a large number of subordinate genes that further subdivide the head epidermis. Thus, the initial gradient system controls the final location of the segmental borders and the correct number of head segments. Surprisingly, although all insect heads have the same segmental composition, neither the bcd gene, nor the role and regulation of hb and otd is conserved in other insect species [3,6]. It appears, therefore, that the evolutionarily conserved architecture of insect heads is produced by an as yet comparatively unexplained diversity of developmental genetic mechanisms. This phenomenon also extends to other arthropods: recent work in the spider Achaearanea tepidariorum demonstrated novel head formation mechanisms involving dynamic gene expression [10]. Understanding head development in arthropods will thus shed light on the flexibility and evolvability of the gene network underlying the patterning of this ancient and evolutionarily conserved structure.

Head segmentation mechanisms not only differ between species, but between adjacent head segments within a species. Developmental studies in Drosophila have shown that the embryonic head consists of two parts that use different mechanisms for segmentation [1,11]. The anterior head part (or procephalon) uses a set of head gap genes to establish the location of the segments [7–9]. The posterior head part (or gnathocephalon) uses the pair-rule genes for segmentation and thus closely resembles the establishment of the trunk segments [2,12]. The segment that abuts this border between the two segmentation mechanisms is the mandible segment. It is the anterior-most gnathocephalic segment that (together with the posterior part of the intercalary segment)
The homeobox gene *Distal-less* (*Dll*) is well known for its evolutionarily conserved role in distal appendage formation in spiders. However, we also find that the *Dll* gene of *A. tepidariorum* is expressed unexpectedly early in the L1 region, before the formation of the germ band and the specification of the appendages, and concurrent with the dynamic gene expression in the prechephalon. This early expression of *Dll* is also seen in the spider *Pholcus phalangioides*, which is only distantly related to *A. tepidariorum*. We demonstrate in *A. tepidariorum* that this early expression of *Dll* in the L1 region is necessary for the specification of the entire L1 segment. Our results show that loss of *At-Dll* function leads to the complete loss of the L1 segment in adult spiders, and the loss of this segment in the embryo is preceded by the loss of segmentation gene expression and increased cell death.

Thus, *At-Dll* functions similarly to the head gap genes that pattern the procephalic head segments in *Drosophila* [7,9], implicating *At-Dll* as a novel head gap gene in spider head segmentation. Our results suggest that in contrast to the procephalic segments of this spider, the L1 segment is specified in a manner mechanistically similar to procephalic segment specification in *Drosophila* (i.e. involving the action of stably expressed head gap genes). The gene expression data from the cellar spider *P. phalangioides* suggest that the head gap gene role is more widely conserved in spiders. It is interesting to note that in insects and other mandibulate arthropods the expression levels of *Dll* in the homologous segment are reduced [19,20] and we discuss possible implications of the divergent roles of *Dll* in this segment for the evolution of the mandible segment in the arthropods.

**Results**

Unexpected early *Dll* expression in the spider head

The anterior (“head”) segments of the spider *A. tepidariorum* are specified very early in embryonic development, at the germ disc stage when the embryo still has radial symmetry, and well before the transition into the bilateral symmetric germ band [10,14,21,22]. The *Dll* gene of *A. tepidariorum* is strongly expressed in a static ring in presumptive anterior cells of the L1 region already at early stage 5 (staging after [21]) (Figure 1A). This is surprising because this expression occurs very early in development, long before the specification of the limb primordia, and concurrent with the general anterior-posterior patterning of the head region [10,14]. The ring of *At-Dll* expression persists through late stage 5 (Figure 1B) and opens during the transition from germ disc to germ band formation at stage 6 (Figure 1C). Subsequently, as the germ band forms during early stage 7, the ring of expression is transformed into a broad stripe in the L1 segment (Figure 1D, 1E). In addition, a weaker stripe of expression is detected in the second walking leg segment (L2). Expression at later stages is found predominantly in the limb buds and other domains known for *Dll* homologs in other arthropods, (e.g. [17,18]) (Figure 1F–1H).

