The *Sp8* zinc-finger transcription factor is involved in allometric growth of the limbs in the beetle *Tribolium castaneum*

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Summary

Members of the Sp gene family are involved in a variety of developmental processes in both vertebrates and invertebrates. We identified the ortholog of the *Drosophila Sp-1* gene in the red flour beetle *Tribolium castaneum*, termed *T-Sp8* because of its close phylogenetic relationship to the vertebrate *Sp8* genes. During early embryogenesis, *T-Sp8* is seen in segmental stripes. During later stages, *T-Sp8* is dynamically expressed in the limb buds of the *Tribolium* embryo. At the beginning of bud formation, *T-Sp8* is uniformly expressed in all body appendages. As the limbs elongate, a ring pattern develops sequentially and the expression profile at the end of embryogenesis correlates with the final length of the appendage. In limbs that do not grow out like the labrum and the labium, *T-Sp8* expression remains uniform, whereas a two-ring pattern develops in the longer antennae and the maxillae. In the legs that elongate even further, four rings of *T-Sp8* expression can be seen at the end of leg development. The role of *T-Sp8* for appendage development was tested using RNAi. Upon injection of double stranded *T-Sp8* RNA, larvae develop with dwarfed appendages. Affected *T-Sp8*^{RNAi} legs were tested for the presence of medial and distal positional values using the expression marker genes *dachshund* and *Distal-less*, respectively. The results show that a dwarfed *T-Sp8*^{RNAi} leg consists of proximal, medial and distal parts and argues against *T-Sp8* being a leg gap gene. Based on the differential expression pattern of *T-Sp8* in the appendages of the head and the thorax and the RNAi phenotype, we hypothesise that *T-Sp8* is involved in the regulation of limb-length in relation to body size – a process called allometric growth.

Key words: *Tribolium*, *Sp8*, Leg elongation, Allometric growth, *Serrate*

Introduction

The body appendages of insects, spiders and crustaceans show a high degree of variation between species. The numerous modifications of the appendages contributed substantially to the evolutionary success of the arthropods. Within an individual, limbs are also morphologically distinct depending on their function in sensory perception, feeding or locomotion. Form and length of an appendage is the result of the specification of limb identity and the modulation of its growth. Segment identity together with appendage identity is controlled by the Hox genes (Averof and Patel, 1997; Morata, 2001; Morata and Sanchez-Herrero, 1999) but little is known about genes controlling the shape of the serially homologues limbs (Stern, 2003). Differences in the size of an organ – such as the limb – in relation to body size or to other organs is defined as allometry (Stern and Emlen, 1999). Hox genes have been described to contribute to the allometric growth of specific insect limbs (Stern, 2003). Likely candidates for genes that play a general role in the process of allometric growth should be expressed prior to or from the beginning of appendage formation onwards in all limb buds. Later, these genes should be differentially expressed in appendages of different length.

The molecular basis of limb development has been studied intensively in *Drosophila*. The anlagen of the adult appendages develop during embryonic and larval stages as flat, set-aside cell nests, the imaginal discs. Initially, they become subdivided by the signalling proteins Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg). Gradients of Dpp and Wg activate its downstream target genes *homothorax* (*hth*) in the proximal, and *Distal-less* (*Dll*) and *dachshund* (*dac*) in the distal part of the leg disc (Morata, 2001). At present, the leg gap genes *Dll*, *dac* and *hth* are thought to be sufficient for the formation of all the adult leg segments in *Drosophila* by activating their target genes (Rauskolb, 2001). Recently, a *Sp*-like gene has been isolated in *Drosophila* (*D-Sp1*) that is expressed in a *Dll* like fashion in the leg imaginal discs (Wimmer et al., 1996). This raises the question whether possibly more genes are required early for the process of leg formation. In contrast to *Drosophila*, the red flour beetle *Tribolium* (Sokoloff, 1972) and many hemimetabolous insect species, such as the cricket, the grasshopper and gryllus (Inoue et al., 2002; Jockusch et al., 2000; Niwa et al., 2000; Panganiban et al., 1994), show a more ancestral mode of appendage formation. In these species, the formation of an appendage starts already early during embryogenesis as a small outgrowth from the body wall, the limb bud (Brown et al., 1994). As development proceeds, the leg continuously elongates and the leg segments differentiate before hatching. Despite the morphological differences, a

hierarchical subdivision of the appendage anlage takes place in both species *Drosophila* and *Tribolium* (Prpic et al., 2001; Rauskolb, 2001) and orthologs genes are required for this process (Abzhanov et al., 2001; Beermann et al., 2001; Prpic and Tautz, 2003; Prpic et al., 2001).

We describe the isolation, the expression pattern and the function of the *D-Sp1* ortholog from the beetle *Tribolium*, termed *T-Sp8*. We found that *T-Sp8* belongs to the Sp-class of zinc-finger transcription factors that have been isolated from nematodes, insects and vertebrates (Kaczynski et al., 2003). Functionally, Sp-like genes are involved as transcriptional regulators in the segmentation process, growth control, tissue differentiation and neoplastic transformation. Members of this protein family share a highly conserved Cys2-His2 zinc-finger protein motif that has been shown to bind to GC-rich promotors (Kaczynski et al., 2003). We show that *T-Sp8*, together with its *Drosophila* ortholog *D-Sp1*, can phylogenetically be grouped close to the vertebrate *Sp7* and *Sp8* genes. In the embryo, *T-Sp8* expression was observed in segmental stripes prior to the formation of the appendages and, like the gene *Distal-less* (Beermann et al., 2001), during the complete process of limb development. Double staining of *T-Sp8* with *Dll* revealed co-expression of both genes in more distal regions, and exclusive expression of *T-Sp8* in the proximal leg. In the early limb bud, *T-Sp8* was shown to be uniformly expressed. As the limb elongated, ring-like expression domains developed sequentially. The number of *T-Sp8* expression domains correlated with the final length of the appendage. We observed a remaining uniform expression in short appendages, such as the labium, whereas in the antennae and in the legs, two and four *T-Sp8* expression domains, respectively, developed. A knock-down of *T-Sp8* function via RNAi led to a shortening of the appendages from the head and the thorax. Affected legs retained proximal, medial and distal values, and therefore *T-Sp8* cannot be designated as a leg gap gene. Adult beetles that displayed the *T-Sp8* RNAi phenotype as larvae, also had dwarfed legs and shortened antennae. We conclude that *T-Sp8* is required for the differential outgrowth of the body appendages and thus contributes to shape the insect limb.

Materials and methods

Breeding beetles, collection of eggs and pupae

Beetles were reared on wholewheat flour supplemented with 5% yeast powder and 0.5% Fumidil at 33°C (Berghammer et al., 1999). For injection of double-stranded RNA into embryos (embryonic RNAi), eggs were collected from a 3 hour egglay and injected during nuclear divisions. For parental RNAi, young female pupae were selected from the stock and injected with double-stranded RNA as described (Bucher et al., 2002).

