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Tarsal-less peptides control Notch signalling through the Shavenbaby transcription factor

Jose I. Pueyo, Juan Pablo Couso *

School of Life Sciences, University of Sussex, Falmer, Brighton, UK

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ABSTRACT

The formation of signalling boundaries is one of the strategies employed by the Notch (N) pathway to give rise to two distinct signalling populations of cells. Unravelling the mechanisms involved in the regulation of these signalling boundaries is essential to understanding the role of N during development and diseases. The function of N in the segmentation of the *Drosophila* leg provides a good system to pursue these mechanisms at the molecular level. Transcriptional and post-transcriptional regulation of the N ligands, Serrate (Ser) and Delta (Dl) generates a signalling boundary that allows the directional activation of N in the distalmost part of the segment, the presumptive joint. A negative feedback loop between *odd-skipped*-related genes and the N pathway maintains this signalling boundary throughout development in the true joints. However, the mechanisms controlling N signalling boundaries in the tarsal joints are unknown. Here we show that the non-canonical *tarsal-less (tal)* gene (also known as *pri*), which encodes for four small related peptides, is expressed in the N-activated region and required for joint development in the tarsi during pupal development. This function of *tal* is both temporally and functionally separate from the *tal*-mediated tarsal intercalation during mid-third instar that we reported previously. In the pupal function described here, N signalling activates *tal* expression and reciprocally Tal peptides feedback on N by repressing the transcription of *Dl* in the tarsal joints. This Tal-induced repression of *Dl* is mediated by the post-transcriptional activation of the Shavenbaby transcription factor, in a similar manner as it has been recently described in the embryo. Thus, a negative feedback loop involving Tal regulates the formation and maintenance of a Dl+/Dl− boundary in the tarsal segments highlighting an ancient mechanism for the regulation of N signalling based on the action of small cell signalling peptides.

Introduction

The organisation of cells in complex tri-dimensional structures relies to a great degree on efficient communication between cells. Cell–cell interactions coordinate patterns of cell division, survival, migration and differentiation, giving rise to the formation of the final organ. Despite the vast variety of cell types and cell communication events, only a small number of cell signalling pathways have been characterized. One of these is the Notch (N) pathway, which consists of a single transmembrane receptor, N, that is activated by binding to the transmembrane DSL ligands (named after Delta (Dl), and Serrate (Ser) and LAG-2) from the neighbouring cells (Bray, 2006; Fleming et al., 1997). Upon binding the N receptor undergoes two consecutively proteolytic cleaves by the ADAM-metalloprotease and γ-secretase complexes respectively, releasing the N intracellular domain (N^{icd}) (Bray, 2006; Fortini, 2009). Consequently, the N^{icd} translocates into the nucleus where it binds to the CSL (named after CBF1, Su(H) and LAG-1) transcriptional complex and activates the transcription of target genes (Bailey and Posakony, 1995; Bray, 2006; Lecourtois and Schweisguth, 1995).

The fundamental role of the N pathway in different developmental processes from lateral inhibition to formation of patterning boundaries is to control cell fate choices between neighbouring cells (Artavanis-Tsakonas et al., 1999; Bray, 2006). N-mediated cell fate determination relies on the differential expression of ligand and receptor in opposing cells. This differential distribution, between ligand in the signal cell and receptor in the responsive cell, is accomplished by a negative feedback loop by N signalling that represses ligand expression in the responsive cell (Artavanis-Tsakonas et al., 1999; Fortini, 2009). Reciprocally, ligand expressing cells lose their own ability to respond to Notch, by transcriptional or post-transcriptional repression of N (Becam et al., 2010; Fortini, 2009; Miller et al., 2009). Thus, feedback loops amplify and reinforce the differential distribution of the ligand and the receptor giving rise to different cell fates within a population of competent cells. It appears that these feedback loop mechanisms controlling N signalling differ depending on the developmental context (Heitzler et al., 1996; Huppert et al., 1997). Therefore, unravelling these mechanisms is key to

* Corresponding author at: School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK.
E-mail address: j.p.couso@sussex.ac.uk (J.P. Couso).

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understanding how N signalling regulates these developmental processes, and hence diseases where N signalling is deregulated, such as cancer (Rizzo et al., 2008; Roy et al., 2007; Stylianou et al., 2006; Weng et al., 2004).

The legs of *Drosophila* are a good system in which to study N signalling as the genetic cascade controlling leg development and the subsequent morphogenesis is well understood (Fristrom and Fristrom, 1993; Galindo and Couso, 2000; Kojima, 2004; Manjon et al., 2007; Mirth and Akam, 2002). *Drosophila* legs are composed of segments separated by flexible specialized structures called joints (Fig. 1E–F) (Fristrom and Fristrom, 1993). There are two types of joints according to their structure and function (Bishop et al., 1999; Fristrom and Fristrom, 1993; Mirth and Akam, 2002; Tajiri et al., 2010): the true joints, which correspond to the coxa, trochanter, femur and tibia segments and pretarsus, are attached by muscles and each one has a unique morphology (Fig. 1E); the joints of the tarsal segments have an identical ball and socket structure and do not develop muscle attachments (Fig. 1F).

