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RESEARCH ARTICLE

The evolutionarily conserved transcription factor Sp1 controls appendage growth through Notch signaling

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ABSTRACT

The appendages of arthropods and vertebrates are not homologous structures, although the underlying genetic mechanisms that pattern them are highly conserved. Members of the Sp family of transcription factors are expressed in the developing limbs and their function is required for limb growth in both insects and chordates. Despite the fundamental and conserved role that these transcription factors play during appendage development, their target genes and the mechanisms by which they participate in control limb growth are mostly unknown. We analyzed here the individual contributions of two *Drosophila* Sp members, *buttonhead* (*btd*) and *Sp1*, during leg development. We show that *Sp1* plays a more prominent role controlling leg growth than does *btd*. We identified a regulatory function of Sp1 in Notch signaling, and performed a genome-wide transcriptome analysis to identify other potential Sp1 target genes contributing to leg growth. Our data suggest a mechanism by which the Sp factors control appendage growth through the Notch signaling.

KEY WORDS: Sp1, Buttonhead, Appendage, Growth, Notch, *Drosophila*

INTRODUCTION

Understanding the molecular mechanisms that control the specification and acquisition of the characteristic size and shape of organs is a fundamental question in biology. Of particular interest is the development of the appendages of vertebrates and arthropods, i.e. non-homologous structures that share a similar underlying genetic program to build them, a similarity that has been referred to as 'deep homology' (Shubin et al., 2009). Some of the conserved genes include the *Dll/Dlx* genes (Panganiban et al., 1997), *Hth/Meis* (Mercader et al., 1999) and the family of Sp transcription factors (Bell et al., 2003; Estella et al., 2003; Schock et al., 1999; Treichel et al., 2003). The Sp family is characterized by the presence of three highly conserved Cys2-His2-type zinc fingers and the presence of the Buttonhead (BTD) box just N-terminal of the zinc fingers (Suske et al., 2005).

Members of the Sp family have important functions during limb outgrowth in a range of species from beetles to mice (Beermann et al., 2004; Bell et al., 2003; Haro et al., 2014; Kawakami et al., 2004; Schaeper et al., 2009; Schock et al., 1999). In vertebrates, Sp6, Sp8 and Sp9 are expressed in the limb bud and are necessary for *Fgf8* expression and, therefore, for apical ectodermal ridge (AER) maintenance (Bell et al., 2003; Haro et al., 2014; Kawakami et al., 2004; Treichel et al., 2003). Moreover, Sp6/Sp8 phenotypes have been related to the split-hand/foot malformation phenotype (SHFM) and, in the most severe cases, to amelia (the complete loss of the limb) (Haro et al., 2014; Talamillo et al., 2010).

In *Drosophila*, two members of this family, *buttonhead* (*btd*) and *Sp1*, are located next to each other on the chromosome and share similar expression patterns throughout development (Estella and Mann, 2010; Estella et al., 2003; Schock et al., 1999; Wimmer et al., 1996, 1993). Recently, another member of the family, *Spps* (*Sp1-like factor for pairing sensitive-silencing*) has been identified with no apparent specific function in appendage development (Brown and Kassis, 2010; Schaeper et al., 2010). The phenotypic analysis of a *btd* loss-of-function allele and of a deletion that removes both *btd* and *Sp1* led to the proposal that these genes have partially redundant roles during appendage development (Estella and Mann, 2010; Estella et al., 2003). However, the lack of a mutant for *Sp1* has prevented the analysis of the specific contribution of this gene during development.

In *Drosophila*, leg development is initiated in the early embryo by the expression of the homeobox gene *Distal-less* (*Dll*) in a group of cells in each thoracic segment (Cohen, 1990). Later on, *Dll* expression depends on the activity of the Decapentaplegic (Dpp) and Wingless (Wg) signaling pathways, which, together with *btd* and *Sp1*, restrict *Dll* expression to the presumptive leg territory (Diaz-Benjumea et al., 1994; Estella and Mann, 2008, 2010; Lecuit and Cohen, 1997). Therefore, the early elimination of *btd* and *Sp1* completely abolishes leg formation and, in some cases, causes a leg-to-wing homeotic transformation (Estella and Mann, 2010). As the leg imaginal disc grows, a proximo-distal (PD) axis is formed by the differential expression of three leg gap genes, *Dll*, *dachshund* (*dac*) and *homothorax* (*hth*), which divides the leg into distal, medial and proximal domains, respectively (reviewed by Estella et al., 2012). Once these genes have been activated, their expression is maintained, in part through an autoregulatory mechanism, and no longer relies on Wg and Dpp (Diaz-Benjumea et al., 1994; Estella et al., 2008; Galindo et al., 2002). Meanwhile, the distal domain of the leg is further subdivided along the PD axis by the activity of the epidermal growth factor receptor (EGFR) signaling pathway through the activation of secondary PD targets such as *aristaless* (*al*), *BarH1* (*B-H1*) or *bric-a-brac* (*bab*) (Campbell, 2002; Galindo et al., 2002) (reviewed by Kojima, 2004). During these stages, *btd* and *Sp1* control the growth of the leg but are no longer required for *Dll* expression (Estella and Mann, 2010). How *btd* and *Sp1* contribute to the shape and size of the leg and the identity of their downstream effector targets is unknown.

One important consequence of the PD territorial specification is the generation of developmental borders that activate organizing

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molecules to control the growth and pattern of the appendage. In the leg, PD subdivision is necessary to localize the expression of the Notch ligands Delta (Dl) and Serrate (Ser), which in turn activate the Notch pathway in concentric rings at the borders between presumptive leg segments (Bishop et al., 1999; de Celis et al., 1998; Rauskolb, 2001; Rauskolb and Irvine, 1999). Notch directs the formation of all leg joints and promotes leg growth (de Celis et al., 1998; Kerber et al., 2001; Rauskolb and Irvine, 1999). However, it is still unknown how Notch controls leg growth and how the localization of its ligands is regulated.

In the present study, we have generated a specific *Sp1* null mutant, which, in combination with the *btd* mutant and a deletion that removes both *btd* and *Sp1*, allowed us to analyze the individual contributions of these genes to leg development. We find that *Sp1* plays a fundamental role during patterning and growth of the leg disc, and that this function is not compensated by *btd*. The growth-promoting function of Sp1 depends in part on the regulation of the expression of *Ser* and, therefore, on Notch activity. In addition, we identified other candidate targets of Sp1 affecting leg growth and morphogenesis. Intriguingly, some of these Sp1 potential downstream targets are ecdysone-responding genes. Our results highlight a mechanism by which *btd* and *Sp1* control the size and shape of the leg, in part through regulation of the Notch pathway.

RESULTS

Expression pattern of *Sp1* and *btd* during leg development

In order to compare the expression pattern of *Sp1* and *btd* during leg development, we used the *btd-Gal4* line and a recently generated Sp1 antibody (see Materials and Methods). Although *btd* and *Sp1* expression is very similar and covers almost the entire first instar leg disc, later in development both genes present remarkable differences in their expression patterns (Fig. 1A-C). In early third instar leg discs, both genes are expressed throughout the medial and distal regions, but are excluded from the body wall and the distal tip of the leg. Interestingly, Sp1 protein levels are slightly reduced in the medial domain compared with *btd* expression. In prepupal leg discs, *Sp1* expression is restricted to the tarsal segments and specifically to the inter-joint regions (see below). *btd* is also expressed in the tarsal segments although it extends more proximally and presents a gap in the region that corresponds to the tibia. These results indicate that as development progresses, *Sp1* and *btd* expression patterns diverge, and this differential expression suggests that these genes might play specific roles during leg growth.

Individual contributions of *Sp1* and *btd* to leg development

Our previous analyses of a deletion of both *btd* and *Sp1* [*Df(btd, Sp1)*], a *btd* null mutation (btd^{XG81}) and RNA interference (RNAi) against Sp1 suggested that *Sp1* plays a major role during leg development (Estella and Mann, 2010). In order to identify the individual contributions of *btd* and *Sp1*, we compared the phenotype of legs developing in the absence of each gene independently or when both genes are deleted. For this purpose, and considering that the RNAi causes only a partial loss of function of *Sp1*, we generated an *Sp1* null allele ($Sp1^{HR}$) by replacing the third exon, where the Sp1 DNA-binding domain is located, with an *attP* integration site using homologous recombination (Fig. 1D). All mutant flies show a leg phenotype characterized by a strong reduction in the overall size of the leg and defects in the joints, phenotypes that are consistently more severe in the tarsal domain (compare Fig. 1G with 1E). As a control, we reintegrated the deleted *Sp1* exon in $Sp1^{HR}$ flies, which led to an almost complete rescue of the mutant phenotype (Fig. S1). As btd^{XG81} and *Df(btd,Sp1)* homozygous mutants die as embryos, we deleted their function in the legs using *Dll-Gal4*; UAS-*flp* and the *Minute* (*M*) technique