Isolation and cloning of *T-Sp8* and *T-Serrate*

PCR fragments of the Sp family have been obtained using degenerate PCR primer directed against a part within the zinc-finger region (Sp-f, 5′CAYATHGGN GARMGNCCNTTYMARTG 3′; and Sp-r, 5′TGNRTYTTCATRTGYTTYTTNARR TGRTC 3′). The resulting 176 bp PCR products were cloned in TA-vectors (Invitrogen) and sequenced. The complete cDNA sequence was generated with 3′ and 5′ RACE reactions using SMART technology (Clontech). The *Serrate* ortholog has been isolated from *Tribolium* using the degenerate PCR primer DL2 and DL2re/DL3re as described (Stollewerk, 2002). The PCR products (597 bp) were subcloned and verified by sequencing. The GenBank Accession Numbers for *T-Sp8* and *T-Serrate* are AY316682 and AY453651, respectively.

Sequence alignment and phylogenetic analysis

Clustal W alignments were obtained with the LASERGENE-DNASTAR® package and phylogenetically analyzed using TREE-PUZZLE (Strimmer and von Haeseler, 1996).

The amino acid positions for the alignment shown in Fig. 1B are (GenBank Accession Number/position of amino acids): *Tribolium castaneum* T-Sp8, AY316682/287-398; *Drosophila melanogaster D-Sp1*, AAF46519/333-444; *Homo sapiens* Sp8-Hs, XP_166519/290-401; *Gallus gallus* Gg, CAC84905/amino acids 586-694; *Mus musculus* Sp3Mm, AAC16322/amino acids 540-648; ostMm: NP_569725/263-374; HsSp4, NP_003103/621-730; HsSp1, AAF67726/597-706; UKLF, AB015132/197-302; TIEG1, U21847/amino acids 341-452; TIEG2, AF028008/amino acids 366-477; EZF, U70663/365-470; BTEB2, D14520/112-218; btdDmel, NP_511100/303-414; SP7Hs, NM_152860/366-377; SP5mm, NP_071880/270-379; and SP6, XP_064386/300-413. The amino acid positions for the alignment shown in Fig. 1C are: Tc Sp, pos 27-72; Dm Sp1, 14-59; Hs Sp 8, 7-56; Rn Sp 8, 149-198; Hs Sp 7, 7-53; Mm Sp5, 23-72; Hs Sp4, pos 39-88; Hs Sp 2, 19-66; Hs Sp 1, 52-101.

The amino acid sequences used in the alignment shown in Fig. 6 are (GenBank Accession Number/position of amino acids): Dm-btd, NP_511100/30-414; Tc-Sp, AY316682/30-398; Dm-Sp1, AAF46519/17-444; Hs-Sp1, AAF67726 + P08047/196-706; Hs-Sp2, M97190/1-490; Gg-Sp3, CAC84905/190-694; Mm-Sp3, AAC16322 + XP_130306/143-648; Hs-Sp4, CAA48535 + NP_003103/191-730; Mm-Sp5, NP_071880/1-379; Mm-Sp6, XP_064386/11-413; Hs-Sp7, NM_152860/10-377; Mm-*osterix*, NP_569725/10-374; Hs-Sp8, NM_182700/10-456; *Rattus norvegicus* Rn-Sp8, XP_234724/113-513; Hs-TIEG1, U21847/58-452; Hs-TIEG2, AF028008/65-477; Hs-UKLF, AB015132/38-302; Hs-EZF, U70663/41-470; and Hs-BTEB2, D14520/1-218.

Nomenclature

Sp genes have been named after sephacryl and phosphocellulose columns used in the original purification protocol (Kadonaga et al., 1987). The described *Tribolium Sp* gene was assigned *T-Sp8* because the DNA sequence is more similar to the vertebrate *Sp8* than to the *Sp7* genes. To name *T-Sp8* '*T-Sp1*' would imply an incorrect structural relationship to the vertebrate *Sp1* (see also Fig. 6). Therefore the fly *D-Sp1* gene represents the *Sp8* ortholog.

RNA interference

Double-stranded (ds) RNA corresponding to nucleotide position 1-1325 of the *T-Sp8* gene was prepared and injected at a concentration of 500 ng/μl into pupae or embryos as described (Bucher et al., 2002; Schröder, 2003). The same dsRNA preparation was used for parental and embryonic RNAi experiments.

Cuticle preparation and in situ hybridisation for analysing embryos, larvae and adults

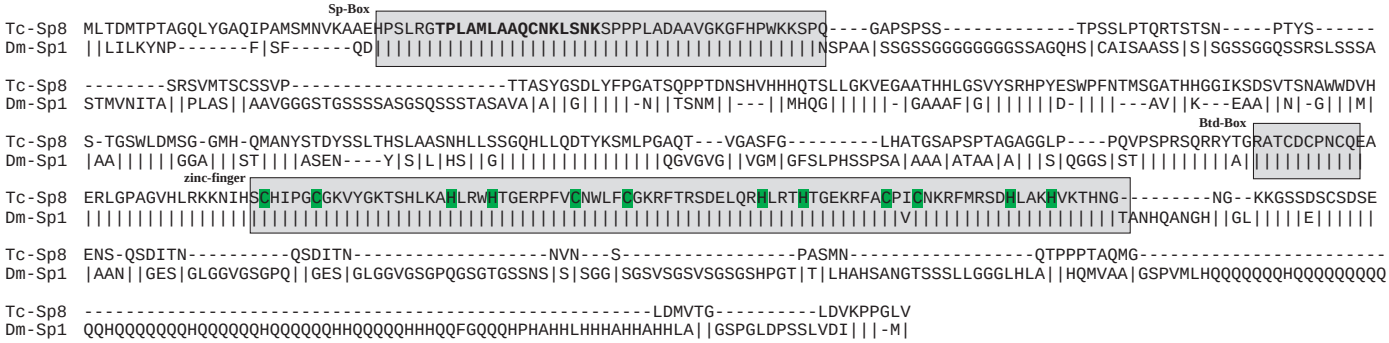
Larval and adult cuticles were embedded in Hoyer's medium (Van der Meer, 1977). Adult beetles were boiled in 10% KOH prior to embedding. In situ hybridisation was carried out as previously described (Tautz and Pfeifle, 1989) using labelled riboprobes (Klingler and Gergen, 1993).