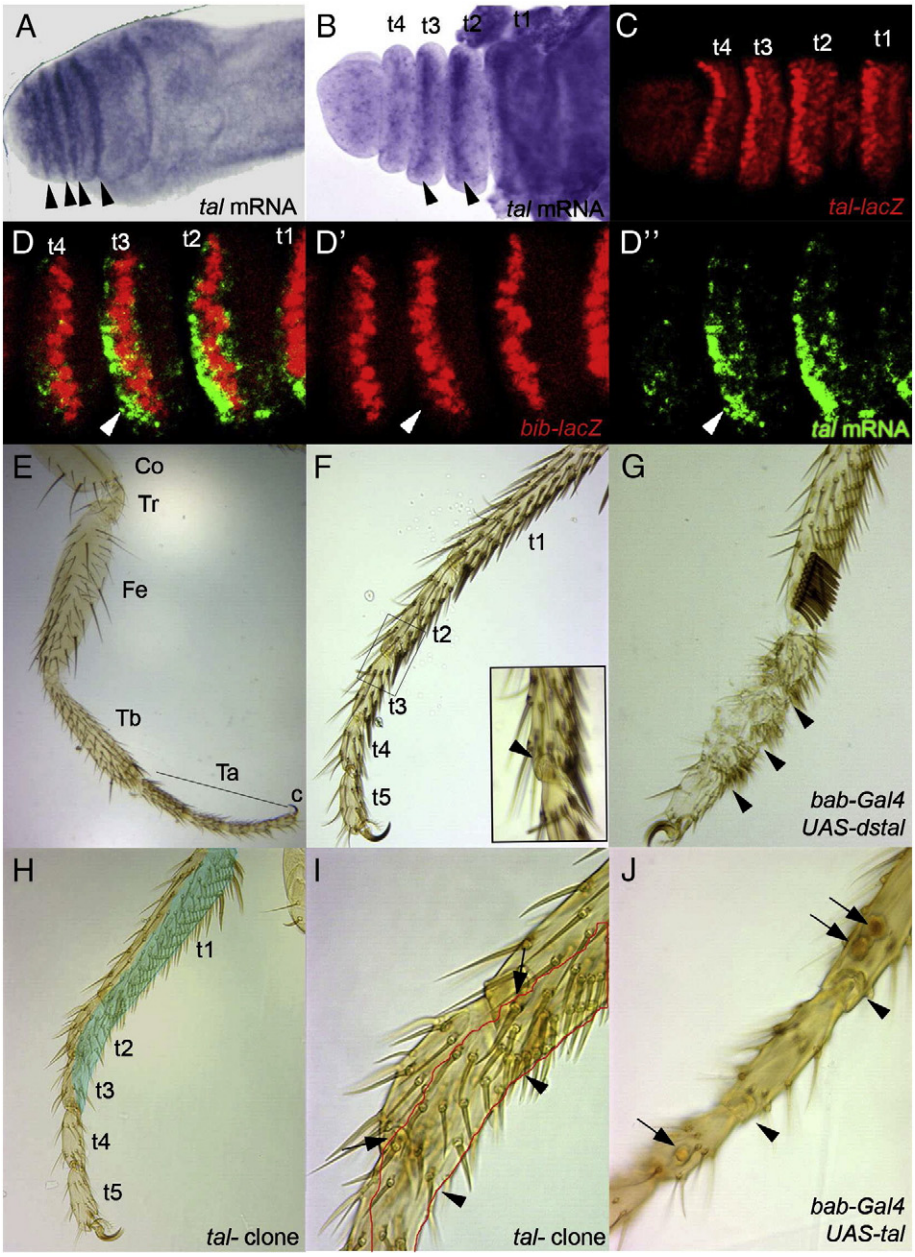

Fig. 1. Tal is required for joint development in the tarsal segments. A — pupal leg (4 h after puparium formation (APF)) showing stripes of *tal* mRNA expression in the tarsus (arrowheads). The leg is starting to evert; distal to the left. B — *tal* mRNA localisation in a pupal leg at 6 h APF. *tal* is expressed in the distal part of the tarsal (t1–t4) segments (arrowheads) near the joint constrictions. C — distal part of a *tal-lacZ* pupal leg (8 h APF) showing strong *tal* expression in the distalmost part of each tarsal segment. D–D″ — everted pupal leg (8 h APF) showing *tal* mRNA (green, arrowhead) and *bib-lacZ* (red) expression patterns. *bib-lacZ* and *tal* mRNA are expressed adjacent to each other in the distalmost part of the tarsal segments (D). red channel showing *bib-lacZ* expression (D′). *tal* mRNA expression (arrowhead) (D″). E — leg of a wild-type fly, showing the true segments (Coxa (Co), Trochanter (Tr), Femur (Fe), Tibia (Tb) and Pretasus (c)), and the tarsal segments (Ta). F — distalmost part of a wild-type leg showing the tarsal segments (t1–t5) separated by naked joint tissue. Inset denotes the stereotypical ball and socket tarsal joint (arrowhead). Proximal to the top, distal to the bottom. G — tarsal region of a *bab-Gal4;UAS-dstal* leg. The tarsal segments are misshapen, lacking joints (arrowheads). H — *tal* null clone induced between 96 and 120 h AEL that runs along the ventral part of the tarsi marked with *forked* (blue). Note that every tarsal segment is still present. I — high magnification of H. In the *tal* null homozygous clone the joints do not form (arrowheads). However, some *forked* mutant bristles lacking *tal* can form part of remaining joint structures, revealing the non-autonomous nature of *tal* function (arrows). J — *bab-Gal;UAS-tal* tarsi showing ectopic joint structures (arrows) adjacent to the proper joint (arrowheads).

Leg segmentation and subsequent joint development are controlled by the spatially regulated activation of the N signalling pathway and its ligands (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). During leg development a complex regulatory gene network of proximodistal (PD) patterning genes induces the activation of *Ser* and *Dl* expression in the distalmost region of each segment just proximal to the presumptive joint region (Rauskolb, 2001; Shirai et al., 2007; St Pierre et al., 2002). Different regulators such as Fringe and the PCP (Planar Cell Polarity) and Ras pathways are involved in restricting Ser and Dl signalling to the distal side of the *Dl* and *Ser* expression domains, generating a signalling boundary (Bishop et al., 1999; de Celis et al., 1998; Galindo et al., 2005; Shirai et al., 2007). The activation of downstream genes, such as *activator protein-2 (AP-2)*, *Enhancer of Split complex* (*E(spl)*), *disconnected* and *big brain* (*bib*) in these distal cells initiates the joint developmental programme (Bishop et al., 1999; de Celis et al., 1998; Kerber et al., 2001). Maintaining the spatial asymmetry between ligand expression and N activation is vital for leg segmentation, as ectopic expression of ligands in the N responsive region represses joint formation through post-transcriptional down regulation of N (Bishop et al., 1999; Rauskolb and Irvine, 1999).

It has been shown that sharp N signalling boundaries in the true joints are maintained by a negative feedback loop between the N signalling pathway and the "odd-skipped *(odd)*-related" *drumstick (drm)-lines-bowl* genes cassette (Greenberg and Hatini, 2009). In cells expressing *Dl*, the Lines protein is active and nuclear leading to the destabilisation of the Bowl protein and its subsequent degradation (Greenberg and Hatini, 2009; Hatini et al., 2005). In the adjacent joint cells, N signalling activates the expression of *drm*, and as a result the Drm protein binds to Lines thus preventing Bowl degradation; consequently, Bowl can accumulate in the nucleus where it represses *Dl* expression (Greenberg and Hatini, 2009). This elegant mechanism reinforces a Dl+/Dl− signalling boundary and ensures its maintenance through joint development. Although AP-2 contributes also to the repression of *DSL* ligands in the true joints, possibly in parallel to the Drm–Lines–Bowl mechanism, and in the tarsal joints (Ciechanska et al., 2007), it appears that there must be other, currently unknown, factors contributing to the generation and maintenance of the Dl+/Dl− boundary in the tarsal segments in an analogous way to the *drm–lines–bowl* cassette at work in true segments (Ciechanska et al., 2007).

Here we show that the non-canonical *tarsal-less* (*tal*) gene (also known as *polished rice*) is the crucial factor for the establishment and maintenance of a Dl+/Dl− signalling boundary in the tarsal joints. *tal* produces a single polycistronic transcript that encodes for several small related peptides required for the development of embryonic ectodermal structures, such as the denticle belts and trachea and also for leg tarsal development (Galindo et al., 2007; Kondo et al., 2007; Pueyo and Couso, 2008). Interestingly, the 11 amino acid long Tal peptides act non-autonomously in each developmental context explored, indicating that Tal peptides could be a new type of cell signal (Kondo et al., 2007; Pueyo and Couso, 2008). A recent study on the function of Tal during denticle formation has revealed that Tal peptides are able to switch the Shavenbaby (Svb) transcription factor, a master protein involved in denticle formation, from a repressor to an activator (Kondo et al., 2010; Payre, 2004). This change in behaviour of Svb is associated with a change in its nuclear distribution; the Svb repressor form is localized in nuclear foci, whereas the Svb activator appears diffused throughout the nuclei. Tal triggers Svb activation by the induction of a post-translational modification of Svb, giving rise to an amino-terminal end (Nt) truncated Svb short form which is similar to the germline Svb short variant OvoB. Although Tal peptides control the activation of Svb and denticle formation, it is important to note that other Tal functions during embryonic development are independent of Svb (Kondo et al., 2010).

