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

Sergio Córdoba1, David Requena1, Aurelie Jory2,*, Almudena Saiz1,‡ and Carlos Estella1,§

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), Hh/Hes1 (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 (Sp-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 aristless (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
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 (DI) 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^{G81}) 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^{G81} and Df(btd,Sp1) homozygous mutants die as embryos, we deleted their function in the legs using Dil-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^{G81} 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 Dil-Gal4; UAS-flp to induce btd^{G81} 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.
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 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


mRNA mutant clones do not show any remarkable phenotype, with the exception of some cuticular abnormalities in the tibia (Fig. 3A). By contrast, Sp1 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 mutant clones generated near the distal end of a segment could induce the formation of small outgrowths (Fig. 3B). Similar phenotypes to Sp1


mutant clones generated 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).

The Notch pathway is activated in a row of cells adjacent and distal to the cells expressing the ligands Dll 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


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


mutant legs 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


mRNA mutant clones do not show any appreciable defect in the expression of these genes (Fig. 3I). 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 dysfusin (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


technique. Both Sp1 and btd misexpression induced Ser activation in the tarsal
Fig. 3. Sp1 loss-of-function affects Notch signaling and causes defects in joint formation. (A-C) Adult leg phenotypes caused by btd^{GEG1} (A), Sp1^{RR} (B) and Df(btd, Sp1) (C) yellow-marked clones generated 72-96 h AEL. (A) btd^{GEG1} clones only caused minor cuticular defects in the tibia (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). (B, 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). (F-H) 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-Sp1 or UAS-btd are misexpressed in the ptc domain (green arrows). 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). (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; tubGal80ts′ 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

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; tubGal80ts′ 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; tubGal80ts′ driver. Ectopic expression of Sp1 and btd disrupted joint formation in the tarsal region (H,I; insets).

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^{RR} 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.
Transcriptome analysis of Sp1 mutant leg discs and identification of new potential targets

Although some of the Sp1HR 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. Therefore, to test this hypothesis, we compared the transcriptome of male third instar leg imaginal discs of Sp1HR 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; Garcell et al., 2012). We confirmed this upregulation by using an eGFP trap in the gene (dilp8MO00727) to follow dilp8 expression in Sp1HR mutants and comparing it with control leg discs (Fig. 6B). Another confirmed upregulated gene is the antennal-specific distal antenna-related (dann) (Emerald et al., 2003; Suzanne et al., 2003), expression of which increases in the distal domain of Sp1HR mutant discs (Fig. 6C). The downregulated genes in Sp1HR 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 Sp1HR 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 Sp1HR 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 length:width ratio in control discs is approximately 0.8 whereas that in Sp1HR 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 (Dil-Gal4212; 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, Ppeck, 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
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
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 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; Vasiliasuakas 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 Sp1HR mutant flies by homologous recombination**

To generate Sp1HR mutant flies, we followed a homologous recombination protocol described by Baena-Lopez et al. (2013). Briefly, we cloned 2.3 kb
and 4.7 kb sequences that flank the third exon of Sp1 to act as ‘homology arms’ in the pTyCherry 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 Sp1IR/Sp1Ylz+ mutant embryos. See Table S3 for primers and restriction enzymes.

**Drosophila strains**

btu[1-2001], Df(btd;Sp1), UAS-btd, UAS-Sp1, btd-Gal4; UAS-GFP, Sp1 RNAi and Dll-LT-lacZ have been described previously (Estella et al., 2010; Estella et al., 2008). The Dil-Gal4 line 212, dpp-Gal4; ptc-Gal4, tubGal80p and the Sp1-GFP line (P{Bac(Sp1-EGFP.S)}VK00033) are all available at Bloomington Stock Center. Dllm-Gal4 (Dll-Gal4/12, UAS-flp; act-FRT-stop-FRT-Gal4, UAS-GFP, bish-lacZ, al-lacZ, B-HI-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). dpp5’672-EGFP and dam-Gal4; UAS-GFP were described by Garel et al. (2012) and Suzanne et al. (2003). The RNAi lines, listed in Table S4, were crossed with the Dllm-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/PY (control) and Sp1IR/Y (Sp1IR). A total of 1 µg of RNA per dissection was extracted using the RNA Microprep and RNA Clean and Concentrator Kits (Zymo Research). RNA-seq experiments, using the HiSeq2000 instrument (Illumina). Between 27.5 and 40.4 millions of reads were obtained, of which 85-87% of cDNA synthesis. Each library was sequenced using TruSeq SBS 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 RNA-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 Sp1IR 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-Dll (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-Hh (1/2000; Estella et al., 2008), and rabbit anti-Ap (1/1000; Bieli et al., 2015). Phallloidin-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 of the Y chromosome that covers the btd and Sp1 genes (Dp(1;Y) +ve ) (Schock et al., 1999); yw btu[1-2001] FRT19A/ubi-GFP M(1)oosp FRT19A; Dil-Gal4, UAS-flp, yw Sp1IR FRT19A/ubi-GFP M(1)oosp FRT19A; Dil-Gal4, UAS-flp, yw Df(btd;Sp1) FRT19A/ubi-GFP M(1)oosp FRT19A; Dil-Gal4, UAS-flp.

For loss-of-function clonal analysis we used the following genotypes: yw btu[1-2001], Sp1IR or Df(btd;Sp1) FRT19A/tubGal80 hsflp FRT19A; act-Gal4, UAS-C8 GFPl.

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-tubGal80p system, which allowed temporal restriction of UAS-Sp1 and UAS-btd expression to mid-third instar stage. ptc-Gal4; tubGal80p 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-muc-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 Sp1IR, Sp1-GFP genotype, which completely rescues the Sp1IR 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-β-Gal (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 pdeh 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**


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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

**References**