Results

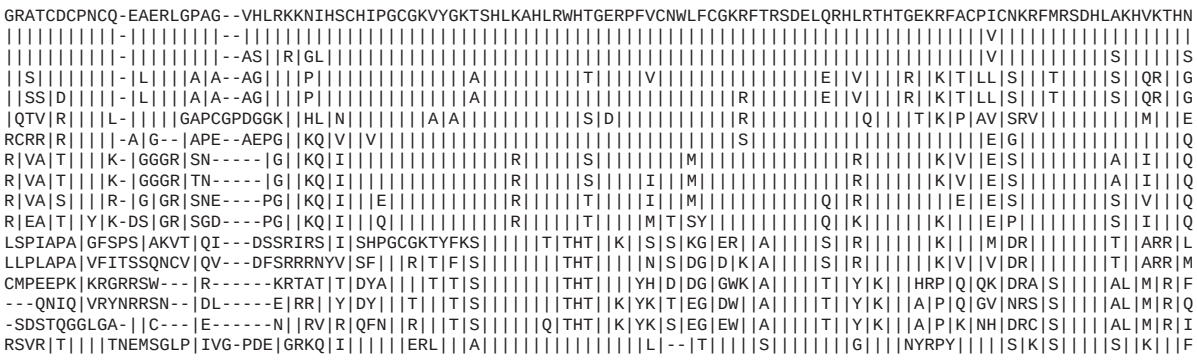
Isolation, structure and phylogenetic position of the *T-Sp8* gene within the *Sp* gene family

We isolated a Sp-like gene from *Tribolium* (*T-Sp8*) using a PCR approach with degenerate oligonucleotides that corresponded to evolutionary conserved regions of the zinc

A



B



Tc: aa 287-398; Dm: AAF46519 (aa 333-444); Hs: NM_182700 (aa 346-457); Gg: CAC84905 (aa 586-694); Sp3Mm: AAC16322 (aa 540-648); ostMm: NP_569725 aa 263-374; HsSp4: NP_003103 (aa 621-730); HsSp1: AAF67726 (aa 597-706); UKLF: AB015132 (aa 197-302); TIEG1: U21847 (aa 341-452); TIEG2: AF028008 (aa 366-477); EZF: U70663 aa 365-470: BTEB2:D14520 (aa 112-218); btdDmel: NP_511100 (aa 303-414); SP7Hs: NM_152860 (aa 366-377); SP5mm: NP_071880 (aa 270-379); SP6: XP_064386 (aa 300-413).

C

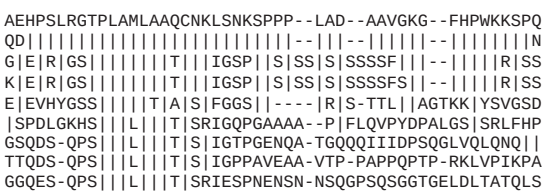


Fig. 1. Sequence alignment of Sp-like proteins. (A) Comparison of the Sp8 orthologs from *Tribolium* and *Drosophila*. Serines and threonines are only moderately enriched and glutamine rich regions are not present in the deduced T-Sp8 protein. These regions are present in the *Drosophila* ortholog and are thought to be targets for post-translational modification (serines/threonines) or to be essential for transcriptional activation (glutamines) (Bouwman and Philipsen, 2002). (B) Alignment of the Btd-box and the adjacent zinc-finger region of different species; the cysteines (C) and histidines (H) structuring the zinc fingers are marked in green in A. (C) A comparison of the N-terminal Sp-box reveals a closer relationship of the insect sequences to *Sp8* than to *Sp7*. The Sp-box is suggested to function in regulating the proteolysis of Sp factors or alternatively as a motif for interaction with a repressor (Bouwman and Philipsen, 2002). | indicates identical amino acid positions, – refers to gaps. Hs, *Homo sapiens*; Gg, *Gallus gallus*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*.

fingers. Cloning and sequencing of the 165 bp fragment identified the *T-Sp8* sequence as an ortholog of the *Drosophila Sp1* (*D-Sp1*) gene (Wimmer et al., 1993). The complete cDNA sequence was generated in 3′ and 5′ RACE reactions using cDNA made from 1- to 3-day-old embryos that comprise all embryonic stages. *T-Sp8* encodes a 456 amino acid protein as deduced from its DNA sequence (Fig. 1A). The T-Sp8 protein is considerably shorter than the *Drosophila* ortholog (691 amino acids) because of the lack of the long glycine and serine repeats in the N-terminal and the glutamine-rich regions in the C-terminal part of the protein (Fig. 1A). An alignment of the amino acid sequences from these two species reveals high conservation not only of the zinc-finger region but also of two other protein motifs in the *Sp* genes, the Sp-box and the Buttonhead (Btd)-box. The Sp-box is a 13 amino acid motif located at the N terminus of the protein, whereas the Btd-box has been described as a 10 amino acid box situated immediately N terminal to the zinc-finger domain. At the moment, the roles of these motifs for the function of the Sp protein is unknown. They are suggested to be involved in transactivation activity (Btd-box) or as an interaction-site with a repressor (Sp-box) (Bouwman and Philipsen, 2002). The comparison to the *D-Sp1* sequence reveals a conserved stretch of 43 amino acids in the Sp-box region of both insect species that include the described Sp-box core (bold in Fig. 1A). The alignment of the Sp-box to the vertebrate orthologs shows that *T-Sp8* is slightly more similar to the yet functionally uncharacterized human *SP8* gene (Bouwman and Philipsen, 2002; Ravasi et al., 2003) than to the *osterix*/*Sp7* gene (Nakashima et al., 2002) that has been shown to be required for bone development in the mouse. The Btd-box of *Tribolium-Sp8*, *Drosophila Sp1* and human *SP8* is identical