In this report we show that *tal* is expressed in the N responsive region of the tarsal joints and is required for their development. Dl signalling activates *tal* expression in adjacent cells and subsequently Tal peptides repress *Dl* expression in these cells, generating a sharp signalling boundary. Tal mediated repression of *Dl* is achieved through the post-transcriptional activation of the Svb transcription factor. Therefore, a negative feedback loop involving Tal regulates the formation and maintenance of a Dl+/Dl− border. Thus, our work highlights a new mechanism for the regulation of N signalling, based on the action of small cell signalling peptides.

Materials and methods

Fly stocks and genetics

Drosophila stocks were raised at 25 °C on a standard cornmeal/agar/yeast medium. The following fly strains have been used: *tal-lacZ*, *tal^{S18}FRT82B* (Galindo et al., 2007), *UAS-dstal* (this work); *svb^{P107}-lacZ*, *y svb^{R9}FRT19A,UAS-ovoB*, *UAS-svb*, *UAS-ovoD* (Delon et al., 2003); *UAS-Svb-GFP* (Kondo et al., 2010); *N^{ts},Df(3)Dl Bx12*, *UAS-N^{intra}*, *UAS-N^{ecd}* and *UAS-Dl* (Bishop et al., 1999); *bib^{E1}-lacZ*, *Gbe+Su(H)-lacZ* (Furriols and Bray, 2001) and *E(spl)mβ1.5-lacZ* (Cooper et al., 2000). The following Gal4 lines *dpp-Gal4*, *omb-Gal4*, *bab-Gal4*, *ptc-Gal4, and Dll-Gal4* were used for ectopic expression (Brand and Perrimon, 1993) and their expression patterns were described in Galindo et al.(2007) and Pueyo and Couso (2008). Several lines carrying FRT chromosomes have been used: *w hsFLP122 Ubi-RFP FRT19A*; *y w f^{36a}hsFLP122;M(3) f^{+}(w^{+})FRT82B/TM6B* (G.Bellido); and *w hsFLP122;M(3) Ubi-GFP FRT82B/TM6B*.

For induction of clones we have utilized the FRT/FLP system (Xu and Rubin, 1993). For *tal^{S18}* loss of function clones, larvae were heatshocked for 45 min at 37 °C between 96 and 120 h after egg laying (AEL). *svb^{R9}* loss of function clones were induced as above between 48 and 72 h AEL. Gain of function flip-out clones were generated by crossing different UAS lines to *y w hsFLP122; Act5>y+>Gal4; UAS-GFP* cassette and heatshocking the offspring for 20 min at 37 °C at 96–110 h AEL.

The *N^{ts}* temperature-sensitive allele was crossed to null alleles and then shifted from the permissive temperature 18 °C to the restrictive temperature 25 °C at 96–110 h AEL to give rise to individuals with almost total loss of function in the tarsi (Bishop et al., 1999).

Generation and expression of double stranded tal construct (dstal)

A 400 pb fragment corresponding to the 5′UTR of the *tal* transcript was amplified by PCR using the following primers: dstalF 5′CACCTGCAGATCACCAGCTAAAAGAAA3′ and dstalR 5′CGTATGCCGTGTATTGACCAAAAATAC3′. The PCR fragment was cloned between the attL1 and attL2 recombination sites in the pENTR-TOPO vector (Invitrogen). Subsequently, *in vitro* recombination using the LR clonase (Invitrogen) was induced between the pENTR-dstal-TOPO vector and the pRISE-ftz vector with two inverted sequences flanked by the attR1 and attR2 recombination sites separated by the *ftz* intron (Kondo et al., 2006). Selection of the colonies with the appropriate pRISE-dstal vector was performed as described in Kondo et al. (2006). The efficiency of the dstal construct in knocking down *tal* expression was tested in S2R+cells by monitoring the Tal1A-GFP expression in S2R+cells transfected with the pRISE-dstal construct (Suppl. Figs. 1F,G; Galindo et al., 2007). Transgenic flies carrying the pRISE-dstal construct were generated following standard procedures (Vanedis injection Service).

To knock down *tal* function in flies we expressed the *UAS-dstal* constructs together with the *UAS-Dicer* constructs as *Dicer* overexpression makes more efficient the production of small double-stranded RNAs.

Immunocytochemisty, in situ hybridisation and microscopy

Pupae at the appropriate stage were collected and dissected as described in Bishop et al. (1999). Standard procedures for immunohistochemistry were followed and the following antibodies were used: mouse anti-AP2 (Kerber et al., 2001), mouse anti-Dl (DSHB), and rabbit anti-β-galactosidase (Cappel). For the detection of the Svb-GFP we have used rabbit anti-GFP (Molecular Probes) and we have amplified the signal using tyramide signal amplification system (Perkin Elmer) (Kondo et al., 2010). Secondary antibodies conjugated to different fluorophores were used to 1:100 (Jackson ImmunoResearch). DAPI (Invitrogen) has been used to label nuclei. Standard protocol for *in situ* hybridisation was followed with minor changes (Galindo et al., 2005). For the fluorescent *in situ* hybridisation and antibody assays we followed the standard *in situ* protocol with the following changes. The proteinase K treatment step was avoided and replaced by a hot hybridisation step at 72 °C in Hybrix solution. After hybridisation washes we proceed with the standard immuno-staining protocol. The DIG-labelled probe was detected with anti-DIG antibody coupled to horseradish peroxidase (Roche) followed by tyramide signal amplification reaction (Perkin Elmer). The labelled *Dl* riboprobe was synthesized by digesting *Dl* LD21369 pOT2 construct (DGRC) with EcoRI restriction enzyme and using it as a template for the Sp6 promoter RNA synthesis with DIG labelled ribonucleotides (Roche). For the *svb* riboprobe, Svb LD47350 pOT2 construct (DGRC) digested with EcoRI restriction enzyme was used as a template for Sp6 RNA production and labelled as above. Images were acquired with a Leica DRBM microscope and a Zeiss LSM 510 confocal microscope, and processed with QWin, LMS and Photoshop software.

Results

Tarsal-less is required non-autonomously for tarsal joint development

We have previously shown that *tal* has a role in the determination of the presumptive tarsal region in early third instar larvae (Galindo et al., 2007; Pueyo and Couso, 2008). At this time, a single domain of *tal* expression is intercalated between the expression domains of the *dachsund* (*dac*) and *Bar* (*B*) genes. Next Tal represses *B* and *dac* through the activation of the Rotund (Rn) and Spineless (Ss) transcription factors, thus generating a new territory of presumptive tarsal cells defined by the presence of Rn and Ss and the absence of Dac and B. (Pueyo and Couso, 2008). We have identified a new role for *tal* in later stages of leg development. During early pupal development, both *tal* mRNA and *tal-lacZ* reporter are expressed in stripes of cells in the distal part of each tarsal segment (Figs. 1A–C). These stripes of cells correspond to the joint region because *tal* expression is distally adjacent to the expression of the N target gene *bib* (Figs. 1D–D″), which identifies the proximal side of the presumptive joint (de Celis et al., 1998; Galindo et al., 2005).