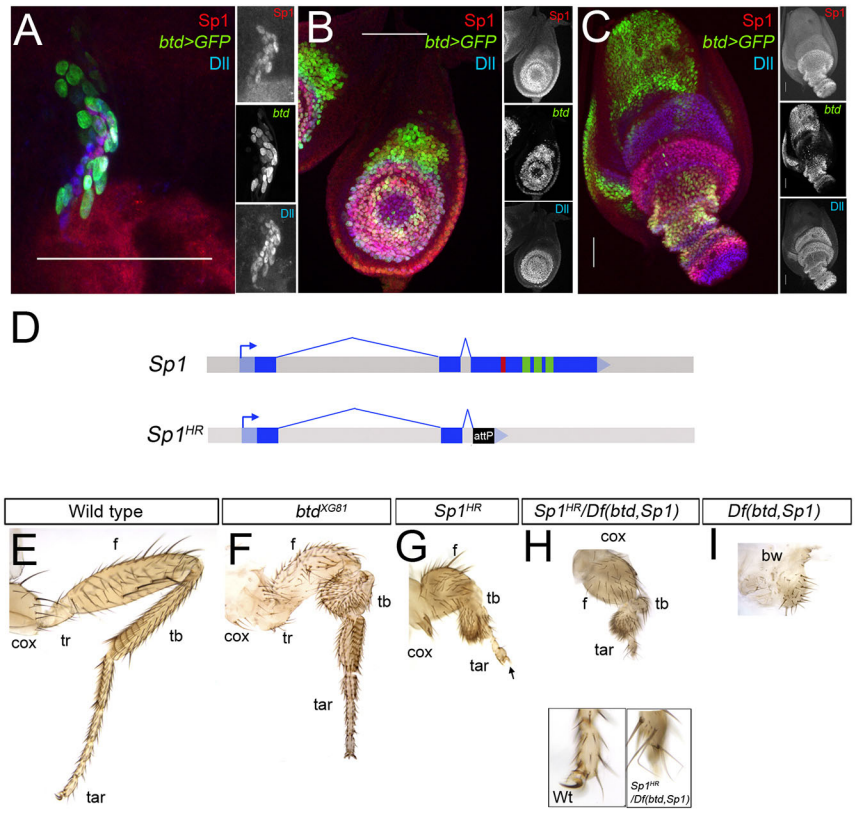

Fig. 1. Expression pattern of *btd* and *Sp1* in prepupal leg discs and adult phenotypes of Sp family mutants. (A-C) Expression pattern of Sp1 (anti-Sp1, red) and *btd* (*btd-Gal4*, UAS-*GFP*, green) in L1 disc (A), early L3 disc (B) and prepupal leg disc (C). Scale bars: 50 µm. (D) Schematic of the $Sp1^{HR}$ null allele, in which an *attP* integration site substitutes the third exon of *Sp1*. Within the third exon, the three zinc fingers (green) and the BTD-box (red) are highlighted. (E-I) Adult legs of: wild type (E), btd^{XG81} mutant (F), $Sp1^{HR}$ mutant (G), heterozygous $Sp1^{HR}$ over *Df(btd, Sp1)* background (H) [arista-like structures appear in the distal tip of the leg (inset)] and *Df(btd, Sp1)* mutant (I). Legs shown in F and I were generated using *Dll-Gal4*$_{212}$; UAS-*flp* to induce btd^{XG81} M^+ or *Df(btd,Sp1)* M^+ clones that cover the entire leg. bw, body wall; cox, coxa; f, femur; tar, tarsus; tb, tibia; tr, trochanter.

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(Morata and Ripoll, 1975; Fig. 1F,I). According to our previous results, removing *btd* function from the entire leg caused, in the most extreme cases, a fusion between the femur and the tibia and a reduction in the size of these two segments, whereas the rest of the leg remains apparently normal (Fig. 1F). By contrast, $Sp1^{HR}$ mutant legs display a much more dramatic phenotype in which leg segments are dramatically reduced in size and present fusions (Fig. 1G). Animals in which both copies of the *Sp1* gene and one copy of the *btd* gene were mutant [$Sp1^{HR}$/*Df(btd,Sp1)*] developed a similar phenotype to homozygous $Sp1^{HR}$ legs. However, in this case we also observed long bristles at the tip of the leg that resembles arista-like structures from the antenna (Fig. 1H; see also below). Removing both copies of *Sp1* and *btd* resulted in flies with no legs or with a small stump of residual leg tissue (Fig. 1I).

Next, we analyzed the individual contribution of *btd* and *Sp1* to *Dll* regulation in the embryo. Consistent with the experiments described above, only the deletion of both *Sp1* and *btd* eliminated the activity of the *Dll*-LT enhancer (Estella et al., 2008; McKay et al., 2009) and failed to maintain *Dll* expression (Fig. S2). In summary, these results suggest that the contribution of *btd* and *Sp1* to early *Dll* activation is mostly redundant, whereas during leg development *Sp1* plays a more prominent role than does *btd*. Nevertheless, *btd* is capable of contributing to some extent to leg growth when *Sp1* function has been compromised.

Leg PD pattern is maintained in *Sp1* mutants

The size reduction and leg morphology defects observed in *Sp1* mutants could be explained by an inappropriate expression of the leg PD patterning genes. The leg disc is initially divided into proximal, medial and distal domains by the differential expression of the leg gap genes *hth*, *dac* and *Dll*, respectively. Later on, the distal part of the leg is further subdivided in nested expression patterns in part by the activity of the EGFR pathway at the distal-most tip of the leg (Fig. 2A) (reviewed by Kojima, 2004). Therefore, we decided to test the expression of these PD genes in $Sp1^{HR}$ mutant legs. Surprisingly, all genes analyzed (eight in total), either in *Sp1* mutant leg discs or in discs with reduced Sp1 function in a particular leg domain using interference RNA, are still expressed and correctly localized along the PD axis, although their domains of expression are slightly reduced (Fig. 2B-G). In summary, these results suggest that the PD gene expression pattern is maintained in *Sp1* mutants, and that their observed narrower expression domain is consistent with the overall reduction of leg size.

Sp1 regulates the Notch pathway through its ligand Ser

We have described here that mutation of *Sp1* results in legs with joint defects and reduced size, phenotypes that resemble *Notch* mutant legs. Therefore, we decided to study in detail the potential genetic relationship between *Sp1* and *btd* and the Notch pathway. First, we generated marked loss-of-function clones for *btd* and/or *Sp1* and analyzed them in adult legs. btd^{XG81} mutant clones do not show any remarkable phenotype, with the exception of some cuticular abnormalities in the tibia (Fig. 3A). By contrast, $Sp1^{HR}$ mutant clones that span leg segments, such as the tibia and first tarsus or several tarsi, result in fusions between them, and are usually associated with size reduction of those segments. In addition, $Sp1^{HR}$ mutant clones generated near the distal end of a segment could induce the formation of small outgrowths (Fig. 3B). Similar phenotypes to $Sp1^{HR}$, although stronger, were observed in *Df(btd, Sp1)* mutant clones (Fig. 3C). These phenotypes are very similar to loss-of-function clones of components of the Notch pathway (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

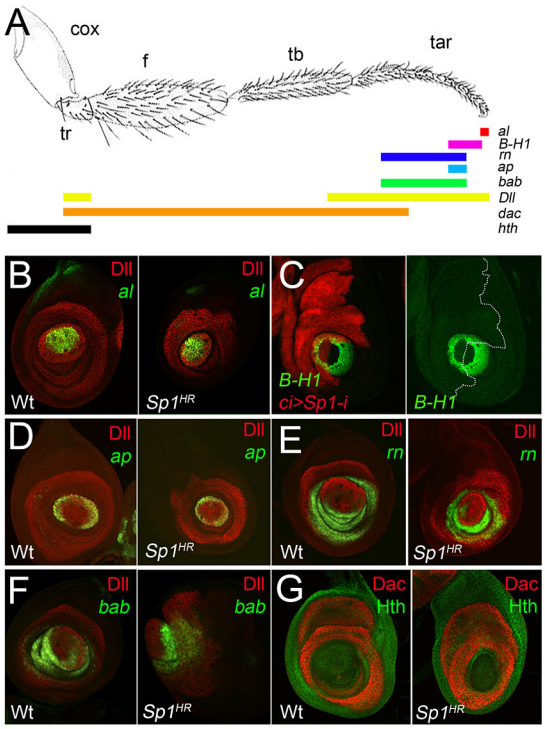

Fig. 2. Expression of leg patterning genes in $Sp1^{HR}$ mutants. (A) Schematic of the transcription factors that pattern the leg along the PD axis. (B) *Dll* (red) and *al* (green) expression in wild type (Wt) and in $Sp1^{HR}$ mutant L3 leg discs. (C) Knock down of Sp1 in the anterior compartment (*ci*>UAS-*Sp1* RNAi, red) of an L3 leg disc and *B-H1* expression in green. (D-G) *Dll* (red) and *ap-lacZ* (green in D), *rn-Gal4*; UAS-*GFP* (green in E), *bab-Gal4*; UAS-*GFP* (green in F), *hth* (green in G) and *dac* (red in G) expression in Wt and in $Sp1^{HR}$ mutant L3 leg discs. cox, coxa; f, femur; tar, tarsus; tb, tibia; tr, trochanter.