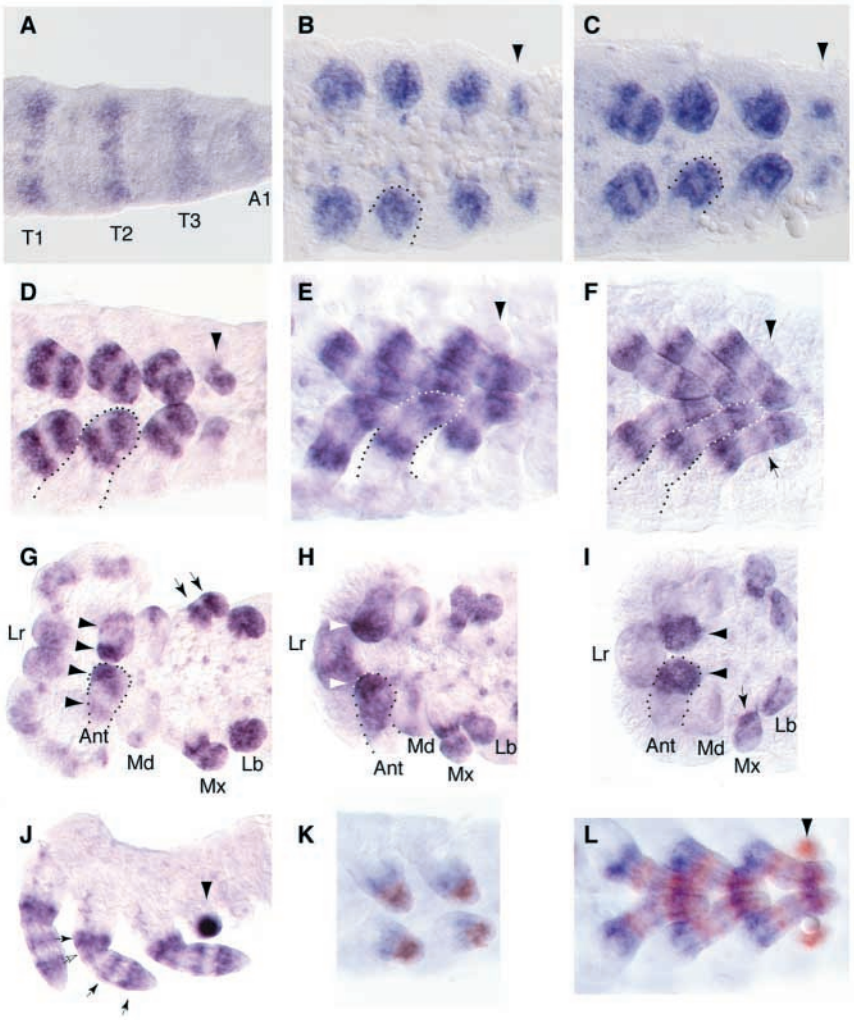


Fig. 2. *T-Sp8* expression in wild-type *Tribolium* embryos. Successively older stages are shown from A-F. (A) *T-Sp8* is expressed in segmental stripes in the complete embryo prior to limb bud formation. Only the thoracic segments (T1-3) and abdominal segment 1 (A1) are shown. (B) *T-Sp8* is uniformly expressed in the young limb bud (arrowhead indicates the pleuropodium, the appendage of abdominal segment 1). (C) *T-Sp8* resolves into two rings but is still seen at the limb tip (arrowhead indicates the pleuropodia). (D) *T-Sp8* begins to retract from the limb tip (arrowhead indicates the pleuropodia). (E) The process of leg elongation continues. No *T-Sp8* expression is detectable in the pleuropodia (arrowhead). (F) A third, slightly weaker *T-Sp8* domain (arrow) intercalates between the primary rings. (G) *T-Sp8* expression in the head (same embryo as in D). Two ring-shaped expression domains can be seen in the antennae (arrowheads) and the outer branch of the maxillae, the telopodit (arrows). *T-Sp8* expression is strong in the labial buds (Lb) and diffuse in the labrum (Lr). One small distal positioned *T-Sp8* spot is visible in the mandibles (Md) that presumably marks a sensory organ. (H) *T-Sp8* expression in the head (same embryo as in E). Strong *T-Sp8* expression is seen at the distal tip of the antennae (arrowhead) whereas in the rest of this limb, *T-Sp8* expression is diffuse. (I) *T-Sp8* expression in the head (same embryo as in F). In the antennae, only the distal tip shows *T-Sp8* expression (arrowhead). In the maxillae the proximal *T-Sp8* domain is still visible (arrow). (J) The three rings of *T-Sp8* expression are now fully developed (solid arrows) and a fourth ring appears (outlined arrow). The staining in the pleuropodia (arrowhead) is due to a known artefact. (K,L) Double labelling of *T-Sp8* (in blue) and *Distal-less* (*Dll*, in red) expression reveals that the proximal *T-Sp8* ring lays outside of the distal *Dll* expression domain (arrowhead indicates the pleuropodia in J).

and 2/10 amino acids are different in the Btd-box of human *SP7*.

T-Sp8 is dynamically expressed in the appendages during the complete process of limb formation

Prior to the formation of the limb buds, *T-Sp8* is expressed in every segment in an embryo undergoing germ band elongation. Like the *Dll* ortholog in *Tribolium* (Beermann et al., 2001), *T-Sp8* is expressed in the body appendages from the beginning of bud formation onwards (Fig. 2). During the successive stages of leg growth, *T-Sp8* expression resolves into two ring domains: one proximal (*T-Sp8*^{prox}) and one distal (*T-Sp8*^{dist}). The distal domain initially also covers the leg tip (Fig. 2B-D), but retracts quickly to establish a subterminal ring that persists until the end of leg growth. During further leg outgrowth, a third, slightly weaker *T-Sp8* expression domain (*T-Sp8*^{med}) intercalates between the primary rings (Fig. 2F). At the end of the leg elongation process, *T-Sp8*^{prox} has stretched out and a weak fourth ring appears at its distal boundary (Fig. 2J). *T-Sp8*/*Dll* double labelling reveals that both genes are expressed at the same time in the growing (Fig. 2K) and in the fully elongated leg (Fig. 2L). *T-Sp8*^{dist} and *Dll* are partially co-expressed in the distal part of the leg. The other expression domains of both genes border each other with no considerable overlap (Fig. 2L). In the head appendages of the *Tribolium* embryo, *T-Sp8* expression characteristically reflects the length and fate of the respective limb. *T-Sp8* is strongly expressed in the antennae, the maxillae and the labium but only weakly, diffuse and transiently in the labrum and the mandible (Fig. 2G). As in the leg, an initially uniform expression pattern develops into ring domains in the antennae and the telopodite of the maxillae. Until the end of embryogenesis, these limbs stretch out and develop into ‘long’ appendages. In contrast to the even longer thoracic legs, no additional *T-Sp8* domain develops in the antennae or maxillae. Rather, the proximal antennal *T-Sp8* domain ceases early (Fig. 2G-I) and only the distal domain remains strong until the antenna reaches its final size. The distal domain in the antenna is different to that in the leg in that it never retracts from the tip, whereas the distal *T-Sp8* domain in the maxilla forms as a subterminal ring (Fig. 2G). In the ‘short’ head appendages – the labial palps – uniform *T-Sp8* expression remains at high levels and does not resolve into ring domains. The two labial buds do not grow out but fuse to build the