To characterize Tal function during joint development without disrupting its earlier function we have performed mosaic analysis. In legs with *tal* loss of function clones induced after tarsal intercalation, all the tarsal segments are present indicating that tarsal intercalation has proceeded normally (Figs. 1H–I). However, these clones are not phenotypically normal, as no joint structures are formed in the middle of large clones covering the distal part of the tarsal segments (Fig. 1I). Joint loss is prefigured in the developing pupal legs by the loss of *bib-lacZ* reporter which is a marker of joint cell fate (Figs. 2A–A′, B–B′) (de Celis et al., 1998; Shirai et al., 2007). As in other developmental contexts *tal* acts non-autonomously, some *tal* mutant cells develop joint structures (Fig. 1I). Similarly, *bib-lacZ* expression can be observed in *tal* mutant cells, which are up to 3–4 cell diameters away from the *tal*-expressing cells, but it is lost in *tal* mutant cells located further away (Figs. 2B–B′). These observations are in agreement with the non-autonomous range of action of Tal peptides

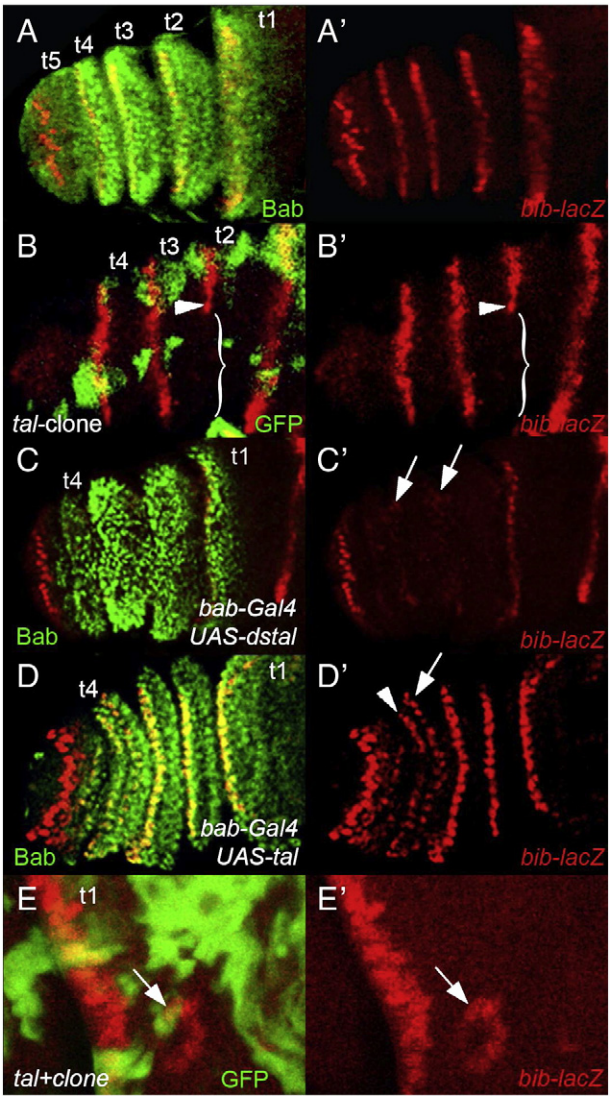

Fig. 2. Tal regulates N target genes non-autonomously in the tarsal joint. A–A′ — expression of the joint marker *bib-lacZ* (red) in the tarsal region covered by Bab protein (green) in a pupal leg (6 h APF) (A). *bib-lacZ* expression is limited to a single row of cells in the distal part of the tarsal segments (A′). B–B′ — distal part of a *bib-lacZ* pupal leg (5 h APF) containing *Minute + GFP-*, *tal-* null clones. GFP expression labels the *tal+* tissue. *bib-lacZ* expression (red) is absent in the middle of a large *tal* mutant clone (brackets). Note that *tal* acts non-autonomously in *bib-lacZ* regulation (arrowhead) (B). Expression of *bib-lacZ* (B′). C–C′ — a *bib-lacZ* (red) pupal leg (5 h APF) expressing *UAS-dstal* in the tarsi using the *bab-Gal4* driver (green) (C). Strong reduction of the *bib-lacZ* reporter is observed (arrows) (C′). D–D′ — ectopic joints in a *bab-Gal4;UAS-tal* pupal leg (6 h APF) showing Bab (green) and *bib-lacZ* (red) patterns of expression (D). A duplicated row of *bib-lacZ* expressing cells is observed (arrowhead: endogenous; arrow: ectopic) (D′). E–E′ — *tal* gain of function clones (green) induce ectopic expression (arrow) of the *bib-lacZ* reporter (red) in the distal part of the first tarsal segment (E). *bib-lacZ* expression (E′).

in other developmental processes (Kondo et al., 2007; Pueyo and Couso, 2008).

To further prove the essential role of Tal peptides in tarsal joint development we have expressed a *tal* construct that produces double stranded *tal* RNA, which efficiently knocks out *tal* expression in the tarsus using the *Dll-Gal4* (not shown; (Calleja et al., 1996)) and *bab-Gal4* (Cabrera et al., 2002) drivers (Suppl. Figs. 1A–A″, D, E). In these *UAS-dstal* RNAi flies, the tarsal segments are misshapen and lack joint structures and they also exhibit necrotic tissue and ectopic bristles (Fig. 1G and Suppl. Fig. 1C). In addition, a significant reduction of *bib-lacZ* expression can be observed in tarsal segments in the *bab-Gal4;*

UAS-dstal pupal legs (Figs. 2C–C′). Altogether these results show that *tal* is required for tarsal joint development.

To define the role of *tal* in joint development further, we mis-expressed *tal* in the tarsal region. Ectopic *tal* expression throughout the tarsi produces extra joint structures proximal to the endogenous ones (Fig. 1J and Suppl. Table 1). In pupal *bab-Gal4;UAS-tal* legs, a proximal ectopic stripe of cells expressing *bib-lacZ* or AP-2 can be observed (Figs. 2D–D′; Suppl. Figs. 2A–A″,B–B″). Induction of ectopic joints is not a consequence of the early role of *tal* in regulating *B* and *dac* genes since the expression patterns of these genes are not affected (Suppl. Figs. 2G–G″,H–H″). Similarly, *tal* gain of function clones are only able to activate *bib-lacZ* ectopically proximal to the joint, in a non-autonomous manner (Figs. 2E–E′). Thus, Tal-mediated induction of ectopic joints seems to require a factor(s) located in the cells proximal to the endogenous joint.

Tal function requires N signalling during tarsal joint formation

The formation of the joints depends on a complex gene interaction network that ensures N signalling activation in the distalmost part of the segment (Bishop et al., 1999; de Celis et al., 1998; Galindo et al., 2005; Shirai et al., 2007). Given that *tal* is expressed and required in the N responsive domain, and that *tal* induces ectopic joints in the region proximal to the endogenous joints where N ligands are expressed (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999), we have searched for genetic interactions between *tal* and N signalling during joint formation.