The Notch pathway is activated in a row of cells adjacent and distal to the cells expressing the ligands Dl and Ser (Fig. 3D). We compared the expression pattern of *Sp1* with *big brain* (*bib*), a known Notch target that is activated specifically in a row of cells in the presumptive joints of all segments (de Celis et al., 1998). In prepupal leg discs, *Sp1* is expressed in all five tarsal segments and is excluded from the cells that activate *bib* expression and therefore where the Notch pathway is activated (Fig. 3E). To test whether Sp1 regulates this pathway, we generated $Sp1^{HR}$ loss-of-function clones and examined the expression of *Ser* and *bib* in the tarsal segments of prepupal leg discs. We observed a strong reduction of Ser levels in $Sp1^{HR}$ mutant cells that was associated with a disruption of *bib* expression (Fig. 3G). Consistently, clones of cells that lack *btd* and *Sp1* function also downregulated *Ser* and *bib* expression, whereas btd^{XG81} mutant clones do not show any appreciable defect in the expression of these genes (Fig. 3F-H). The regulation of the Notch pathway by *Sp1* was also confirmed after knocking down Sp1 in the fourth tarsal segment in *ap-Gal4*; UAS-*Sp1* RNAi flies (Fig. S3). In these leg discs, *Ser* and *dysfusion* (*dys*; *dysf*– FlyBase), a direct target of the Notch pathway (Cordoba and Estella, 2014), were strongly downregulated with the consequent failure to induce the joint between tarsi 4 and 5 (Fig. S3).

To confirm whether Sp1 and Btd are sufficient to induce *Ser* expression in the leg, we ectopically expressed these genes in an anterior row of cells along the PD axis of the leg disc using the *patched* (*ptc*)-*Gal4* line. We restricted *Sp1* and *btd* ectopic expression to the third instar stage using the $Gal80^{ts}$ technique. Both *Sp1* and *btd* misexpression induced *Ser* activation in the tarsal

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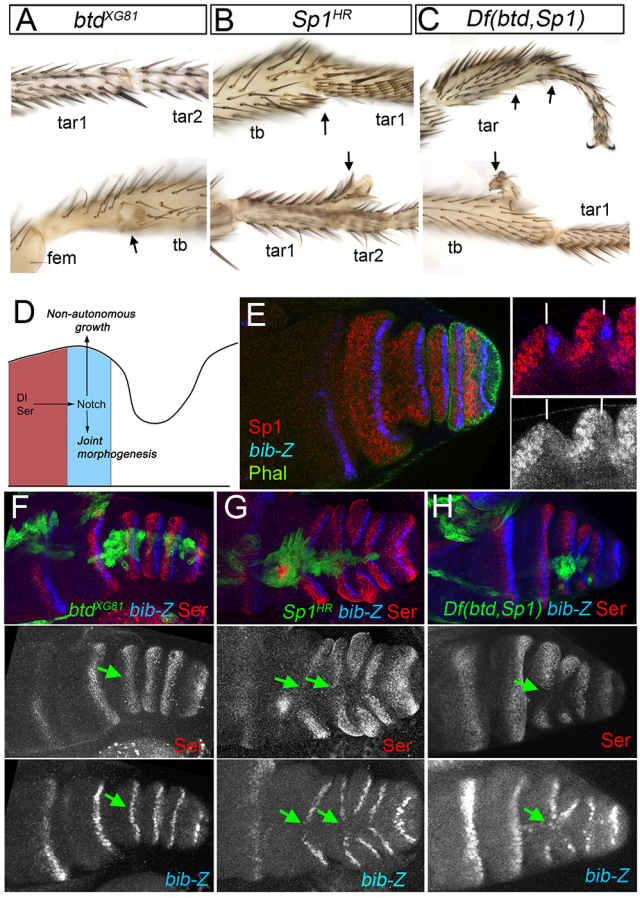

Fig. 3. *Sp1* loss-of-function affects Notch signaling and causes defects in joint formation. (A-C) Adult leg phenotypes caused by btd^{XG81} (A), $Sp1^{HR}$ (B) and *Df(btd, Sp1)* (C) *yellow*$^{-}$ marked clones generated 72-96 h AEL. (A) btd^{XG81} clones only caused minor cuticular defects in the tibia (arrow). (B) $Sp1^{HR}$ clone that spans two segments produced joint defects (upper arrow). Other clones generated close to the distal end of a segment, are associated with tissue outgrowths composed by mutant and wild-type tissue (lower arrow). (C) *Df(btd, Sp1)* clones are also associated with joint defects and non-autonomous outgrowths (arrows). (D) Diagram of Notch signaling in the leg joints. (E) Sp1 staining in prepupal leg discs (red and gray in inset) is detected at high levels in the interjoint regions of the distal leg, whereas its levels decrease in *bib-lacZ*-expressing cells (blue; white bars). Phalloidin staining is shown in green. (F-H) Prepupal leg discs presenting clones of btd^{XG81} (F), $Sp1^{HR}$ (G) and *Df(btd, Sp1)* (H) generated 72-96 h AEL and marked with GFP (green) and indicated by arrows. Ser staining is in red and gray. *bib-lacZ* is shown in blue and gray as indicated. fem, femur; tar, tarsus; tb, tibia.

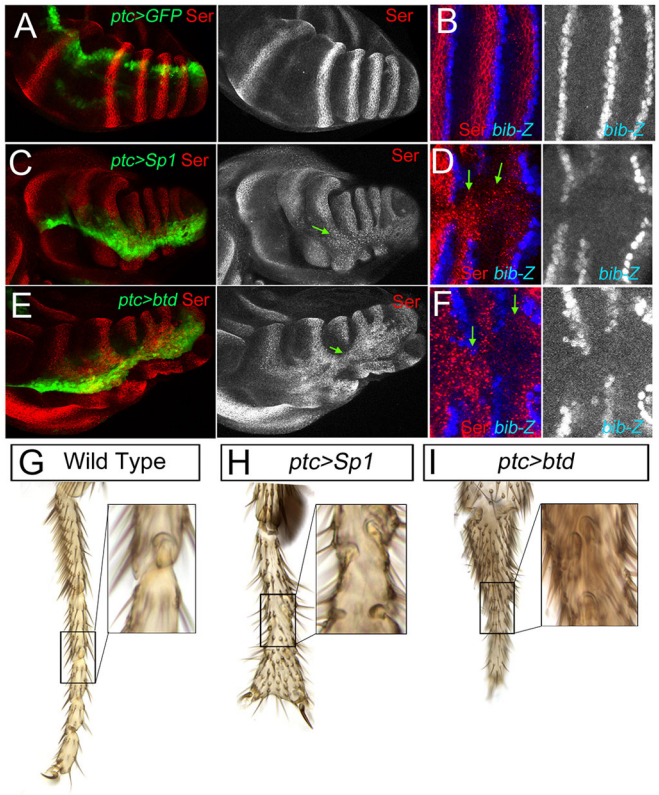

Fig. 4. Sp1 and Btd activate *Ser* expression in the leg disc. (A-F) Prepupal leg discs stained for Ser (red and gray in A,C,E) or Ser and *bib-lacZ* (blue and gray in B,D,F) where either UAS-*GFP* (A,B), UAS-*Sp1* (C,D) or UAS-*btd* (E,F) were ectopically expressed starting from third instar larval stage using the *ptc-Gal4; tubGal80*^{ts} driver (green in A,C,E). Green arrows indicate misexpression of Ser that was restricted to the tarsal region. (B,D,F) Detailed view of leg discs of the same genotypes as in A, C and E, showing *bib-lacZ* expression that is disrupted when *Sp1* or *btd* are misexpressed in the *ptc* domain (green arrows). (G-I) Adult wild-type leg (G), and phenotypes of misexpression of UAS-*Sp1* (H) or UAS-*btd* (I) starting from third instar larval stage using the *ptc-Gal4; tubGal80*^{ts} driver. Ectopic expression of *Sp1* and *btd* disrupted joint formation in the tarsal region (H,I; insets).

region of the leg, and as a consequence *bib* expression was lost in those cells (Fig. 4A-F). As expected by the loss of *bib* expression, *Sp1* or *btd* misexpression in the *ptc* domain disrupts joint formation in adult legs (Fig. 4G-I). From these results, we concluded that *Sp1* is necessary for correct Notch pathway activation at the tarsal joints through the regulation of its ligand *Ser*.

Sp1 regulates *Ser* expression through specific tarsal *cis*-regulatory elements

Ser expression is regulated by multiple *cis*-regulatory elements (CREs) distributed all over the *Ser* genomic locus (Bachmann and Knust, 1998; Rauskolb, 2001), but none of these reproduced the endogenous pattern of Ser in the tarsal region. In an attempt to identify the CREs that mediate Sp1 regulation of *Ser* in the leg, we searched for open chromatin regions identified by formaldehyde-assisted isolation of regulatory elements (FAIRE seq) in the *Ser* locus (McKay and Lieb, 2013) (Fig. 5A). We cloned three regions, two corresponding to the previously identified V-1.9 and I-2.2 elements and one immediately 3′of *Ser* (named *Ser* 3.7). Interestingly, only the *Ser* 3.7 CRE partially reproduced the expression of *Ser* in the distal domain of the leg and it is restricted to the fourth tarsal segment where its activity overlaps with *Sp1* and *ap* expression (Fig. 5B-D). This CRE is also active in some proximal leg rings of Ser and in the wing, and its sequence overlaps with the *Ser* minimal wing enhancer (Yan et al., 2004). These results are consistent with a model in which Sp1, in combination with tarsal-specific transcription factors such as Ap, might be regulating *Ser* expression through different CREs (*Ser* 3.7 in this case). Accordingly, we found a strong downregulation of *Ser* 3.7 CRE activity in $Sp1^{HR}$ mutant leg discs and in discs in which the Ap activity repressor dLMO (Bx – FlyBase) was expressed in the *ptc* domain (Fig. 5E,F). Next, we performed chromatin immunoprecipitation (ChIP) to test whether Sp1 was bound to a smaller version of the *Ser* 3.7 CRE (named *Ser* 2.3) using an *Sp1-GFP* line and an anti-GFP antibody. We found that anti-GFP was able to immunoprecipitate this CRE specifically, but not a control region (Fig. 5G). Interestingly, the immunoprecipitated region coincides with the location of putative Sp1/Btd-binding sites.