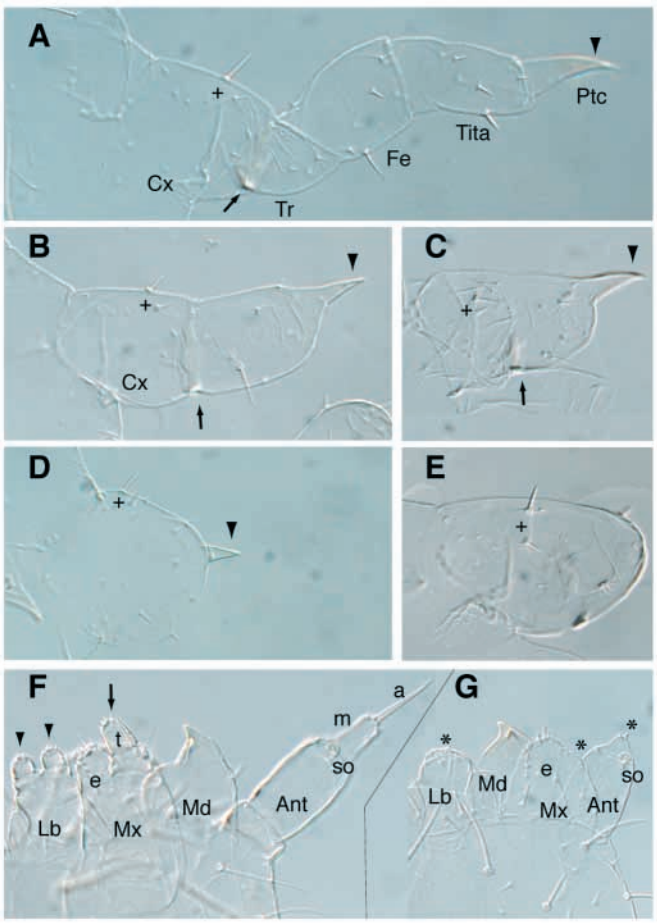


Fig. 3. Cuticle preparations of wild-type and *T-Sp8* RNAi larval legs. (A-E) The joint between coxa and trochanter is easily identifiable by its heavy cuticularized structure (arrow). + indicates the presence of two bristles of different length (‘mother and father’) that are indicative for the coxa. The arrowhead indicates the tip of the pretarsal claw. (A) The leg of a wild-type first instar larvae is subdivided into the coxa (Cx), the trochanter (Tr), the femur (Fe), the tibiotarsus (Tita) and the pretarsal claw (Ptc). (B) Cuticle of a *T-Sp8*^{RNAi} leg: weak phenotype. The coxa (Cx) is not affected; the segment distal to the coxa appears to be of mixed character. (C) Cuticle of a *T-Sp8*^{RNAi} leg: strong phenotype. The coxa is shorter than in the wild type, where the joint has developed partially. (D) Cuticle of a *T-Sp8*^{RNAi} leg: very strong phenotype; only part of the pretarsal claw present (arrowhead). (E) Cuticle of a *T-Sp8*^{RNAi} leg: very strong phenotype, no pretarsal claw present. Note that the coxa is also proximally shortened (C-E). (F) Cuticle of a larval head (wild-type) Lb: labium, arrowheads indicate the palps of the labium, the arrow indicates the telopodite (t) of the maxilla (Mx); e, endite of the maxilla. Ant, antenna; so, sense organ of the antennae; m, middle part; a, arista. (G) Effect of *T-Sp8* RNAi on the head appendages. The asterisk marks the affected limbs: the distal palps of the labium are missing, the telopodite of the maxilla is strongly reduced. In weaker affected embryos, a reduction of the length has been observed (not shown). In the antenna, the middle segment is strongly reduced in size. No effect was seen in the mandibles. Only half of the head is shown in F,G.

unpaired labium at the end of embryogenesis. *T-Sp8* is only faintly expressed in the pleuropodia, the appendages of the first abdominal segment with leg-like character (Lewis et al., 2000) (Fig. 2A-D). Expression in the pleuropodia ceases at a stage when the thoracic legs reach ~70% of their final length (Fig. 2E). In contrast to the legs, the pleuropodia do not grow out further and do not contribute to a cuticular structure of the larva. Instead, these limbs become internalized later during development and function as a hatching gland.

Table 1. *T-Sp8*^{RNAi} leg phenotypes

Embryonic *T-Sp8* RNAi: analysis of the larval leg phenotype (*n*=576; *N*=96)

	%/*n*		%/*n*		%/*n*
Very strong	79/453	Strong	14/81	Weak	7/42
+ claw	31/142	+ claw	91/74	+ claw	98/41
– claw	69/311	– claw	9/7	– claw	2/1

Parental *T-Sp8* RNAi: analysis of the larval leg phenotype (*n*=708; *N*=118)

	%/*n*		%/*n*		%/*n*
Very strong	14.5/103	Strong	63.5/443	Weak	23/162
+ claw	92/95	+ claw	100/443	+ claw	100/162
– claw	8/8	– claw	–/–	– claw	–/–

Parental *T-Sp8* RNAi: analysis of the adult leg phenotype (*n*=192; *N*=32)

	%/*n*		%/*n*
Strong	22/42	Weak	78/150

T-Sp8 RNAi leads to dwarfed legs. The ‘very strong’ effect is seen significantly more often in larvae that were injected as embryos (embryonic RNAi). In addition, the loss of the pretarsal claw occurs with a higher frequency in embryonic than in parental RNAi experiments. In the first egg lay of parental RNAi experiments the same phenotypic classes could be obtained; however, the ‘very strong’ phenotype was observed less often.

In control experiments with *orthodenticle* double stranded RNA injected in either female pupae (*N*=111) or embryos (*N*=100), no leg specific phenotype was observed (Schröder, 2003).

n, number of analysed legs; *N*, number of analysed larvae/adults.

Functional analysis of *T-Sp8*

The homogenous expression of *T-Sp8* in short (labium) and the sequential development of two or four *T-Sp8* rings in short or long appendages (antenna, leg) led us to hypothesize that *T-Sp8* is involved in the process of appendage elongation. To test this theory, we applied RNA interference (RNAi) to reduce or to abolish *T-Sp8* function in the developing *Tribolium* embryo. Indeed, upon injection of double stranded *T-Sp8* RNA, embryos develop short appendages. The effect is most evident in the legs but is also seen in all of the head appendages except the mandibles. The defects in the legs (*T-Sp8*^{RNAi} legs) range from very strong to weak phenocopies (Fig. 3; Table 1). In a strongly affected larval leg, the pretarsal claw, as the most distal pattern element, is shortened and fused to a jointless leg stump (Fig. 3D). Even stronger phenocopies display only the leg stump (Fig. 3E) lacking the claw structure. This indicates that *T-Sp8* acts upstream of *Distal-less*, which is required for the formation of the most distal structures of an appendage. It is not possible to assign individual segment identity to such a strongly affected *T-Sp8*^{RNAi} leg. However, the arrangement and number of bristles on the stump suggest that it is composed of several leg segments. In the wild-type leg, the individual bristles are more widely spaced. Slightly weaker affected legs are composed of pattern elements from the

coxa, the trochanter and the claw (Fig. 3C). Between the parts of the trochanter and the claw, further bristles that probably represent intermediate pattern elements are found, but no further segment is built. In very weak phenocopies, only the distal tibia is affected (not shown). This suggests the requirement for high *T-Sp8* doses in this part of the leg appendage.