To test whether *tal* is a downstream target of N signalling in tarsal joints first we used N^{ts} thermosensitive mutants shifted to the restrictive temperature before tarsal segmentation takes place at the end of third larval instar. The tarsal joints do not form in these N^{ts} mutants (Suppl. Fig. 1B; Bishop et al., 1999) and this is correlated with the loss of *bib-lacZ* expression (Figs. 3A–A′). Similarly, *tal-lacZ* expression is lost from the N^{ts} mutant tarsal joints (Figs. 3B–B′) indicating that N signalling is required for *tal* expression. Next, we ectopically expressed a N dominant negative form (N^{ecd}) that knocks out N signalling, using the *omb-Gal4* driver, which is expressed in the dorsal part of the pupal legs. As a result, these flies have deformed legs with incomplete joints (not shown) and *tal-lacZ* expression is lost or reduced in the dorsal part of the pupal legs (Figs. 3C–C′). Thirdly, we activated the N pathway by ectopically expressing the constitutively active form of Notch (N^{icd}) or its ligand Dl. Ectopic expression of *Dl* or N^{icd} using the *ptc-Gal4* driver or in flip-out clones induced ectopic *tal-lacZ* expression (Figs. 3D–D′; Suppl. Figs. 3A–A′) and flies with shorter legs and ectopic joints (not shown). Importantly, N- mediated activation of *tal* expression is limited to this developmental stage since ectopic expression of N^{icd} or *Dl* in mid third instar leg discs does not induce ectopic *tal-lacZ* expression (Suppl. Figs. 3B–B′, C–C′).

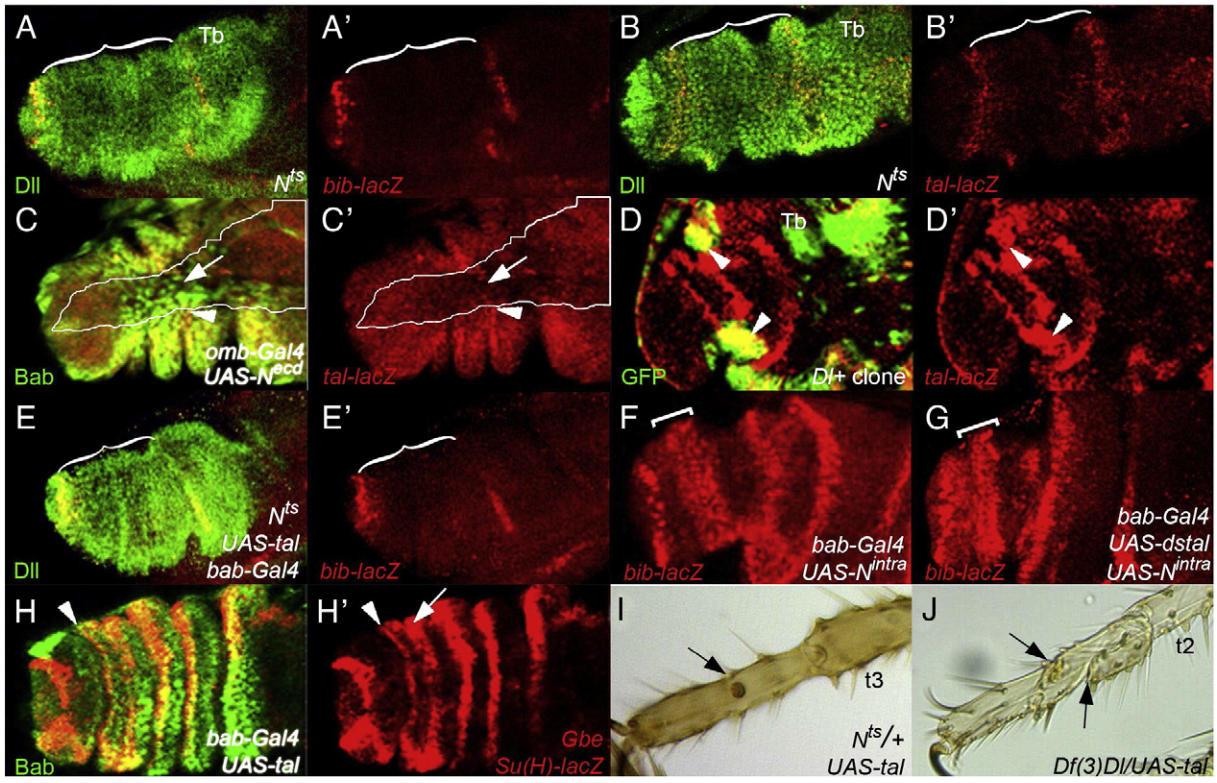

Fig. 3. N signalling functions upstream and downstream of Tal in joint development. A–A′ — distal part of a N^{ts} mutant pupal leg (4 h APF) shifted to the restrictive T[a] at late third instar, showing the unaffected expression of the Dll gene (green), that is not regulated by *N*, and *bib-lacZ* (red). No *bib-lacZ* expression is detected in the tarsus (brackets) (A). red channel (A′). B–B′ — N^{ts} mutant pupal leg (6 h APF) treated as in A) showing *tal-lacZ* (red) and Dll (green) expression patterns (B). *tal-lacZ* expression is missing in the tarsus (brackets) (B′). C–C — ectopic expression of a dominant negative N form (N^{ecd}) using *omb-Gal4* driver in a pupal leg (6 h APF) showing *tal-lacZ* (red) and Bab (green). Note that only Bab is expressed in the dorsal part of the disc (arrow) and *tal-lacZ* expression is repressed from this domain and only detected in the lateral parts (arrowhead) outside of *omb-Gal4* domain (outline) (C). *tal-lacZ* expression (C′). D–D′ — *tal-lacZ* (red) pupal leg (4 h APF) containing *Dl* gain of function clones (green). Ectopic expression of *tal-lacZ* is observed in the *Dl*-expressing clones (arrowheads) (D). *tal-lacZ* expression (D′). E–E′ — overexpression of *tal* in a N^{ts} mutant background pupal leg (4 h APF) showing Dll (green) and *bib-lacZ* (red) expression patterns (E). *bib-lacZ* expression is lost in the tarsal segments (brackets) (E′). F– — pupal leg (5 h APF) expressing N^{intra} in the tarsal region. N^{intra} expands *bib-lacZ* expression in the tarsus (bracket). G — ectopic co-expression of N^{intra} and *UAS-dstal* in the tarsi of a pupal leg (5 h APF). *bib-lacZ* still appears expanded in the tarsus (bracket). H–H′ — a 6 h APF *bab-Gal4;UAS-tal* pupal leg showing the expression of an enhancer regulated directly by N^{intra} (*Gbe+Su(H)-lacZ*) (red) and Bab (green). Ectopic expression of *Gbe+Su(H)-lacZ* is observed in t4 in a row of cells (arrow) more proximal to the endogenous pattern (arrowhead); compare with Fig. 2D and Suppl. Fig 2E (H). *Gbe+Su(H)-lacZ* expression (H′). I — distal part of a *bab-Gal4;UAS-tal* leg in a N^{ts} heterozygous background. No ectopic joint structures are detected. Instead some tarsus display defective joints (arrow). J — tarsi of a leg over-expressing *tal* in a heterozygous background for *Dl* (*Df(3)*Dl^{BX12}/+). As in I), no ectopic joint tissue is observed and some tarsal joints are defective, leading to tarsal segment fusions (arrows).

Altogether these results indicate that N signalling activates *tal* expression during joint development.