*Dll* is required for the formation of a head segment

The unexpected early ring of *At-Dll* expression in the germ disc suggests that *At-Dll* plays a role in early head development in *A. tepidariorum*. We therefore tested the role of *At-Dll* in early head development using parental RNA interference (RNAi) (Table S1). RNAi led to a down-regulation of *At-Dll* transcripts in the cytoplasm in the early ring of anterior expression below the level of detection via in situ hybridisation (Figure S1A, S1B). In 64% of embryos, the RNAi effect was permanent, but in 19% the effect was no longer observed after stage 7 and thus led to a partial restoration of *At-Dll* transcripts in the subsequent stages of development (Figure S1D). In both cases, RNAi resulted in embryos lacking one entire leg-bearing segment, and in a few embryos with permanently down-regulated *At-Dll* expression, the loss of two entire leg-bearing segments was also observed. As expected, RNAi also affected appendage development resulting in severely truncated limbs in permanently affected embryos (Figure 2A–2C). In the other embryos, the development of the remaining legs was not affected owing to restoration of *At-Dll* expression in later stages. Importantly, this indicates that the patterning function of the early ring of *At-Dll* expression is required much earlier than the onset of appendage development and is necessary for segment formation.
While the permanently affected embryos did not survive, embryos where only the early *At-Dll* ring is down-regulated survived to reach the first larval instar. These juvenile spiders had only three pairs of walking legs (Figure 2E) or, in few cases, two pairs of walking legs (Figure 2F). The chelicerae, pedipalps, and the remaining legs were normal. The loss of some walking legs does not cause a discernible gap between the remaining appendages and this indicates that not only the legs, but also the corresponding segments are missing (see also below). The four-legged larvae did not survive, but the six-legged larvae survived and moulted normally and even reached adulthood (Figure 2G, 2H). Thus, under laboratory conditions the loss of a complete body segment does not appear to significantly compromise the viability and feeding behaviour of the animals (Video S1, Video S2).

The *L1* segment, the mandible segment homolog, is missing in *Dil* RNAi animals

In order to establish the identity of the missing walking leg segments, we compared morphological and structural landmarks between wild type and the six-legged adult *Dil* RNAi spiders. Adult spiders have a thick cuticle plate (the sternum) on the ventral side of their anterior body part (prosoma). The sternum in the wildtype has a characteristic shape with three pointed edges, the anterior most edge being the most pronounced (Figure 2I). In *A. tepidariorum*, like in other spider species [23], each walking leg segment except for *L4* is represented on the surface of the sternum by a characteristic slit sense organ (Figure S2A). The slit sense organ of *L1* and *L3* is divided into two separate slits, whereas the slit sense organ of *L2* comprises a group of three slits. In *At-Dil* RNAi animals the anterior most edge of the sternum is missing (Figure 2J), Figure S3A, S3B, S3C) and the bipartite slit sensory organ of *L1* is lacking (Figure S2B). We conclude that the *L1* segment is missing in these animals.

As an additional means of confirming the identity of the missing walking leg segments, we have used the expression of the Hox gene *Sex combs reduced* (*Scr*) as a marker [14]. This gene is expressed differentially in the four walking leg segments (*L1* to *L4*) (Figure 3A). It is not expressed in *L1*, weakly expressed in *L2* and *L4*, and strongly expressed in *L3*. The expression level of *Scr* thus allows the four walking leg segments to be distinguished in the embryo. In six-legged *At-Dil* RNAi embryos, the weakly expressing *L2* and *L4*, and the strongly expressing *L3* remain, thus confirming that *L1* is missing (Figure 3B). In four-legged *At-Dil* RNAi embryos only the strongly expressing *L3* and the weakly expressing *L4* remain, thus confirming that *L1* and *L2* are missing (Figure 3C). There is no indication of homeotic transformation of the identity of the segments, rather the *L1* or *L1* and *L2* segments, respectively, are absent. These results suggest that the early expression of *At-Dil* in the germ disc is required for the formation of the *L1* and *L2* segments and that the lack of early *At-Dil* expression leads to a gap gene-like phenotype in the anterior body region of *A. tepidariorum*.