Surprisingly, the *T-Sp8* RNAi phenotype can also be seen in the legs and the antennae of adult beetles that showed a strong *T-Sp8* phenotype as larvae (Fig. 4B,C; Table 1). The strong adult leg phenotype resembles the strong larval phenotype. In these, the distal-most structure, a pair of claws, is connected with a joint to a leg stump that itself is attached via a joint to the body wall (Fig. 4C). Such a leg and the claws still move in a coordinated way, indicating that the involved muscles are innervated properly. Owing to the lack of cuticular markers, it cannot be decided which leg segments contribute to the jointless leg stump. Weaker affected legs are longer. In these, the claws and a club-like structure that shows characteristics of a tibia are attached to the femur (Fig. 4B). These tibiae are rotational symmetric and covered with ventral bristles, indicating that *T-Sp8* might be also involved in organizing dorsoventral polarity of the growing leg. The occurrence of the adult leg phenotype could be explained by the requirement for Sp function in the larva or the pupae in cells that will give rise to the adult structures. At the moment, the position of such cells is not known in *Tribolium*, but histoblasts that possibly contribute to the adult legs have been described for the related beetle species *Tenebrio* (Lenoir-Rousseaux and Huet, 1976).

The adult antennae also display a *T-Sp8* phenotype. Wild-type antennae consist of a base with the scape and the pedicel, followed by the six segments of the middle region and three distinct segments of the terminal club (Fig. 4D). Affected antennae are shorter than those of the wild type, owing to the loss and/or the fusion of the middle antennal segments (Fig. 4E). Out of 22 analysed antennae, 10 showed four and 12 showed five segments only. Neither the base nor the terminal segments were affected. This phenotype resembles the antennal phenotype seen in the weak *Distal-less* allele *sa* (Beermann et al., 2001), showing a common function of *T-Sp8* and *Dll* in elongating the adult antennae.

Dwarfed *Sp*^{RNAi} legs are composed of proximal, medial and distal positional values

At the cuticle level, the strong *T-Sp8* RNAi phenotype shows a severe shortening of the proximodistal axis. It remains unclear which parts of the wild-type leg contribute to the *T-Sp8*^{RNAi} leg. Therefore we analysed the expression of the leg gap genes *dachshund* and *Distal-less* in the developing embryonic leg. The expression pattern of these genes in the wild type can be used as marker for proximal (*dachshund*, *dac*) (Prpic et al., 2001), medial (*dachshund*) (Dong et al., 2001; Mardon et al., 1994) and distal (*Distal-less*) (Cohen et al., 1989; Sunkel and Whittle, 1987) positional information. A small spot of *Dll* expression at the tip of the *T-Sp8*^{RNAi} leg proves the presence of the most distal limb fate (Fig. 5B). The proximal (S-spot) and part of the distal (P-region) *dac* expression domains in such legs represent proximal and medial positions along the proximodistal axis (Fig. 5D) (Prpic et al., 2001).

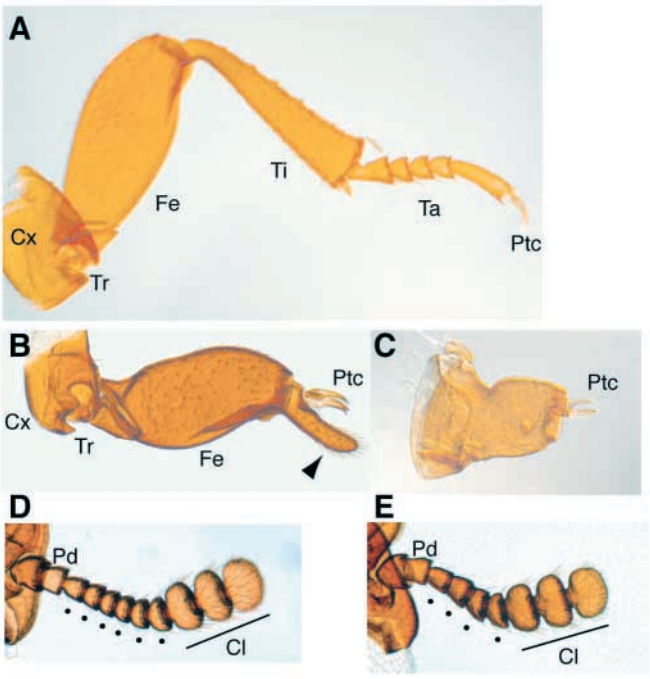


Fig. 4. Cuticle preparations of wild-type and *T-Sp8* RNAi adult legs and antennae (proximal points to the left, distal to the right). (A) The adult leg is composed of the same leg segments as the embryo except that the tibia (Ti) and the tarsal segments (Ta) are distinct. (B) *T-Sp8* RNAi weak phenotype. The leg lacks the tarsus. The pretarsal claw (Ptc) together with the club shaped tibia-like structure (arrowhead) is fused to a shortened femur. (C) *T-Sp8* RNAi strong leg phenotype. Only the pretarsal claw (Ptc) is connected with a joint to the otherwise jointless leg stump. (D) In the wild-type antenna, six segments (dots) form the middle region between pedicel (Pd) and the club (Cl). (E) Adult *T-Sp8* RNAi antennae frequently show a reduction in the number of middle segments. In this example, four segments (dots) can be seen. Cx, coxa; Tr, trochanter; Fe, femur.

Discussion

The *Tribolium* and the *Drosophila Sp* genes are the orthologs of the vertebrate *Sp8* genes

Many genes have been described to be required for appendage formation. We describe a further component of this process, the *Sp*-like zinc-finger transcription factor of the beetle *Tribolium* (*T-Sp8*). We determined the phylogenetic position of *T-Sp8* to be close to the *Sp7*/*Sp8* genes within the *Sp5-Sp8* group of the *Sp* gene family (Fig. 6). All three conserved amino acid stretches of the protein, the Sp-box, the Btd-box and the zinc-finger region of *T-Sp8* are more similar to the *Sp8* gene than to *Sp7/osterix*. In *Drosophila-Sp1* or in human *SP8*, a serine-threonine rich region exists between the Sp- and the Btd-box. Similarly, there are moderately more serines and threonines in this region in *T-Sp8*, but they do not occur in a highly repetitive pattern as in the *Drosophila* ortholog *D-Sp1* (Fig. 1A). In the mammalian *Sp7* gene, the respective region is instead proline rich (Bouwman and Philipsen, 2002). In summary, the structural similarities between *T-Sp8*, *D-Sp1* and the vertebrate *Sp8* genes indicate a close relationship between these genes, and strongly argue for their orthologous relationship.