However, further genetic tests reveal a more complex scenario. Firstly, the ectopic joint phenotype produced by over-expression of UAS-driven Tal peptides in the tarsi is suppressed by *N* or *Dl* haploinsufficiency (Figs. 3 I, J). Since *tal* expression in these experiments is regulated by the *bab-Gal4* driver and thus does not depend on N, the observed phenotypic suppression must be due to a post-transcriptional interaction between Tal peptides and the N pathway. Secondly, UAS-driven expression of *tal* does not rescue the N^{ts} mutant phenotype and does not restore *bib-lacZ* expression (Figs. 3E–E′; compare with A–A′), suggesting that Tal peptides are unable to induce joints in the absence of N. Finally, the phenotype caused by ectopic expression of the N^{icd} is epistatic over the loss of joint markers induced by ectopic expression of the *UAS-dstal* RNAi construct (Figs. 2C–C′, 3F, G). Thus, although N signalling is required for the activation of *tal* expression, these results suggest that N signalling also acts downstream of *tal*. One possible explanation is that Tal peptides interact with the product of a gene regulated by N in a feed-forward mechanism; however, it may be also possible that Tal function feeds back on the N pathway.

To test whether Tal function involves direct interaction with the N signalling pathway and not a downstream gene product, we have used reporters activated by direct binding of N^{intra} and Suppressor of Hairless (Su(H)) to their regulatory sequences. The *grainyhead Gbe+Su(H)-lacZ* reporter is expressed in the joint regions (Suppl. Fig. 2E; Furriols and Bray, 2001) and is activated ectopically by over-expression of *tal* in the tarsi (Figs. 3H–H′) in a similar manner to *bib-lacZ* (Figs. 2D–D′). In addition, *tal* gain of function clones also induce ectopic *E(spl)mβ-lacZ* expression non-autonomously proximal to the endogenous tarsal joint expression (Suppl. Figs. 2C, F–F′; Cooper et al., 2000). Reciprocally, *E(spl)mβ-lacZ* expression is lost in *tal* mutant clones in a non-autonomous manner (Suppl. Figs. 2C, D–D′). Thus, these observations indicate that Tal peptides act upstream of the direct transcriptional targets of N and therefore directly on the N pathway itself.

Altogether these genetic interactions suggest that N signalling is acting at two different levels in relation to *tal* function. On the one hand, N signalling is upstream of the *tal* gene to activate its transcription. On the other hand, N signalling is essential for the joint promoting function of the Tal peptides. Thus, we surmise that Tal peptides act as feedback regulators of the N signalling pathway in the formation of tarsal joints.

Tal regulates N signalling by establishing a Dl+/Dl− boundary

To understand the mechanism by which Tal peptides signal back onto the N pathway, we compared the expression patterns of *tal-lacZ* to those of different components of the Notch pathway. The ligand Dl is expressed at low levels in the proximal and medial parts of the leg segments, with a stripe of high level of expression just proximal to distalmost region, where little or no Dl expression can be detected (Bishop et al., 1999). This pattern forms a sharp Dl+/Dl− signalling boundary that triggers N activation in the Dl-negative cells (Figs. 4A–A″). *tal-lacZ* is expressed a few rows of cells further away from the Dl expressing domain (Figs. 4A–A″); given the signalling function of Tal peptides, this suggests that a negative feedback loop between Tal and Dl may exist.

Several results support this hypothesis. First of all, in large *tal* loss of function clones in which *bib-lacZ* expression is lost, Dl expressing cells are ectopically found in the distalmost part of the segment (Figs. 4B–B′). However, Dl and *bib-lacZ* expression are not affected in smaller *tal* mutant clones, indicating that Tal represses Dl expression non-autonomously (Figs. 4C–C"). Secondly, ectopic expression of *tal* with the *dpp-Gal4* driver represses Dl expression (Figs. 4E–E′). This repression inhibits the formation of the joints in the *dpp* region which is corroborated by the loss of joint markers (Figs. 4G–G′). However, in some instances Tal-mediated repression of Dl also allows the creation of new Dl+/Dl− signalling boundaries that activate N signalling at the edges of the *dpp* expression domain and induce the expression of *bib-lacZ* proximally to the endogenous joint territory (Figs. 4G–G′). Finally, this regulation of Dl is at the level of transcription as *Dl* mRNA is down-regulated in *dpp-Gal;UAS-tal* pupal legs (Figs. 4 D, F). Thus the Tal peptides in the joint region repress *Dl* transcription, generating a signalling border which is essential for the activation of N downstream targets.

Tal-mediated activation of the transcription factor Svb represses Dl expression

tal is a non-canonical gene that encodes four small related peptides which behave genetically as cell signals but their mechanisms of action are still not well understood. Recently, Kondo and colleagues have demonstrated that Tal peptides trigger a functional switch in the Svb transcription factor during embryonic epidermal differentiation. In the absence of Tal, Svb protein acts a repressor of denticle formation, but in the presence of Tal, Svb protein is converted into an activator. We have explored whether the role of Tal in the transcriptional regulation of *Dl* is mediated by Svb. We observe that the *svb* mRNA is expressed in the presumptive joint regions in late third instar (not shown) and pupal legs (Fig. 5A). Similarly, a svb^{PL107} (*svb-lacZ*) enhancer trap, which reproduces the endogenous *svb* pattern during embryogenesis (Bourbon et al., 2002), is also expressed in stripes of cells mostly distal to the Dl expression domain in pupal legs (Figs. 5H–H″). Crucially, expression of a Svb-GFP construct in the tarsus shows that Svb-GFP is localized throughout the nucleus in the distal part of the segments near *bib-lacZ* expression whereas it is localized in nuclear puncta structures in more proximal parts (Figs. 5G–G″). This result suggests that, as has been observed in the embryo, Svb post-transcriptional activation takes place where Tal peptides are present. These results indicate that Svb may indeed be the effector of *tal* function for the transcriptional repression of *Dl* in the tarsi. Supporting this view, escapers of the *svb* hypomorphic allele, svb^{P107}, show incomplete joint formation in some tarsal segments (Fig. 5B). To further explore this hypothesis we have performed mosaic analysis using a svb^{R9} null allele. Quantification of the number of *svb* mutant clones running through joints between true segments and between the tarsi shows that the number of *svb* clones crossing the tarsal joints is lower than expected (Suppl. Table 2). From these tarsal joint-crossing svb^{R9} clones, those being two or more rows of bristles wide (>25%) produced an autonomous loss of joint tissue (Fig. 5C) (Suppl. Table 2). Thus, we conclude that *svb* is expressed and required for tarsal joint development.