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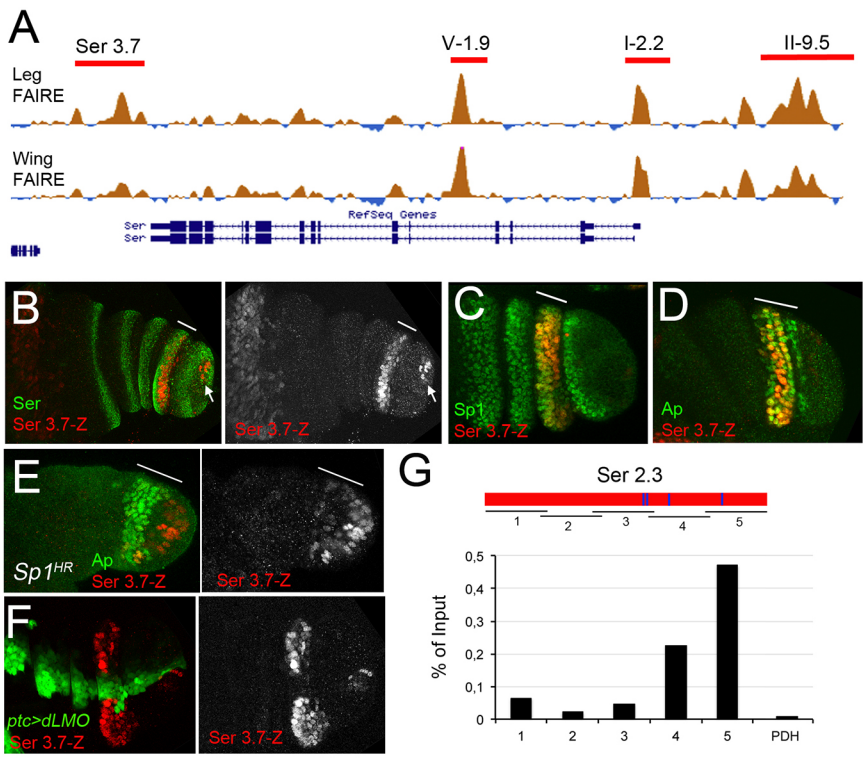

Fig. 5. *Ser* expression in the fourth tarsal segment depends on Sp1 and Ap. (A) Schematic of the *Ser* genomic locus in which open chromatin regions, identified by FAIRE seq for leg and wing larval imaginal discs, are indicated by orange peaks. Data obtained from McKay and Lieb (2013). The previously identified *Ser* CREs and the *Ser* 3.7 are indicated by red bars. (B) Ser staining (green) and *Ser* 3.7-*lacZ* reporter (red and gray) expression in a prepupal leg disc. Note that Ser and *Ser* 3.7-*lacZ* overlap in the fourth tarsal segment (white bar). Arrow indicates a few cells that activate *Ser* 3.7-*lacZ* in the pretarsus. (C,D) *Ser* 3.7-*lacZ* (red) overlaps with Sp1 (C) and Ap (D) in the fourth tarsal segment (white bars). (E) In an $Sp1^{HR}$ mutant prepupal leg disc, *Ser* 3.7-*lacZ* expression (red and gray) is highly reduced in the *ap* domain (green; white bars). (F) Expression of the Ap activity repressor dLMO using the *ptc-Gal4* driver (green) causes the cell-autonomous loss of *Ser* 3.7-*lacZ* activity (red and gray). (G) ChIP of Sp1-GFP at the smaller version of *Ser* 3.7 CRE, named *Ser* 2.3 (red bar), and a control locus (PDH). The five amplicons tested are represented and the blue lines indicate Sp1/Btd candidate binding sites. Representative enrichment values with respect to a 'mock' sample (see Materials and Methods) are shown for a representative single ChIP that was conducted in three replicates.

Transcriptome analysis of *Sp1* mutant leg discs and identification of new potential targets

Although some of the $Sp1^{HR}$ mutant leg defects could be explained by Sp1 regulation of the Notch pathway, other genes might contribute to the leg phenotype and be misregulated in this mutant condition. In order to investigate this hypothesis, we compared the transcriptome of male third instar leg imaginal discs of $Sp1^{HR}$ mutants with the corresponding control discs by RNA-seq. We identified a total of 337 genes that were significantly differentially expressed between these two genotypes (Table S1). From these 337 genes, we selected those with a log2 fold change higher than one (absolute value) and with a minimal expression level (see Materials and Methods). Following these criteria, a total of 30 upregulated and 53 downregulated genes were selected (Table S2). Several upregulated genes encode proteins that are involved in sensory perception, cuticle formation or imaginal disc morphogenesis, or are related to the Ecdysone pathway (Fig. 6A). The most upregulated gene is *Insulin-like peptide 8* (*dilp8*; *Ilp8* – FlyBase), which is activated in conditions of growth impairment (Colombani et al., 2012; Garelli et al., 2012). We confirmed this upregulation by using an eGFP trap in the gene ($dilp8^{MI00727}$) to follow *dilp8* expression in $Sp1^{HR}$ mutants and comparing it with control leg discs (Fig. 6B). Another confirmed upregulated gene is the antennal-specific *distal antenna-related* (*danr*) (Emerald et al., 2003; Suzanne et al., 2003), expression of which increases in the distal domain of $Sp1^{HR}$ mutant discs (Fig. 6C). The downregulated genes in $Sp1^{HR}$ mutants belong to different biological categories, including heat shock proteins (Hsp), sensory perception, Ecdysone pathway, leg/antenna morphogenesis, metabolism and cuticle formation. Here, we have described the narrower expression of the tarsal PD patterning genes *bab* and *B-H1* in $Sp1^{HR}$ mutant discs or in Sp1 knockdown conditions, and consistently their expression appears reduced in the RNA-seq experiment. Of special interest is the downregulation of *dys* and *Stubble* (*Sb*), which are required for tarsal joint formation and leg eversion, respectively (Appel et al., 1993; Beaton et al., 1988; Condic et al., 1991; Cordoba and Estella, 2014). *dys* is a direct target of the Notch pathway in the leg and, in accordance with our previous results, the expression of *dys* is highly reduced in $Sp1^{HR}$ mutant discs (Fig. 6D). *Sb* encodes for a type II transmembrane serine protease required for leg disc elongation, and its expression is regulated by Ecdysone (Appel et al., 1993; Beaton et al., 1988; Condic et al., 1991). Leg disc elongation occurs in part by a change from anisometric to isometric cell shape starting in the first hours after puparium formation (APF) (Condic et al., 1991). We measured the width and length of the fourth tarsal segment cells (Ap positive) at 4 h APF in $Sp1^{HR}$ mutant and control legs (Fig. S4). Tarsal $Sp1^{HR}$ mutant cells are significantly wider and shorter along the PD axis than control cells. The length:width ratio in control discs is approximately 0.8 whereas that in $Sp1^{HR}$ mutants is 0.57. Interestingly, the apical area of the cell is maintained in both genotypes (Fig. S4). These defects in cell shape changes are similar to those described in *Sb* mutant leg discs (Condic et al., 1991).

To investigate further the contributions of several of the downregulated genes to leg morphogenesis, we tested their functional requirements by analyzing the consequences of reducing their expression. To this end, we expressed in the leg disc interference RNA (*Dll-Gal4*$_{212}$; UAS-*dicer2*; UAS-RNAi) against most of the downregulated genes (40 in total) with the exception of most of the Hsp family genes or those for which a phenotype has already been described in the leg, such as *bab*, *dys* or *Sb*. Only four genes, *DnaJ-1*, *Pepck*, *Lsp2* and *CG34166* resulted in leg phenotypes in our experimental approach. Interestingly, the knockdown of these genes resulted in legs with tarsal size reduction and joint defects (Fig. 6E).