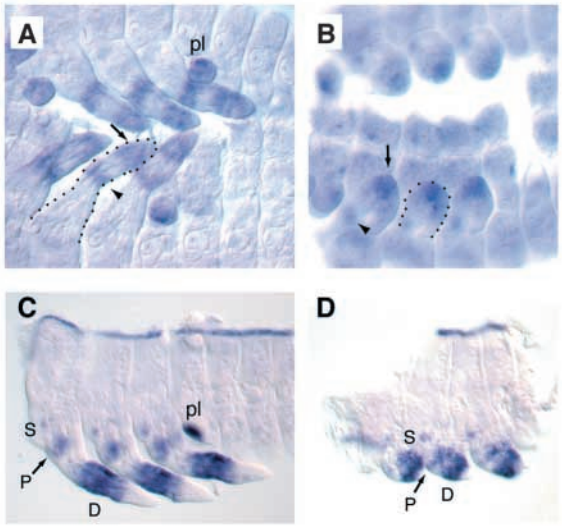


Fig. 5. *Distal-less* and *dachshund* expression in wild-type- and in *T-Sp8* RNAi embryonic legs. The pleuropodium (pl) is nonspecifically stained in A and C. (A) Wild-type embryo at the end of the segmentation process. The long outstretched legs show *Distal-less* expression as a proximal 'ring' (arrowhead) and a distal positioned 'sock' (arrow). (B) In very strongly affected *T-Sp8*^{RNAi} legs, *Distal-less* expression can be seen at the tip of the dramatically shortened leg (arrow). At the base of such a leg a dot of *Distal-less* expression (arrowhead) represents the rest of the 'ring' domain. (C) The *dachshund* gene is expressed in three domains in wild-type embryonic legs: as a spot (S) near the body wall, weakly in a proximal (P) and stronger in a distal (D) domain. (D) All three domains appear to be present in *T-Sp8*^{RNAi} legs. The distal domain (D) is reduced in width. The distal leg tip as in the wild type is free of *dachshund* expression. The embryos shown in B and D most probably represents the very strong phenotype shown in Fig. 3D.

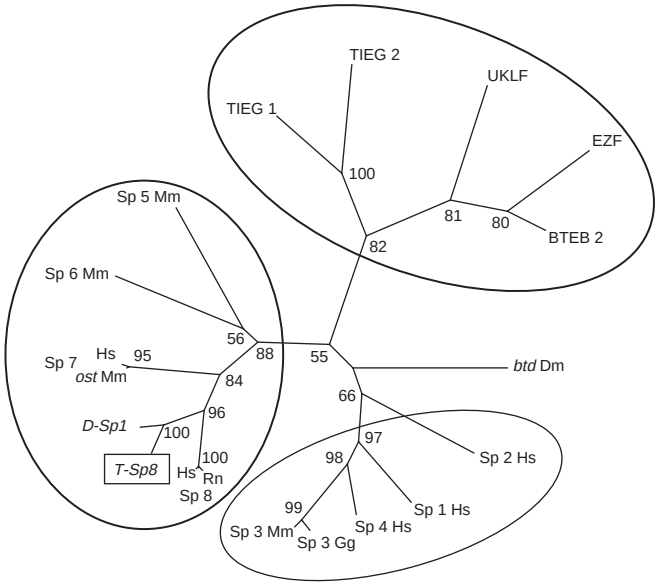


Fig. 6. Phylogenetic analysis of zinc-finger proteins that belong to the Sp class. A Tree-Puzzle analysis based on a Clustal W alignment places the *T-Sp8* gene (boxed) together with its ortholog from *Drosophila melanogaster* (*D-Sp1*) close to the *Sp7*/*osterix* and the *Sp8* (Bouwman and Philipsen, 2002) genes from vertebrates (Clustal W alignment; *btd*-Dm: *buttonhead*/*Drosophila melanogaster* was used as outgroup).

T-Sp8 is required for appendage elongation and is involved in the regulation of allometric growth

T-Sp8 expression starts early during germ band elongation in a segmental pattern even before a limb bud is seen. At the beginning of bud formation, *T-Sp8* expression is uniform in all the appendage anlagen including the mandibles. This suggests an important function of *T-Sp8* for setting up the proximodistal (PD) axis rather than secondarily subdividing the growing appendages like the *dachshund* (*dac*) gene that is expressed for the first time when they are substantially elongated (Abzhanov et al., 2001; Inoue et al., 2002; Prpic et al., 2001). As the limbs start to grow out, segmental expression of *T-Sp8* ceases.

During limb elongation, *T-Sp8* remains to be expressed until the end of appendage differentiation. The development of the *T-Sp8* expression pattern during appendage elongation is different, depending on limb identity. In appendages that do not elongate, such as the labrum and the labium, *T-Sp8* remains uniformly expressed. Despite developing a distinct expression domain, *T-Sp8* contributes to the outgrowth of these limbs: in *T-Sp8* RNAi embryos the region distal to the *dac* expression domain is lost in the labrum (Fig. 7).

In the antennae and the legs, the initial *T-Sp8* domain splits up into two ring-like expression domains. This splitting occurs during further outgrowth of the antenna, the mandible and the leg. Only in the legs does a third domain intercalate between the two primary rings and does a weak fourth ring domain appear at the end of leg elongation (Fig. 2J). Thus, the number of *T-Sp8* rings correlates with the final length of the limb.

The differential expression of *T-Sp8* together with the short leg RNAi phenotype suggests that *T-Sp8* directs the extent of the outgrowth of an appendage. In this way, *T-Sp8* regulates the length of the different limb types of an individual in relation to body size. Therefore, *T-Sp8* can be accounted for as a component of a genetic framework involved in allometric growth (Stern and Emlen, 1999). Such a limb-specific allometry obviously contributes to the phenotype of an individual. During evolution, changes in the regulatory region of genes like *T-Sp8* could result in an altered expression profile (e.g. more rings) and thus lead to changes in limb-length. This phenotypic change could then lead to a change in the ability of using new ecological resources and can be seen as a first step in the evolution towards a new species.

Besides the appendage specific expression, *T-Sp8* transcripts can also be seen in the brain and in cells of the peripheral nervous system. That animals treated with ds*T-Sp8*-RNA hatch and even survive to adulthood can be explained by a redundantly acting *Sp-like* gene in these cells. A likely candidate is a *buttonhead* ortholog that also belongs to the Sp gene family, but has not been isolated from *Tribolium* so far. In *Drosophila*, *D-Sp1* and *buttonhead* are partially functional redundant in the nervous system during post-blastodermal stages of embryogenesis (Wimmer et al., 1996). Redundancy of *D-Sp1* and *btd* also applies to the process of leg formation. Only flies without *D-Sp1* and *btd* function develop a short-leg phenotype (Estella et al., 2003). The fact that such a phenotype has been obtained in *Tribolium* with double stranded *T-Sp8*-RNA alone suggests, that a putative *buttonhead* ortholog in the

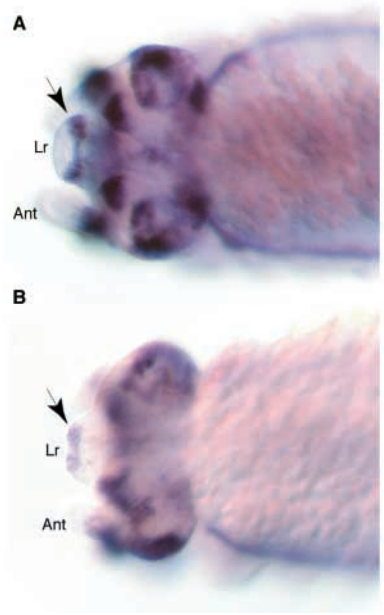


Fig. 7. *dachshund* expression in the labrum of wild-type and *T-Sp8*^{RNAi} embryos. (A) In the wild type, the *dac* expression domain (arrow) is seen in a middle position of the labrum. (B) In *T-Sp8* RNAi embryos the distal part of the labrum is lost and the *dac* domain (arrow) comes to lie at its anterior margin.