To understand the mode of segment loss after early *At-Dil* loss, we have studied the development of the presumptive *L1* and *L2* region in RNAi embryos. During early germ band stages, when the segments first become morphologically visible, in *At-Dil* RNAi embryos, the body portion between the pedipalpal and *L3* segment is bulged and larger than a single normal segment and likely consists of the cells normally comprising both the *L1* and *L2* segments (Figure 4A–4C). Cell death (Figure 4A’–4D’), however, does not occur in this bulged segment before late stage 8 when many dead cells were then detected in this segment (Figure 4C’). These data show that the loss of early *At-Dil* function leads to a malformation of the *L1* and *L2* segments and that a large portion of the cells in this malformed segment is later removed by cell death. Our results suggest that in the majority of cases the presumptive *L2* cells remain and can still form this segment later during development. However, occasionally cell death apparently removes more cells in this region, resulting in the animals lacking both *L1* and *L2*. Thus, the loss of *L2* may not be a specific effect of the loss of the early *At-Dil* ring, but rather a secondary effect of occasional excessive cell death.

*Dll* acts as a head gap gene in the spider

The *Dll* RNAi phenotype in *A. tepidariorum* is reminiscent of the mutant phenotypes of the head gap genes in *Drosophila* (e.g. *empty spiracles* (*ems*) and *buttonhead* (*btd*)) [7], which also show the loss of
anterior segments. One hallmark of these head gap genes is that they are required for the activation of the segment polarity genes (e.g., en, hedgehog (hh)) in the particular head segments they specify [7,8,24]. We therefore investigated the impact of At-Dll RNAi on segment polarity gene expression in A. tepidariorum. We examined the expression of hh and en, both of which are normally expressed in single stripes in the posterior of each segment (Figure 4E, 4F). In At-Dll RNAi animals the expression of these genes was affected in the bulged segment between the pedipalpal and the L3 segment. The stripes of hh and en normally found in the posterior portion of L2 were still present, but the L1 stripe of both genes was missing (Figure 4H, 4I). These data indicate that the activation of these segmentation genes in the L1 segment directly or indirectly requires At-Dll. The lack of the L1 en and hh stripes in the bulged segment is not due to the absence of the cells that normally express these genes, because cell death in this segment
Figure 3. Expression of Scr in At-Dll RNAi embryos. (A–C) Ventral views of stage 10 embryos. The expression levels distinguish the four walking leg segments in control embryos (A) and identify the identity of the remaining leg segments in six-legged Dll RNAi embryos (B) and four-legged Dll RNAi embryos (C). Abbreviations: L, walking leg segment; Pp, pedipalpal segment.

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Figure 4. Effects of At-Dll RNAi. (A–D, A’–D’) Cell death during development of Dll RNAi embryos. (A–D) Morphology of the Dll RNAi embryos at stage 7 (A), early stage 8 (B), late stage 8 (C), and stage 9 (D) photographed under UV light. (A’–D’) These panels correspond to the panels A–D and show the same individuals as the panels A–D, except that the embryos were photographed under white light to document the TUNEL staining. (E–J) L1 specific elements of en and hh expression are deleted in At-Dll RNAi embryos. (E–G) Expression of en (E), hh (F), and twi (G) in control embryos. (H–J) Expression of en (H), hh (I), and twi (J) in Dll RNAi embryos. The asterisk denotes the malformed and bulged segment between pedipalpal and third walking leg segment. Abbreviations: Ch, cheliceral segment; L, walking leg segment; O, opisthosomal segment; Pp, pedipalpal segment.

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occurs later, as described above (late stage 8; see Figure 4C). Moreover, the absence of en and hh expression does not reflect a general lack of gene expression in this malformed segment because the gene twist (twi), which is a mesoderm marker and not related to ectodermal segmentation or patterning [23] (Figure 4G), is still expressed in this bulged segment (Figure 4J).

Recently, it was found that the gene hunchback (hb) is crucial for the development of anterior segments in A. tepidariorum and is required for the formation of the L1, L2 and L4 segments [14]. Since the phenotypic effect of At-hb and At-Dll RNAi is similar with respect to the L1 segment and in part to the L2 segment, we reasoned that there might be an interaction between At-hb and At-Dll in the formation of these segments. However, we could not detect any obvious differences in At-hb expression in At-Dll RNAi embryos at the germ disc stage (Figure S4C, S4D). We express in this bulged segment (Figure 4J).