DISCUSSION

Roles of *Sp1* and *btd* during leg development

The two Sp family members in *Drosophila*, *Sp1* and *btd*, display a similar spatial and temporal expression pattern during embryonic and imaginal development. Previous work suggested that *btd* and *Sp1* have

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A

Up regulated

Ecdysone	
CG1152	**Gld-Glucose dehydrogenase**
CG10121	**SP1173**
CG14059	**Ilp8**
Sensory Perception	
CG1058	**Rpk-ripped pocket**
CG1506	**Ac3**
CG14741	**ATPase 8B**
CG32549	**FBgn0052549**
CG32791	**DIP-a-Dpr-interacting protein α**
CG42541	**CG42541**
Cuticle	
CG3244	**Clect27**
CG8515	**Cpr49Ah**
CG14640	**TwdlV**
CG8927	**CG8927**
Imaginal disc morphogenesis	
CG13651	**danr**
CG31666	**chinmo**

Down regulated

Heat-shock Protein	
CG4463	**Hsp23**
CG4183	**Hsp26**
CG4190	**Hsp67Bc**
CG5436	**Hsp68**
CG31366	**Hsp70Aa**
CG18743	**Hsp70Ab**
CG31449	**Hsp70Ba**
CG31359	**Hsp70Bb**
CG6489	**Hsp70Bc**
CG5834	**Hsp70Bbb**
CG10578	**DnaJ-1**
Sensory Perception	
CG8404	**Sox15**
CG13695	**gk**
CG5171	**CG5171**
CG2297	**Obp44a**
CG9358	**Phk-3**
CG14176	**Or67b**
CG7592	**Obp99b**
Cuticle	
CG14534	**TwdlE**
Ecdysone	
CG17285	**Fbp1**
CG10717	**ImpL1**
CG10160	**ImpL3**
CG9390	**AcCoAS**
CG43934	**Hr4**
CG1342	**Spn100A**
Metabolism	
CG3763	**Fbp2**
CG6806	**Lsp2**
CG17725	**Pepck**
CG32130	**stv**
Leg/antenna morphogenesis	
CG8246	**Poxn**
CG4316	**Sb**
CG9097	**bab1**
CG32632	**Tango13**
CG32474	**dys**
CG5529	**B-H1**

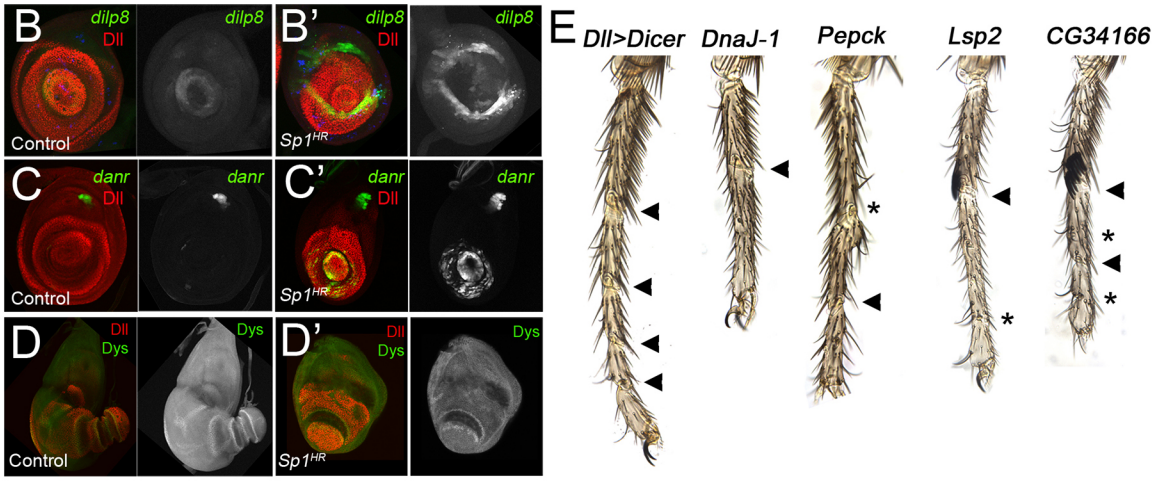

Fig. 6. RNA-seq analysis of *Sp1*^{HR} mutants and identification of putative Sp1 target genes. (A) List of representative upregulated and downregulated genes identified by RNA-seq grouped according to the pathways and morphological processes that they are involved in. (B-D) Validation of identified regulated genes. (B,B′) *dilp8*^{MI00727}*-eGFP* (green and gray) is expressed at very low levels in control leg discs, whereas its expression is strongly enhanced in *Sp1*^{HR} mutant leg discs (B′). (C,C′) *danr-Gal4;* UAS-*GFP* (green and gray) is restricted to the chordotonal organ in control leg discs, whereas in *Sp1*^{HR} mutants its expression is derepressed in the distal region (C′). (D,D′) *dys* (green and gray) is expressed in four rings in the tarsal region of the control prepupal leg disc, and this expression is partially lost in *Sp1*^{HR} prepupal leg disc (D′). In B-D′, Dll staining is shown in red. (E) Downregulation analysis of candidate genes using interference RNA for each gene driven by *Dll-Gal4*$_{212}$; UAS-*dicer2*. Here are depicted the genes for which downregulation cause defects in leg development, compared with control *Dll-Gal4*$_{212}$; UAS-*dicer2* legs. Arrowheads point to correct joint formation and asterisks indicate partial joint defects.

partially redundant functions during development (Estella and Mann, 2010; Estella et al., 2003; Schock et al., 1999; Wimmer et al., 1996). However, the lack of an *Sp1* mutant has prevented the detailed analysis of the individual contributions of each gene. Here, we have generated an *Sp1* null mutant that allowed us to elucidate unambiguously the individual contributions of each of these genes to leg development.

Appendage formation starts in early embryos by the activation of *Dll* (through its early enhancer, *Dll*-304), *btd* and *Sp1* by Wg, and their expression is repressed posteriorly by the abdominal Hox genes (Estella et al., 2003; Vachon et al., 1992). Some hours later, there is a molecular switch from the early *Dll* enhancer (*Dll*-304) to the late enhancer (*Dll*-LT) to keep *Dll* expression throughout the embryo-larvae transition restricted to the cells that will form the leg (reviewed by Estella et al., 2012). At this developmental stage, *Sp1* and *btd* play redundant roles in *Dll* activation, as only the elimination of both genes suppresses *Dll* expression and *Dll*-LT activity in the leg primordia. Once *Dll* expression is activated in the leg disc by the combined action of Wg, Dpp and Btd/Sp1, its expression is maintained in part through an autoregulatory mechanism (Estella and Mann, 2008; Estella et al., 2008; Galindo et al., 2002; Lecuit and Cohen, 1997). At this time point, during second instar, *btd* and *Sp1* are co-opted to control the growth of the leg (Estella and Mann, 2010; Estella et al., 2003). The leg phenotype of *Sp1* and *btd* single mutants demonstrates the divergent

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contributions of each gene to leg growth. Removing *btd* from the entire leg only slightly affects the growth of proximo-medial segments, whereas loss of *Sp1* causes dramatic growth defects along the entire leg. The different phenotypes of *Sp1* and *btd* mutant legs could be a consequence of their distinct expression pattern along the leg PD axis, with *btd* being expressed more proximally than *Sp1*.

The growth defects observed in *Sp1* mutant legs are not due to gross defects in the localization of the different transcription factors that subdivide the leg along the PD axis, nor to defects in the expression of the EGFR ligand *vn* (Fig. S5). By contrast, our results suggest a role for Sp1 in the regulation of the Notch ligand Ser. Notch pathway activation is necessary for the formation of the joints and the growth of the leg, and defects in these two processes were observed in *Sp1* mutant legs. Moreover, our results demonstrate that Sp1 is necessary and sufficient for *Ser* expression at least in the distal domain of the leg and is therefore required for the correct activation of the Notch pathway. These results are consistent with the proposed role of Sp8 in allometric growth of the limbs in the beetle where the number of *Ser*-expressing rings is reduced in Sp8 knockdown animals (Beermann et al., 2004).

The regulation of *Ser* expression is controlled by multiple CREs that direct its transcription in different developmental territories (Bachmann and Knust, 1998; Yan et al., 2004). Interestingly, although the wing and leg are morphologically different appendages and express a diverse combination of master regulators (e.g. Sp1 selects for leg identity whereas Vg determines wing fate), the same set of enhancers are accessible in both appendages, with the exception of the ones that control the expression of the master regulators themselves (McKay and Lieb, 2013). These results imply that appendage-specific master regulators differentially interact with the same enhancers to generate a specific expression pattern in each appendage. Our analysis of *Ser* CREs identified a specific sequence that is active in the wing and in the leg. In the leg, this CRE reproduced *Ser* expression in the fourth tarsal segment and require the combined inputs of Sp1 and Ap. We propose that Sp1, in coordination with the other leg PD transcription factors, interacts with different *Ser* CREs to activate *Ser* expression in concentric rings in the leg. Meanwhile, given the same set of *Ser* CREs in the wing, the presence of a different combination of transcription factors regulate *Ser* expression in the characteristic 'wing pattern'.

Candidate Sp1 target genes

Our transcriptome analysis identified additional candidate Sp1 target genes that contribute to control the size and shape of the leg. Appendage elongation depends on the steroid hormone ecdysone through several of its effectors, such as Sb (Appel et al., 1993; Beaton et al., 1988; Broadus et al., 1999; Condic et al., 1991; Ward et al., 2003). We found that *Sb*, as well as other genes related to the ecdysone pathway, were misregulated in *Sp1* mutant discs. We also observed that the characteristic change in cell shape that normally occurs during leg eversion (Condic et al., 1991) does not happen correctly in these mutants. Other genes identified in our study are the Notch pathway targets *dys* and *Poxn*, which are both required for the correct development of the tarsal joints (Awasaki and Kimura, 2001; Cordoba and Estella, 2014). *dys* and *Poxn* downregulation is consistent with Sp1 regulation of the Notch ligand Ser. Interestingly, the upregulation of the antenna-specific gene *danr* in *Sp1* mutants might explain the partial transformation of the distal leg to antennal-like structures observed when two copies of *Sp1* and one of *btd* are mutated (see Fig. 1H). Interestingly, *btd* and *Sp1* are only expressed in the antenna disc in a single ring corresponding to the second antennal segment whereas in the leg both genes are more broadly expressed (Estella and Mann, 2010; Estella et al., 2003; Schock et al., 1999). Consistent with this, misexpression of *Sp1* in the antenna transforms the distal domain to leg-like structures, suggesting that different levels or expression domains of Sp1 helps distinguish between these two homologous appendages (Fig. S6).