We have undertaken ectopic expression experiments to demonstrate that *Dl* repression induced by Tal peptides in the tarsal joint is mediated by Svb activation. Ectopic expression of *svb* using the *bab-Gal4* driver does not produce phenotypes in the leg (Fig. 5D) which suggest that the encoded Svb protein requires post-transcriptional activation. However, ectopic co-expression of *svb* with *tal* produces loss of joints in the tarsi (Fig. 5E) and a significant reduction of *bib-lacZ* expression in pupal legs (Fig. 5I). This result might seem contradictory with the previous finding of a joint-promoting function for *tal*, and in particular, with ectopic joints in *bab-Gal4;UAS-tal* legs (Figs. 1F, 2D–D′). A possible interpretation is that Tal and Svb are actually repressing N signalling. Corroborating this hypothesis, ectopic expression of a Svb constitutively active form (OvoB) with *bab-Gal4* driver produces shorter tarsi lacking all joints (Fig. 5F), in which *bib-lacZ* expression is highly reduced or absent in pupal legs (Fig. 5J). Similarly, reduction of *bib-lacZ* expression and Dl protein distribution is observed in *dpp-Gal4;UAS-tal;UAS-svb* (not shown) and *dpp-Gal4;UAS-ovoB* (Figs. 5L–L″) pupal legs leading to loss of joint tissue (Suppl. Figs. 3D, E). Finally, this repression of *Dl* expression is at the level of transcription as *Dl* mRNA is downregulated in *dpp-Gal4;UAS-ovoB* pupal legs (Fig. 5K). Therefore, Tal-mediated activation of Svb promotes the repression of *Dl* in the tarsal joint region.

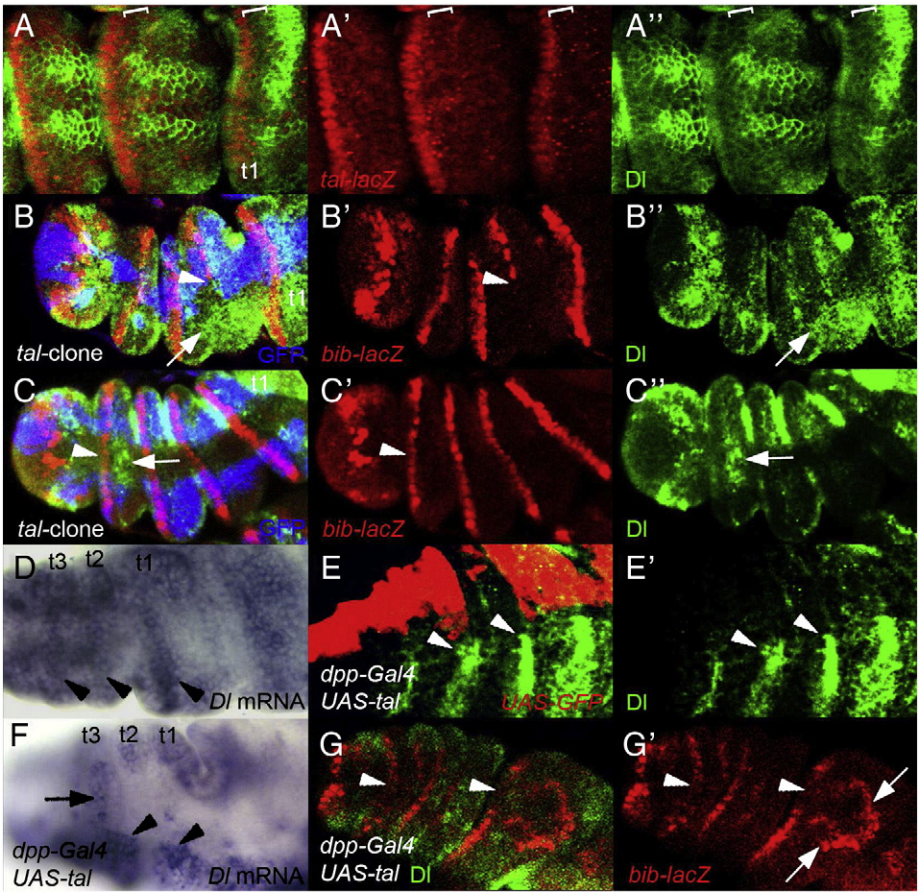

Fig. 4. Tal represses *Dl* transcription in the N-responsive region to form a signalling border. A–A″ — patterns of expression of Dl (green) and *tal-lacZ* (red) in the distal part of a pupal leg (6 h APF). Note that Dl and *tal-lacZ* patterns of expression do not overlap (brackets) (A). *tal-lacZ* expression in the distalmost part of the segment (A′). Dl distribution showing a sharp boundary with non-Dl-expressing cells at the distal part of the segment (brackets) (A″). B–B″ — 6 h APF pupal leg showing a large *Minute* + *tal*- mutant clone of around 10–15 cells wide (marked by lack of GFP, blue) and stained for *bib-lacZ* (red) and Dl expression (green) (B). *bib-lacZ* is lost in the larger area of the clone. Arrowhead denotes the edge of *bib-lacZ* expression. (B′) Dl distribution in this area does not form a boundary and it appears more distally (arrow) (B″). C–C″ — average *Minute* + *tal*- mutant clone in a 6 h APF pupal leg labelled as in B). *bib-lacZ* expression is normal in the *tal* mutant clone (arrowhead) (C′). Dl is localized proximally forming a clear boundary (arrow; compare with B″) (C″). D — *Dl* mRNA distribution in a 5 h APF pupal leg. *Dl* is highly expressed near the distal part of the segment (arrowheads), but is absent or at low concentration in the distalmost part. E–E′ — pupal leg (6 h APF) over-expressing *UAS-tal* and *UAS-GFP* driven by *dpp-Gal4,* showing the GFP distribution in the *dpp* pattern (red) and Dl protein (green) (E). Dl protein (green) is only detected outside of the *tal* over-expressing domain (arrowheads) (E′). F — *in situ* hybridisation using a *Dl* riboprobe in a *dpp-Gal4;UAS-tal* pupal leg 5 h APF. *Dl* transcription is repressed in *tal* over-expressing cells (arrow). Dl expression is detected outside the *dpp* pattern (arrowheads). G–G′ — ectopic expression of *tal* in a 5 h APF pupal leg with *dpp-Gal4* driver represses Dl (green) and *bib-lacZ* (red) in the dorsal part of the disc (arrowheads) but induces ectopic expression domains of *bib-lacZ* at the edges of the *dpp-Gal4* domain (arrows) (G). Expression of *bib-lacZ* (G′).

Thus, Tal peptides appear to promote N signalling by generating a sharp signalling boundary through the transcriptional repression of *Dl* in the presumptive joint region. This repression is mediated by the activation of the Svb transcription factor and allows directional N signalling activation and the formation of the tarsal joints (Fig. 6). Hence in our model, for Tal and Svb to promote joint formation, Tal and Svb must overlap and either overlap or abut high Dl expression. This model explains our experimental data in which perturbations of *svb* and *tal* functions disrupt N signalling and joint formation. Complete depletion of *tal* or *svb* function in the tarsal joints (as in *tal* or *svb* loss of function clones or by ectopic expression of *UAS-dstal* construct; Figs. 1, 2, 4, 5) results in the expansion of *Dl* into the joint region and precludes the formation of Dl+/Dl− sharp boundaries, leading to a loss of tarsal joints. Ectopic expression of activated Svb (by means of *ovo-B* expression or *tal* and *svb* co-expression; Fig. 5) represses *Dl* and hence also eliminates Dl+/Dl− signalling boundaries and joint structures. Finally, when *UAS-tal* is ectopically expressed in the tarsi, ectopic joints only arise in the region where endogenous Svb is present but out of reach of the endogenous Tal source, and yet overlapping the stripe of high Dl: these three conditions are only met in a narrow stripe proximal to the endogenous presumptive joint (Fig. 6). Consequently, repression of *Dl* is achieved in this region and new Dl+/Dl− boundaries and ectopic joints form proximally to the endogenous joint region (Figs. 1J, 2D–E and 4G).