Since the phenotypic effect of At-hb RNAi are similar with respect to the L1 segment and in part to the L2 segment, we reasoned that there might be an interaction between At-hb and At-Dll in the formation of these segments. However, we could not detect any obvious differences in At-hb expression in At-Dll RNAi embryos at the germ disc stage (Figure S4C, S4D). We express in this bulged segment (Figure 4J).

Evidence for a head gap gene role of Dll in Pholcus phalangioides, a haplogyne spider

Our results show that Dll has a role in the formation and development of the L1 segment in A. tepidariorum, but is this a general mechanism for L1 formation in spiders or a novelty of this particular group of spiders like the role of bicoid in higher flies [26]?

To answer this question, we studied Dll expression in the cellar spider Pholcus phalangioides (Figure S5A–S5E). The common house spider A. tepidariorum is a member of the Entelegynae that contain the vast majority of extant spiders. P. phalangioides, however, belongs to the Haplogyneae, which is the sister group of the Entelegynae (Figure S6). Comparisons between A. tepidariorum and P. phalangioides can therefore reveal conserved features that trace from the common ancestor of entelegyne and haplogyne spiders.

The development of P. phalangioides differs somewhat from that of A. tepidariorum. The early stages of P. phalangioides also form a nearly round germ disc that undergoes a transition from a radial symmetric embryo to an axial symmetric embryo, which results in the formation of the germ band. In A. tepidariorum as well as in P. phalangioides the cumulus migrates from the centre of the germ disc to the rim of the disc, the future dorsal pole of the embryo [19,27]. In A. tepidariorum the cumulus disappears after its migration, but in P. phalangioides the cumulus persists much longer and is still visible during early stages of germ band elongation (asterisk in Figure S5C). The earliest stages of P. phalangioides embryos that are accessible for in situ hybridisation are comparable to stage 6 or stage 7 embryos of A. tepidariorum. Pp-Dll in these embryos is expressed in an almost closed ring or stripe close to the anterior rim of the germ disc and forming germ band (Figure S5B, S5C).

This expression is very similar to the early Dll ring that is observed in A. tepidariorum embryos (Figure S5B), and thus argues for the conservation of early Dll function in entelegyne and haplogyne spiders. The late pattern of Pp-Dll is also virtually identical to the pattern in A. tepidariorum (and other spiders) and includes strong expression in the cheliceres, pedipalps, and walking legs (Figure S5D, S5E).

Discussion

While the last common ancestor of all arthropods is generally accepted to have been segmented and, moreover, had a segmented head, the molecular and genetic mechanisms for trunk segmentation and head segmentation are diverse in extant arthropods [6]. Understanding the genetic basis of head development in arthropods thus provides us with insights into the diversification of the underlying gene regulatory networks that nonetheless specify an evolutionarily conserved structure. Here we describe a novel function of the highly conserved Dll gene in head segmentation in the spider Achaearanea tepidariorum and we present evidence that this function is conserved in a second distantly related spider species, the cellar spider Pholcus phalangioides.

A novel role for Dll as a head gap gene in Achaearanea

Previous results from the spider A. tepidariorum showed how positional values for procephalic segmentation genes are specified via the dynamic expression of the conserved head gene old [10]. However, segmentation gene expression in the following walking leg segments (that constitute the gnathocephalon homolog of spiders) is static and does not depend on old expression dynamics [10,14]. The L1 segment has a special position in the spider head as it lies at the boundary between the procephalon and gnathocephalon that divides dynamic and static segmentation gene expression patterns. Our results identify a novel function of the appendage patterning gene Dll as a head gap gene in this particular head segment. An early static activation of At-Dll in L1 is necessary for the specification and formation of the L1 segment, and is required for the subsequent activation of the segmentation genes in this region. In these respects this early function of At-Dll is similar to the head gap genes that mediate procephalic segmentation in Drosophila, e.g. ens and btd [7,8]. Remarkably, this head gap gene function of At-Dll represents a novel function of Dll and has not been observed in other arthropod groups. In addition, Dll function in A. tepidariorum is independent of the conserved early head factor hb and might act in parallel with this gene, whereas in Drosophila the head gap genes interact with hb [2,28,29]. Together, this indicates that the formation of the L1 segment in A. tepidariorum is mechanistically similar to procephalic segmentation in Drosophila (i.e. involving the action of statically expressed head gap genes), but employs some factors that are not used in segmentation in this insect. Indeed, it is currently unclear to what extent the mode of head development in Drosophila is representative of other arthropods.