A considerable group of Hsp-related genes were downregulated in *Sp1* mutant legs. Although their contribution to *Drosophila* leg development is unknown, downregulation of DnaJ-1, the *Drosophila* ortholog of the human HSP40, affects joint development and leg size, suggesting a potential role of these genes during leg morphogenesis.

Evolutionarily conserved growth-promoting functions of *Sp1*

An ancient common mechanism for the formation of outgrowths from the body wall has been suggested (Gonzalez-Crespo et al., 1998; Mercader et al., 1999; Panganiban et al., 1997; Panganiban and Rubenstein, 2002). Members of the Sp family are expressed and required for appendage growth in a range of species from *Tribolium* to mice (Beermann et al., 2004; Bell et al., 2003; Kawakami et al., 2004; Treichel et al., 2003). Consistent with our results, knockdown of *Sp8/Sp9* in the milkweed bug or the beetle generated dwarfed legs with fused segments that maintain the correct PD positional values (Beermann et al., 2004; Schaeper et al., 2009). As is the case for *Drosophila Sp1* mutants, mouse *Sp8*-deficient embryos develop with truncated limbs (Bell et al., 2003; Haro et al., 2014; Treichel et al., 2003). By contrast, loss of function of *Sp6* results in milder phenotypes of limb syndactyly (Talamillo et al., 2010). A progressive reduction of the dose of *Sp6* and *Sp8* lead to increased severity of limb phenotypes from syndactyly to amelia, suggesting that these genes play partially redundant roles (Haro et al., 2014). Our phenotypic analysis of *Sp1* and *btd* are consistent with this model, in which *Sp1* plays the predominant role in appendage growth and the complete elimination of *btd* and *Sp1* together abolish leg formation. Therefore, *Drosophila Sp1* mutants are phenotypically equivalent to vertebrate *Sp8* mutants. In vertebrate *Sp8* mutant limbs, *Fgf8* expression is not maintained and a functional AER fails to form (Bell et al., 2003; Kawakami et al., 2004; Treichel et al., 2003). In *Drosophila*, FGF signaling does not seem to be involved in appendage development. Nevertheless, another receptor tyrosine kinase, EGFR, is activated at the tip of the leg and act as an organizer to regulate the PD patterning of the tarsus (Campbell, 2002; Galindo et al., 2005, 2002). Our results suggest that *Sp1* acts in parallel with the EGFR pathway, as the ligand *vn* and EGFR target genes maintain their PD positional information in *Sp1* mutant legs. However, we cannot rule out a potential relationship between *Sp1* and the EGFR pathway in later stages of leg development (Galindo et al., 2005).

Our results suggest that the Notch ligand Ser is a target of Sp1, and mediates in part the growth-promoting function of Sp1. Interestingly, members of the Notch pathway in vertebrates, including the *Ser* ortholog jagged 2 and notch 1 are expressed in the AER and regulate the size of the limb (Jiang et al., 1998; Sidow et al., 1997; Vargesson et al., 1998; Vasiliauskas et al., 2003). It would be interesting to investigate further the possible relationship between Sp transcription factors and the Notch pathway in vertebrates, and test whether the functional relationship described in this work is also maintained throughout evolution.

MATERIALS AND METHODS

Generation of *Sp1^{HR}* mutant flies by homologous recombination

To generate *Sp1^{HR}* mutant flies, we followed a homologous recombination protocol described by Baena-Lopez et al. (2013). Briefly, we cloned 2.3 kb

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and 4.7 kb sequences that flank the third exon of *Sp1* to act as 'homology arms' in the pTV^{Cherry} vector. Candidate *Sp1* mutants were later confirmed by PCR and sequencing. The third exon of *Sp1* was cloned in the reintegration vector (RIV) and then injected into $Sp1^{HR}/Dp(1;Y)lz+$ mutant embryos. See Table S3 for primers and restriction enzymes.

Drosophila strains

btd^{XG81}, *Df(btd,Sp1)*, UAS-*btd*, UAS-*Sp1*, *btd-Gal4*; UAS-*GFP*, *Sp1* RNAi and *Dll*-LT-*lacZ* have been described previously (Estella and Mann, 2010; Estella et al., 2008). The *Dll-Gal4* line 212, *dpp-Gal4*; *ptc-Gal4*, $tubGal80^{ts}$ and the *Sp1*-GFP line (PBac{Sp1-EGFP.S}VK00033) are all available at Bloomington Stock Center. *Dllm-Gal4* ($Dll\text{-}Gal4_{212}$, UAS-*flp*; *act-FRT-stop-FRT-Gal4*, *UAS-GFP)*, *bib-lacZ*, *al-lacZ*, *B-H1-lacZ*, *ap-lacZ*, *vn-lacZ*, *rn-Gal4*; UAS-*GFP*, *bab-Gal4*; UAS-*GFP* were previously described (Campbell, 2002; de Celis et al., 1998; Galindo et al., 2005, 2002). $dilp8^{MI00727}$-*EGFP* and *danr-Gal4*; UAS-*GFP* were described by Garelli et al. (2012) and Suzanne et al. (2003). The RNAi lines, listed in Table S4, were crossed with the Dll_{212}-Gal4, UAS-*dicer2* line.

RNA-seq and data analysis

A total of 120 male larvae were dissected in six individual dissections of the following genotypes: *FM7-GFP*/Y (control) and $Sp1^{HR}$/Y ($Sp1^{HR}$). A total of 1 µg of RNA per dissection was extracted using the RNA Microprep and RNA Clean and Concentrator Kits (Zymo Research). RNA quality was then analyzed using the RNA Analysis Kit and Bioanalyzer system (Agilent). mRNA libraries were prepared using the mRNA-Seq Sample Preparation kit (Illumina, RS-122-2001x2), according to the manufacturer's protocol. First strand cDNA synthesis by random hexamers and reverse transcriptase was followed by second strand cDNA synthesis. Each library was sequenced using TruSeq SBS Kit v3-HS, in paired end mode with the read length 2×76 bp for the mRNA-seq experiments, using the HiSeq2000 instrument (Illumina). Between 27.5 and 40.4 millions of reads were obtained, of which 85-87% of pairs were aligned against *D. melanogaster* genome (BDGP 5) using TOPHAT2 aligner (http://ccb.jhu.edu/software/tophat/index.shtml) and the differentially expressed genes were identified using the DESeq software (http://bioconductor.org/packages/release/bioc/html/DESeq2.html). A total of 337 genes were identified as being differentially expressed in $Sp1^{HR}$ mutants compared with control discs with a *P*-value ≤0.05. From this list of genes, we selected those for which log2 fold change was higher than 1 (absolute value). Next, we selected those upregulated genes and downregulated genes for which expression was >400 normalized counts in the mutant or control condition, respectively. (The average signal for genes not expressed in the leg disc is <400 normalized counts.)

Immunostaining

Embryos, and larval and prepupal leg discs were stained following standard procedures (Estella et al, 2003). Primary antibodies used were: rabbit and mouse anti-βGal (1/1000, Promega and MP Biomedicals), rabbit anti-Dys (a gift from L. Jiang, Oakland University, 1/200), rat anti-Ser (a gift from Ken Irvine, Rutgers University, 1/1000), rat anti-Sp1 (kindly provided by Richard Mann, Columbia University, 1/50), guinea pig anti-Dll and rabbit anti-Hth (1/2000; Estella et al., 2008), and rabbit anti-Ap (1/1000; Bieli et al., 2015). Phalloidin-Atto was used to stain F-actin (Sigma-Aldrich).

Clonal analysis

To generate flies in which the whole leg is mutant we used the following genotypes, and a duplication on the Y chromosome that covers the *btd* and *Sp1* genes (*Dp(1;Y)lz+*) (Schock et al., 1999):

yw btd^{XG81} FRT19A/*ubi-GFP M(1)osp* FRT19A; *Dll*-Gal4, UAS-*flp*,

yw $Sp1^{HR}$ FRT19A/*ubi-GFP M(1)osp* FRT19A; *Dll*-Gal4, UAS-*flp*,

yw Df(btd,Sp1) FRT19A/*ubi-GFP M(1)osp* FRT19A; *Dll*-Gal4, UAS-*flp*.

For loss-of-function clonal analysis we used the following genotypes:

yw btd^{XG81}, $Sp1^{HR}$ or *Df(btd,Sp1)* FRT19A/*tubGal80 hsflp* FRT19A*; act-Gal4, UAS-CD8 GFP*.

Larvae were heat shocked for 1 h at 37°C 72 to 96 h after egg laying (AEL).