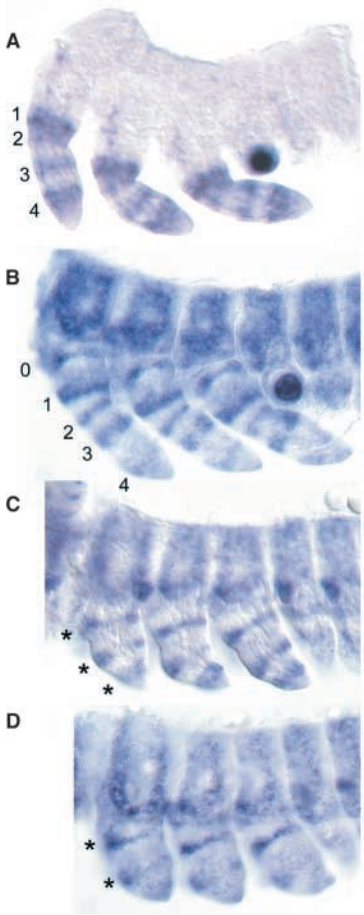


Fig. 8. *T-Serrate* expression in wild-type and *T-Sp8* RNAi legs. The rings mark the positions of the leg joints. 0, body wall/coxa; 1, coxa/trochanter; 2, trochanter/femur; 3, femur/tibiotarsus; 4, tibiotarsus/pretarsal claw. (A) *T-Sp8* expression in wild-type legs (same embryo as in Fig. 2J). No *T-Sp8* expression is seen in the region of the body-wall/coxa joint. (B) *T-Serrate* expression in wild-type legs. (C) *T-Serrate* expression (*) in *T-Sp8*-RNAi legs (weak RNAi effect). (D) *T-Serrate* expression (*) in *T-Sp8*-RNAi legs (strong RNAi effect).

beetle is not involved in leg formation in parallel to *T-Sp8*. This function might have evolved recently during the evolution of the higher diptera.

Regulation of *T-Sp8*

How the *Sp8* orthologs specifically contribute to limb outgrowth in *Drosophila* and *Tribolium* is unclear. Based on the following observations, *Sp8* could be connected to the Notch signalling pathway. In *Drosophila*, activated Notch and its ligand *Serrate* are expressed like *buttonhead* and *D-Sp1* in rings in the leg imaginal disc (de Celis et al., 1998; Estella et al., 2003), suggesting that these genes are functionally related. Furthermore, the impairment of *Drosophila* Notch function results in shortened adult legs (de Celis et al., 1998), as this is seen in legs lacking both *D-Sp1* and *btd* function (Estella et al., 2003). In *Tribolium*, the Notch ligand *Serrate* is expressed in the same way as *T-Sp8* in rings in the fully elongated larval leg (Fig. 8B). It is tempting to speculate that the *T-Sp8* RNAi phenotype could be due to a failure of Notch activation because the control of cell proliferation mediated by Notch signalling has been found in both invertebrates and vertebrates (Kenyon et al., 2003; Rao and Kadesch, 2003). Indeed, we find that the number of *Serrate* rings in *T-Sp8* RNAi legs is reduced (Fig. 8C,D) showing that *T-Sp8* function is directly or indirectly required for initiating the Notch signalling pathway.

In the mouse, the *Sp8* ortholog is also required for the process of limb outgrowth. Here, *Sp8* is required to maintain signalling gene activities in the apical ectodermal ridge (AER) during early stages of limb elongation (Bell et al., 2003; Treichel et al., 2003).

Further targets of *Sp*-like genes involved in the regulation of cell proliferation have been described for other members of the Sp-KLF gene family (Alpy et al., 2003; Black et al., 2001). Indirectly, *T-Sp8* could specify groups of cells that specifically respond to signals like hormones or growth factors by changing their rate of cell proliferation and/or by cell growth.

Based on the analysis of the RNAi leg phenotype, we suggest that one target of *T-Sp8* is the *Distal-less* gene. Our hypothesis is based on the observation, that: (1) the strongest affected *T-Sp8*^{RNAi} leg also lacks the most distal structure, the pretarsal claw that is *Distal-less* dependent (Fig. 3E); and (2) all of the proximal and most parts of the distal *Dll* expression domain are missing in slightly weaker *T-Sp8* legs (Fig. 5B). A dramatic shortening of the limbs therefore might be cause by a failure of *Distal-less* activation. However, *Distal-less* seems not to be required for *T-Sp8* expression, as embryos homozygous mutant for the strongest *Dll* allele *Sa-8* (Beermann et al., 2001) still show *T-Sp8* expression on their leg stumps (not shown). Hox genes that control segment identity are among the candidates acting upstream of *T-Sp8* and could lead to the limb specific modulation of *T-Sp8* expression.

To test this hypothesis, Hox-binding sites within the upstream sequences of *T-Sp8* have to be identified and transgenes with altered binding sites have to be tested in beetles and flies.

Does the Sp^{RNAi}-leg phenotype reflect the evolutionary ground state of a leg?

The evolutionary ground state of a limb has been proposed by Snodgrass as an 'undivided lobe or tubular outgrowth of the body wall, serving as an aid in locomotion' (Snodgrass, 1935). Morphologically, strongly affected *T-Sp8*^{RNAi} legs fulfill these requirements. But according to Snodgrass, a leg representing the ground state is composed of only proximal and distal pattern elements. The *T-Sp8*^{RNAi} legs we have shown to include proximal, medial and distal positional values may therefore reflect the state of a more advanced ground state. We suggest calling such an appendage the 'Ur-limb'. The evolutionary invention of genes such as *Sp*, the co-option of an already existing *Sp8*-like gene for the process of limb-elongation or the addition of 'ring-elements' to the regulatory region of an already existing *Sp* gene required for the process of limb development have contributed to stretch proximal, medial and distal positions apart from each other to result in the elongated 'modern' leg.

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