The specific functions of the maternal coordinate genes (e.g. bcd) are not conserved even within the higher flies (cyclorrhaphan dipterans) [26,30], and in non-dipteran species the role of bcd appears to be assumed by different factors in different species [3,6,31]. The role of the head gap genes is only partially conserved in other insects, because recent results in the beetle Tribolium castaneum show that the btd gene does not have a role in head development [32], and the situation in non-insect arthropods is largely unknown. Thus, paradoxically, the evolutionarily conserved morphological structure of the arthropod head is produced by highly diverse molecular mechanisms. It is generally appreciated that the germ band stage in arthropod development represents the “phylyotypic stage” for the phylum and functions as a bottleneck for the diverse developmental mechanism before and after that stage [33,34]. Indeed, recent work has shown that the transcriptome that is active during the phylyotypic stage is phylogenetically ancient and well-conserved [35,36]. The morphology of the arthropod head is very similar at the germ band stage and thus is an integral part of the arthropod phylyotypic stage. It thus appears that so long as the conserved transcriptome of the head is activated at the phylyotypic stage, then what mechanisms actually activate this transcriptome does not really matter- hence the divergence in early head patterning mechanisms.
Implications for understanding the evolution of the mandibular segment

Our results also have additional evolutionary implications. As mentioned above, the spider L1 segment is homologous to the insect mandibular segment [4,5]. This segment in insects is remarkable for its complete lack of Dll expression, but yet produces the insect typical gnathal appendage, the mandible [20]. In other mandibulate arthropods (crustaceans, myriapods) reduced Dll levels are expressed during mandibular development [17,18,20,37]. In contrast, our results show that Dll is broadly expressed in the mandibular segment homolog of spiders and has an important role in the formation of this segment.

The function of Dll has been studied previously in another spider, the Central American wandering spider Cupiennius salei, but no role of Dll in L1 segment formation has been observed [38]. The lack of gap-like phenotypes in C. salei, however, may have resulted from methodological limitations in this species because parental RNAi is not applicable in C. salei [38], and thus an early function of Dll might have been missed in the embryonic RNAi experiments. Indeed, we have performed embryonic RNAi for Dll in A. tepidariorum embryos identical to the RNAi procedure in C. salei, and obtained similar phenotypes as in C. salei: appendage phenotypes, but no gap phenotypes (Figure S7A–S7C). This class of phenotype is absent in parental RNAi experiments in A. tepidariorum where the appendage phenotypes (if present) are always associated with gap phenotypes (Table S1). These data suggest that the RNAi mechanism requires some time to develop the full effect after the application of the dsRNA, and thus only the parental injections provide the effect early enough, whereas the embryonic injections fail to interfere with the early expression of Dll and thus only lead to appendage phenotypes.

Early embryonic stages of C. salei are not accessible for in situ hybridisation, therefore it is not possible to analyse the early expression of Dll in C. salei. However, we could demonstrate that in another spider, the cellar spider P. phalangioides, Dll is expressed in a similar early anterior ring like in A. tepidariorum. P. phalangioides belongs to the Haplogynae, the sister group of the Entelegynae, the spider, the Central American wandering spider Cupiennius salei, but no role of Dll in L1 segment formation has been observed [38]. The lack of gap-like phenotypes in C. salei, however, may have resulted from methodological limitations in this species because parental RNAi is not applicable in C. salei [38], and thus an early function of Dll might have been missed in the embryonic RNAi experiments. Indeed, we have performed embryonic RNAi for Dll in A. tepidariorum embryos identical to the RNAi procedure in C. salei, and obtained similar phenotypes as in C. salei: appendage phenotypes, but no gap phenotypes (Figure S7A–S7C). This class of phenotype is absent in parental RNAi experiments in A. tepidariorum where the appendage phenotypes (if present) are always associated with gap phenotypes (Table S1). These data suggest that the RNAi mechanism requires some time to develop the full effect after the application of the dsRNA, and thus only the parental injections provide the effect early enough, whereas the embryonic injections fail to interfere with the early expression of Dll and thus only lead to appendage phenotypes.