Gain-of-function experiments

Gain-of-function experiments were performed using the $Gal4\text{-}tubGal80^{ts}$ system, which allowed temporal restriction of UAS-*Sp1* and UAS-*btd* expression to mid-third instar stage. *ptc-Gal4*; $tubGal80^{ts}$ flies were crossed with each UAS strain, and the eggs laid each 24 h were collected and maintained at restrictive temperature (17°C) until mid-third instar stage, when the fly vials were shifted to the permissive temperature (29°C).

Cloning of *Ser* CREs in a *lacZ* reporter vector

V-1.9 and I-2.2 were previously described by Bachmann and Knust (1998) and Rauskolb (2001). *Ser* 3.7 CRE is located 3′ of the *Ser* transcription start. *Ser* 2.3 CRE is a smaller version of *Ser* 3.7 that maintains its activity in the leg and wing. All these sequences were cloned in the *attB-hs43-nuc-lacZ* plasmid vector (Estella et al., 2008). The primers used for cloning each reporter line are described in Table S3.

Chromatin immunoprecipitation

For the Sp1-GFP ChIP at the *Ser* 2.3 CRE we used the $Sp1^{HR}$; *Sp1-GFP* genotype, which completely rescues the $Sp1^{HR}$ phenotype. The leg discs of 25 female larvae were dissected per ChIP and each ChIP was conducted in three replicates as described by Estella et al. (2008). Samples were incubated with rabbit anti-GFP (1:500; Abcam #290) and rabbit anti-βGal (1:1000; MP #08559761) as 'mock' to correct for non-specific immunoprecipitation. ChIP enrichment values were normalized relative to 'mock' enrichment values. Five real-time PCR amplicons that cover the entire *Ser* 2.3 CRE were used to quantify immunoprecipitated chromatin. As a negative control we used an amplicon in the *pdh* gene on the X chromosome. The primers used are described in Table S3.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.E.; Methodology: S.C., D.R., A.S., C.E.; Formal analysis and investigation: S.C., D.R., A.S., C.E.; Resources: S.C., D.R., A.S., A.J., C.E.; Writing - original draft preparation: C.E.; Writing - review and editing: S.C., C.E.; Funding acquisition: C.E.

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Data availability

Transcriptome data sets for RNA-seq are available under accession number PRJEB14788 at http://www.ebi.ac.uk/ena/data/view/PRJEB14788.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.138735.supplemental

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Supplementary Figures

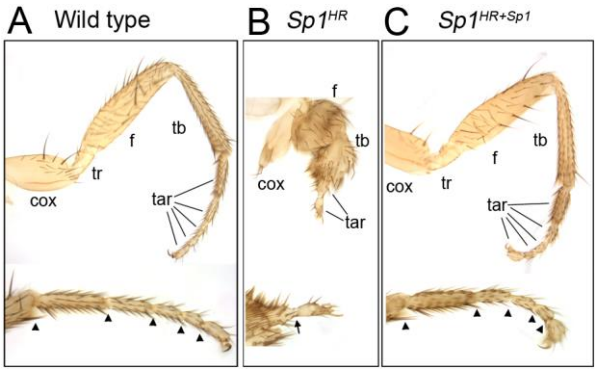

Figure S1: Rescue of *Sp1*^{HR} mutant phenotype by the reintegration of the third exon of Sp1. Adult leg phenotypes of wild type (A), *Sp1*^{HR} (B) and *Sp1*$^{HR+Sp1}$ (C). The inset below displays an amplification of the tarsal region of each genotype. Correct joint formation is indicated with arrowheads .

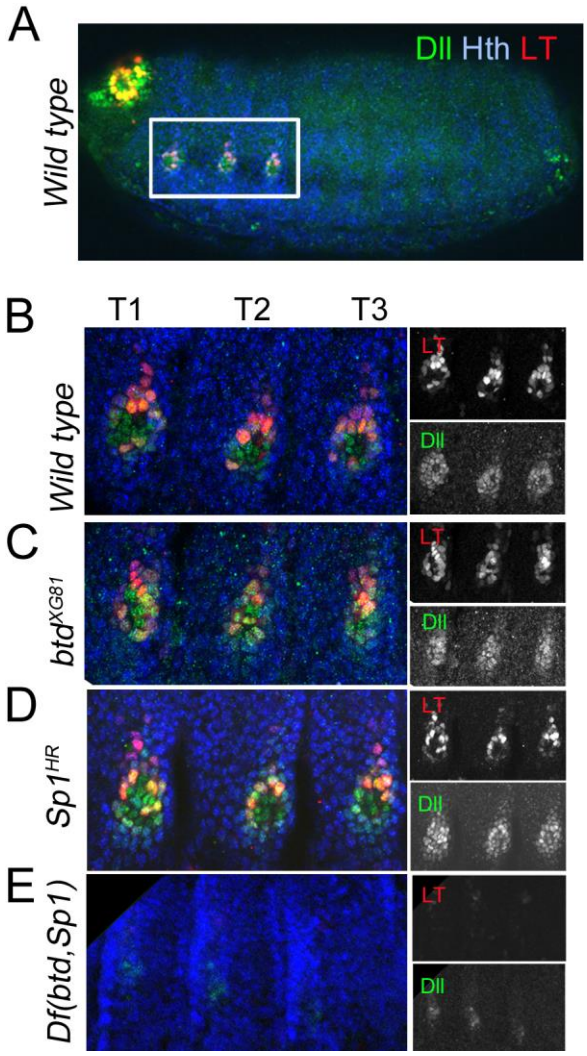

Figure S2: Individual contribution of *btd* and *Sp1* to *Dll* regulation during embryonic development. (A) Wild type embryo (stage 15), stained with antibodies against Dll, Hth, and βgal for the *Dll*-LT-*lacZ* enhancer (green, blue and red, respectively and in all panels). The thoracic region, where the three leg primordia are located, is framed in a white box. (B-E) Magnification of the leg primordia in wild type (B), btd^{XG81} (C), $Sp1^{HR}$ (D) and *Df(btd,Sp1)* (E) mutant embryos. Only when both *Sp1* and *btd* are eliminated, *Dll* and *Dll*-LT fails to be properly activated at the presumptive leg region. T1, T2 and T3 designate first, second and third thoracic segments, respectively. At the right side of each panel is shown in grey the individual signal for Dll and *Dll*-LT for each genotype. All embryos are oriented anterior left and dorsal up.

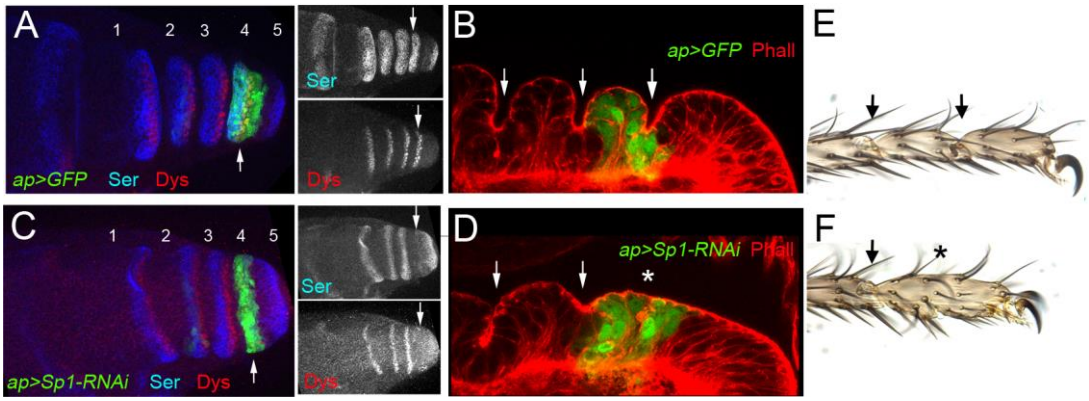

Figure S3: Sp1 loss of function alters Ser positioning causing defects in joint formation. (A) *ap-Gal4*; UAS-*GFP* (green) control prepupal leg disc stained for Ser (blue) and Dys (red). The individual signal for Ser and Dys is displayed in grey at the right of each panel. Arrows indicate the *ap-Gal4* domain. (B) Sagittal view of the leg epithelium (same genotype as in A) stained with Phallodin (Phal, red) and GFP (green). The joints between tarsal segments are marked with arrows. Close view of the adult tarsal joints is shown in (E). (C) Knock-down of Sp1 levels by the expression of UAS-*Sp1* RNAi in the *ap-Gal4* domain (green) cause the loss of Ser (blue) and Dys (red) staining. Reduction of the levels of Sp1 in the *ap-Gal4* domain cause defects in joint formation that can be assessed both in prepupal leg discs and in adult legs (D and F, respectively), as compared with Wt prepupal leg discs and adult leg discs (B and E, respectively). Arrows indicate correct fold or joint morphology, while asterisks indicate defects in these processes. Cell actin cytoskeleton in B and D is stained with Phal (red).