In summary, we present evidence for a gap-like function of Dll in entelegyne and haplogyne spiders in addition to its widely conserved role as an appendage gene. We propose the hypothesis that this dual role of Dll in spiders might have prevented the reduction of Dll expression levels in the spider homolog of the mandibular segment, because this would have led to severe and possibly deleterious phenotypes. By contrast, we propose that in the mandibulates, where Dll has no dual role, the reduction of Dll levels in the mandible segment only led to a distal reduction of the mandibular appendages as can be seen in Crustacea [17,18,37]. This novel type of distally reduced appendage has then further diversified also leading to the fully gnathobasic (i.e. lacking all distal elements) insect mandible. In this way, diversification of Dll function may have driven the diversification of mandible segment morphology and facilitated the evolution of the mandible as a tool to tap into new food resources.

Whether the gap-like role of Dll in the L1/mandible segment is the ancestral state for the arthropods or has evolved later in the chelicerate lineage is difficult to be determined in the absence of functional data from outgroups (e.g. onychophorans (velvet worms), tardigrades (water bears)). However, the presence of early Dll expression in the anterior body region of a mollusc [40], a hemichordate [41] and a cnidarian [42] suggests that Dll is ancestrally involved in anterior development, and it has been proposed recently that the majority of the leg patterning genes (including Dll) originally were involved in setting up the anterior-posterior head axis in the bilaterian animals and were only later co-opted for proximal-distal axis formation of the appendages [43].

Materials and Methods

Animal culture and gene cloning

Embryos and adults of A. tepidariorum were obtained from our laboratory stocks in Gottingen. A partial sequence of the A. tepidariorum Dll gene was cloned previously [44]. Additional sequence was obtained by analysing the embryonic transcriptome (sequenced from RNA isolated from stages 1 to 10) and by RACE PCR using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) and the following primer sets: At-Dll-5’RACE-1: GGCCGAGAGTTGTGGGCTGGGCGG; At-Dll-5’RACE-2: CTTGGG CatGGTGGGGA CACCCGG; At-Dll-3’RACE-1: CTCACTCGGTCACAGGTTGGTCTGATCGCC; At-Dll-3’RACE-2: CGGGCGTCTCGCCTATGTCATCACC. The full At-Dll gene sequence has been deposited with the EMBL sequence database under accession number FM876233.

Adult females of P. phalangioides were collected from several cellars in Gottingen, Frankfurt am Main and Munich. Embryos were fixed as described previously [45].

A fragment of the homeobox of the P. phalangioides Dll gene was isolated using the primers eDP-fw, eDP-bw, iDP-fw, and iDP-bw as described previously [46]. RACE-PCR to obtain the full Pp-Dll sequence was performed using the primers Pp-Dll-5’RACE (CGA GTG ATG CGG CGG GTT CGG CGT) and Pp-Dll-3’RACE (GGT TCC AAA GGA GCG AGT ACC TGG CGC). The full Pp-Dll sequence has been deposited with the EMBL sequence database under accession number HE585694.

RNA interference

Parental RNAi was performed as described previously [47] with minor modifications. To exclude off-target effects caused by the dsRNA injections, either the full length At-Dll fragment or a 1260 bp fragment, both including the homeobox, or two not overlapping fragments of 305 bp and 360 bp, both excluding the homeobox, were injected independently of each other into adult spider females. All injections led to identical phenotypes. For the knock-down of hunchback, dsRNA of a 1015 bp fragment was injected and led to the same phenotypes as described previously [14]. Control spiders were injected with water.