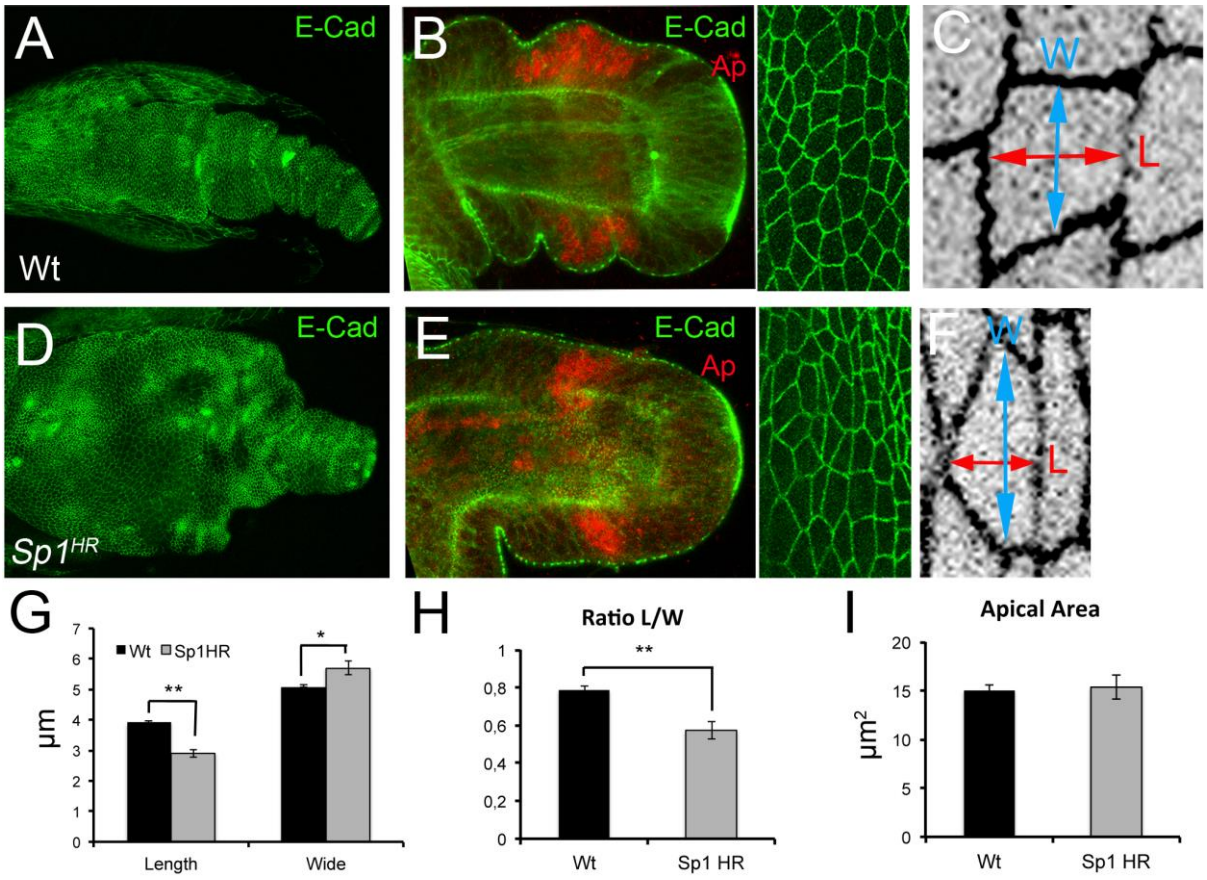

Figure S4: Cell shape changes during leg eversion are impaired in *Sp1^{HR}* mutants. Study of cell shape in control (*FM7-GFP*, referred from now onwards as Wt) (A, B, C) and *Sp1^{HR}* (D, E, F) prepupal leg discs (4hrs APF). E-cadherin-GFP is marked in green in all panels and black in C and F, and Ap protein is shown in red in B and E. (A and D) View of prepupal Wt and *Sp1^{HR}* leg discs, respectively. (B and E) Amplified view of the *ap* domain of the same discs as in A and D, and magnification of the apical region of the Ap domain in the right panels. Note that *Sp1^{HR}* prepupal leg discs are shorter and wider compared to Wt and the defects in fold formation in the *Sp1^{HR}* mutant condition. Length (L, red) and width (W, blue) of control cells (n=77) and *Sp1^{HR}* mutant cells (n=80) (C and F, respectively) within the *ap* domain of prepupal leg discs was measured (G), and L/W ratio is shown in H. Note that cells in *Sp1^{HR}* mutants tend to be shorter and wider along the PD axis than control cells. (I) Apical area was also measured, and it did not differ between control (n=50 cells) and *Sp1^{HR}* (n=62 cells). In G and H (*) indicates significative difference with $p \leq 0{,}05$ and (**) for $p \leq 0{,}005$ with Student's t-test.

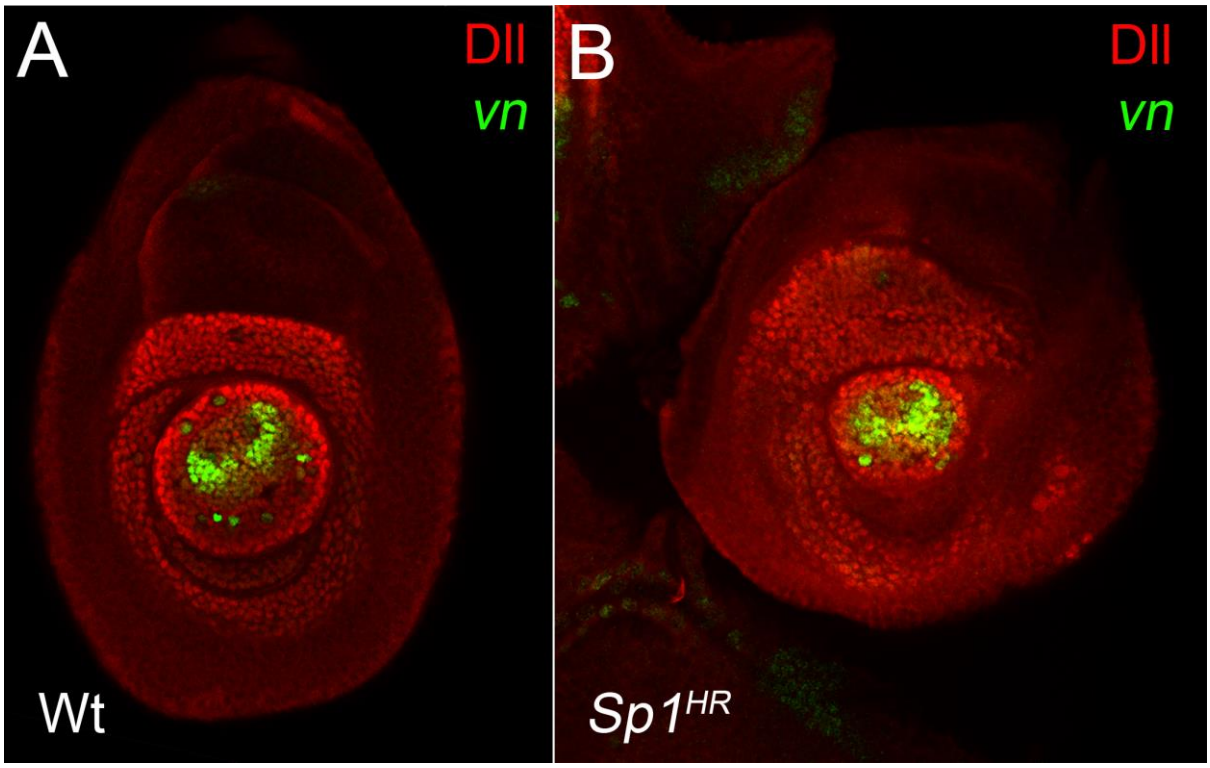

Figure S5: Expression of the EGFR ligand *vn* is maintained in *Sp1^{HR}* mutants. Dll (red) and *vn-lacZ* (green) expression in Wt (A) and *Sp1^{HR}* (B) L3 imaginal leg discs. Note that the expression of the EGFR ligand *vn* in the distal tip of the leg disc is not altered in the absence of *Sp1*.

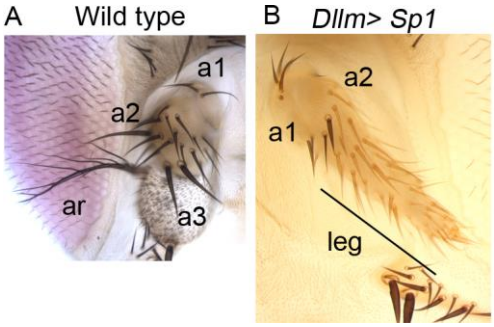

Figure S6: Misexpression of Sp1 in the antenna cause transformation to leg tissue. (A) Wild type antenna, where antennal segments 1, 2 and 3, and arista (ar) are indicated. (B) Misexpression of *Sp1* using the *Dllm-Gal4* driver causes the transformation of a3 and ar to leg-like structures (marked by *yellow*).

Table S1: List of the 337 genes identified (179 up-regulated and 158 down-regulated) that were significantly differentially expressed between $Sp1^{HR}$ and control leg discs.

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Table S2: From the total of genes identified, we decided to select those with a log2 fold change higher than one (absolute value) and that have a minimal expression level (see Materials and Methods). Following these criteria, a total of 30 up-regulated and 53 down-regulated genes were selected.

Click here to Download Table S2

Table S3: Primers used in this study.

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Table S4: List of RNAi lines used in this study.

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