Embryonic RNAi was performed in A. tepidariorum by injections into the perivitelline space as described for Cupiennius salei [38]. A. tepidariorum embryos were injected at stage 4, being the earliest stage that has a perivitelline space that is wide enough for injections, thus sticking to the Cupiennius protocol [38] as closely as possible.

In situ hybridisation, DNA labeling, imaging

In situ hybridisation, TUNEL and nuclear staining were performed as described previously [44,48,49] with minor modifications. For the TUNEL experiments both negative and positive
controls were performed as previously described [49] to confirm the specificity of the obtained stainings (Figure S1B–S1D). Images of spider embryos or adult spiders were either captured with a Zeiss Axiosplan-2 microscope or with a Leica dissection microscope equipped with an Intas digital camera and UV-light. Confocal x-stacks of spider larvae and of the sternum of adult spiders were captured using a Zeiss LSM 510. Before scanning the animals were heat fixed, embedded in Voltalef H10S oil and covered with a cover slip.

Supporting Information

Figure S1  Confirmation of the RNAi with the expression of At-Dll. (A, A') Detection of At-Dll transcripts in the early stripe in a stage 6 control embryo (A) and detailed magnification in (A'). Transcripts are detected in the nuclei and the cytoplasm. (B, B') Detection of At-Dll transcripts in a stage 6 At-Dll RNAi embryo (B) and detailed magnification in (B'). Nascent At-Dll transcripts are detected in the nucleus, but no transcripts are present in the cytoplasm. (C, D) Expression of At-Dll in control (A) and At-Dll RNAi animals (B) at late stage 8. While expression of At-Dll remains permanently downregulated by RNAi in the majority of embryos, in some embryos expression reappears (D), leading to normal expression in later stages. Abbreviations: Ch, cheliceral segment; L, walking leg segment; Pp, pedipalpal segment.

Figure S2  Slit sense organs on the sternum of adult male animals. (A) Control animal. (B) Dll RNAi animal. The slit sense organs are emphasized in green colour. The sense organ of the first walking leg segment (denoted by the white circle) is lacking in At-Dll RNAi animals.

Figure S3  Shape comparison of the sternum in wildtype and Dll RNAi animals. (A, B) Adult wildtype and Dll RNAi spiders (same images as in Figure 2G, 2H) where the sternum has been marked in colour (red and blue, respectively). (C) Alignment of the shape of the Dll RNAi sternum (blue) with the wildtype sternum (red) reveals that the posterior edges (edges 2 and 3) align well, but edge 1 is only present in the wildtype sternum. Abbreviations: L, walking leg.

Figure S4  No interaction between At-Dll and At-hb. (A, B) Early (stage 6) expression of At-hb is virtually identical in control (A) and At-Dll/RNAi animals (B). (C, D) Early (stage 6) expression of At-Dll is virtually identical in control (C) and At-hb RNAi animals (D). The embryos have also been stained with a probe against orthodontic (At-otd) to mark the anterior rim of the embryo. Abbreviations: Ch, cheliceral segment; L, walking leg segment; Pp, pedipalpal segment.

Figure S5  Dll expression in Pholcus phalangioides embryos. (A) An adult Pholcus phalangioides female. (B) Several embryos of P. phalangioides stained for Pp-Dll transcripts. The embryos are in a developmental stage that is comparable to stage 6/7 of Achaearanea tepidariorum embryos. For comparison, an embryo of A. tepidariorum at stage 6 (denoted by the asterisk) and stained for At-Dll transcripts has been placed next to the P. phalangioides embryos. Similar to the expression of Dll in A. tepidariorum also Pp-Dll is expressed in an early anterior ring/strip like domain. (C) Close-up of a Pp-Dll stained P. phalangioides embryo. The asterisk marks the cumulus and the white dotted line indicates the border between the embryonic and the extra-embryonic cells. Note the Pp-Dll expression stripe that is close to the anterior end of the embryo. (D, E) Late embryonic Pp-Dll expression pattern at the beginning of germ band inversion (D) and at the end of inversion (E). Pp-Dll transcripts are detected in the distal parts of all developing appendages. This late expression is virtually identical to Dll expression of other spider species [38,44,50]. Abbreviations: ch, cheliceral segment; L, walking leg segment; pp, pedipalpal segment; O, opisthosomal segment; A, anterior end; P, posterior end.

Figure S6  Overview of spider phylogeny. Spiders (Araneae) comprise a few primitive clades (Mesothelae, Mygalomorphae) and the speciose clade Neocribellatae that comprises the vast majority of all extant spiders. This clade consists of two sister groups, Haplogynae and Entelegynae. So far, all spider species (except for Acanthoscurria geniculata [44]) used for gene expression or gene function studies belong to the Entelegynae. The cellar spider Pholcus phalangioides is the only studied member of the Haplogynae. The similarities in early Dll expression in A. tepidariorum and P. phalangioides indicate that this patterning function of Dll was already present before the split of Haplogynae and Entelegynae. The tree is simplified after [51]. Only representative well-known families (where English common names were available) are shown, and examples (genus names) are given when representatives of the group have been used in gene expression/function studies. The red dots denote the placement of Achaearanea and Pholcus in the phylogenetic tree.

Figure S7  Embryonic RNA interference with At-Dll. (A) Control embryo injected with water. (B) After embryonic RNAi (eRNAi) with At-Dll all appendages are present but reduced in size. While parental RNA interference with At-Dll affects the early (segmental) and late (appendage) gene function (compare to Figure 2), embryonic RNAi only affects the development of the appendages. This result is consistent with the findings in Cupiennius salei in which eRNAi with Cs-Dll is leading to appendage defects, but no segment loss has been observed [38]. (C) Summary of the eRNAi results in A. tepidariorum. The phenotype was assessed at the late inversion stage. “Complete phenotype” refers to embryos with all appendages reduced in size. “Mosaic phenotype” refers to embryos with only some appendage reduced in size. “Developmental arrest” refers to embryos that showed some development, but died before reaching the late inversion stage, whereas “dead” refers to embryos that died at stage 4 and thus at or shortly after injection. “Unspecific” refers to embryos that reached the late inversion stage, but showed injection artefacts already known from eRNAI experiments in Cupiennius salei (i.e. malformation of body parts caused by unintentional injuries during injection). Abbreviations: Ch, cheliceral segment; L, walking leg segment; Pp, pedipalpal segment.

Figure S8  Confirmation of specific TUNEL staining after parental At-Dll knockdown. (A) An At-Dll pRNAi embryo showing enhanced cell death within the affected L1/L2 region. Please note that the L1 segment is already deleted in this embryo. (B) Only a few apoptotic cells are marked in the wild type control TUNEL staining. (C) In the negative control water instead of terminal deoxynucleotidyl transferase was added to the labelling reaction. (D) In the positive control the embryos were treated with DNase I before dig-UTP labelling [49]. Abbreviations: Ch, cheliceral segment; L, walking leg segment; Pp, pedipalpal segment.
Table S1 Summary of the At-Dll RNAi phenotypes. Adult females were injected five times (over a period of 10 days) with 1.5 μl dsRNA (3.0 μg/μl). Control females were treated the same as experimental animals, but injected with water. Female A. tepidarium produce several cocoons during their lifetime. For this summary, embryos of cocoons 2 and 3 have been pooled and randomly chosen embryos have been assessed in detail for morphology and At-Dll expression.

Video S1 Prey-catching behaviour of a wildtype A. tepidarium. The spider has caught a Drosophila fly and immobilizes it using silk threads. The silk threads are mainly manipulated with the legs of the L4 segment. The legs of the different walking leg segments L1 to L4 are highlighted in red in the final frames of the movie.

Video S2 Prey-catching behaviour of an At-Dll RNAi A. tepidarium. The spider has caught a Drosophila fly and immobilizes it using silk threads. Like the control spiders, the RNAi spiders also manipulate the silk threads mainly with the legs of the L4 segment. There is no significant difference in this behaviour between control and RNAi spiders, despite the latter are lacking the L1 leg pair. The legs of the different walking leg segments L2 to L4 are highlighted in red in the final frames of the movie.

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Author Contributions
Conceived and designed the experiments: MP N-MP. Performed the experiments: MP SK NT. Analyzed the data: MP APM WGMD N-MP.

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