Discussion

A distinct role for non-canonical Tal peptides in the generation of patterning and signalling boundaries that allow the specification of new territories of cells in a growing tissue is starting to emerge. The molecular mechanisms employed by Tal peptides seem to vary depending on the developmental context. During development of the tarsal joints, the N signalling pathway activates *tal* expression in each presumptive joint region. Subsequently, Tal peptides activate the transcription factor Svb, which represses *Dl* expression to define a sharp Dl+/Dl− signalling boundary. Thus, a negative feedback loop between Tal and the N pathway produces a spatial asymmetry in the distribution of the ligand Dl, which is essential for the directional activation of N signalling in the joint region. Importantly, Tal peptides act non-autonomously maintaining this border allowing the recruitment of cells into the presumptive joint region as the expression of *Dl* retracts out of range from the Tal domain of action. During tarsal intercalation at mid-third instar, *tal* expression is activated at the border of the *B* and *dac* expression domains (Pueyo and Couso, 2008). Next, *tal*

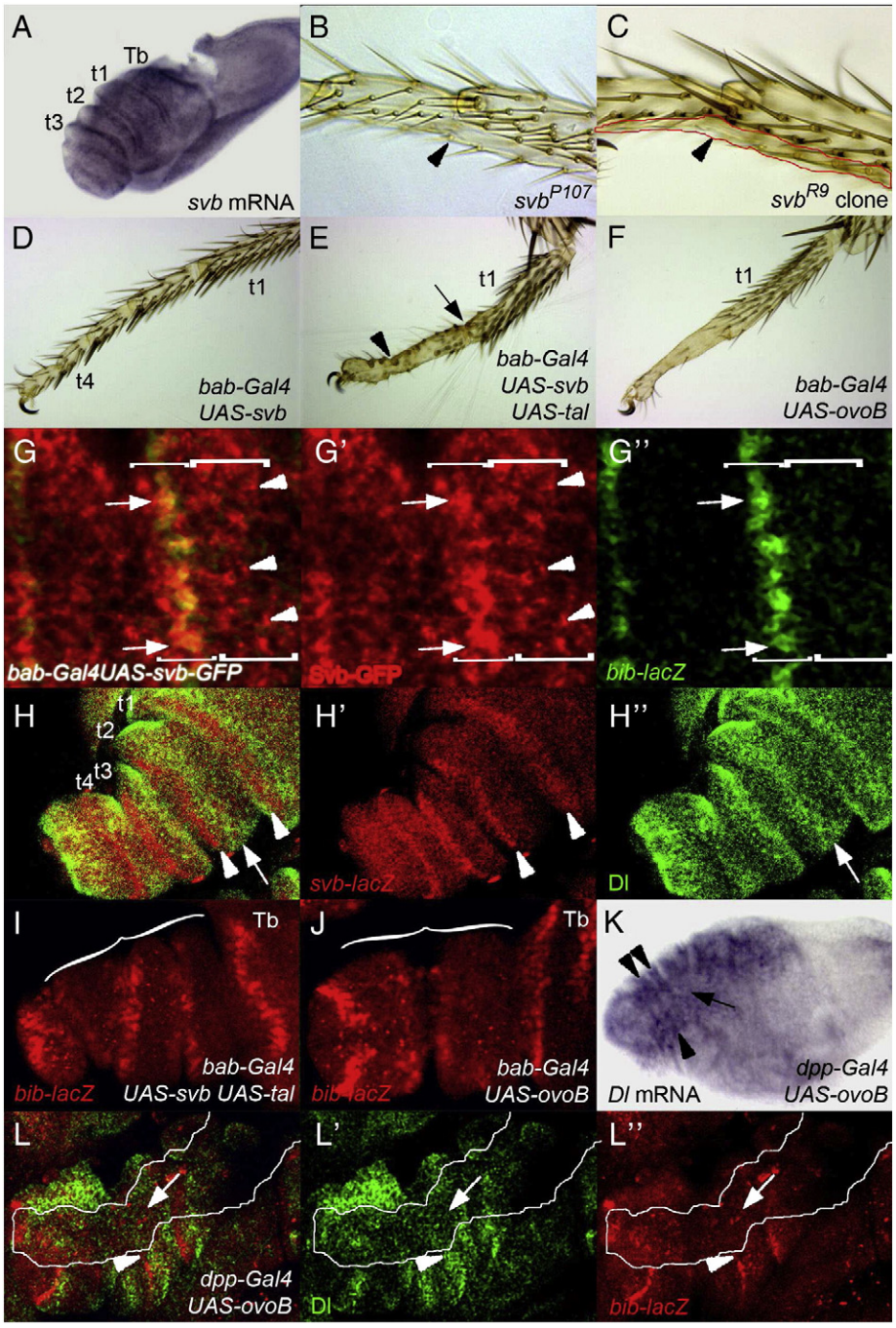

Fig. 5. Tal regulates N signalling through the Svb transcription factor. A — distribution of *svb* mRNA in a 4 h APF pupal leg. *svb* is detected in stripes in the tarsal segments. B — tarsal joints of a svb^{107} mutant escaper displaying an incomplete joint (arrowhead). C — a svb^{R9} mutant clone marked with yellow in the tarsal segments (outlined in red). The cells lacking *svb* do not form autonomously the joint fold (arrowhead). D — leg of a fly over-expressing *UAS-svb* in the tarsi is completely wild-type. E — leg over-expressing both *UAS-tal* and *UAS-svb* using the *bab-Gal4* driver. Apart from an abnormal joint in t1 (arrow) only attempts of joints can be observed in the rest of the tarsus (arrowhead). F — distal part of a leg over-expressing an active Svb form (*ovo-B*) in the tarsi. The tarsal region is reduced and all joints are completely absent. G–G″ — distribution of a GFP-tagged Svb protein (Svb-GFP) (red) in a tarsal segment of a pupal leg using the *bab-Gal4* driver, which is expressed evenly throughout the tarsus. Expression of the *bib-lacZ* (green) indicates the proximal part of the joint region. (G). Svb-GFP is differentially distributed throughout the segment. In the joint region (thin brackets) Svb-GFP is strongly detected in entire nuclei (arrows) whereas in the proximal (non-joint) part of the segment (thick brackets), Svb-GFP is only detected in puncta (arrowheads) (G′). *bib-lacZ* expression (G″). H–H″ — a 5 h APF pupal leg showing *svb-lacZ* (red; arrowhead) and Dl (green; arrow) patterns of expression. Note that these adjacent expression domains are slightly overlapping (H). *svb-lacZ* reporter is expressed in the distal part of the tarsal segments (arrowheads) (H′). Dl protein distribution (arrow) (H″). I — a *bab-Gal4;UAS-tal;UAS-svb* pupal leg (5 h APF) showing a strong reduction of *bib-lacZ* expression in the tarsi (brackets). J — a pupal leg (5 h APF) over-expressing *ovo-B* in the tarsi. The *bib-lacZ* pattern of expression is completely lost or very reduced (brackets). K — *in situ* hybridisation showing the *Dl* transcript pattern in a *dpp-Gal4;UAS-ovoB* pupal leg (4 h APF). *Dl* expression is reduced in the dorsal part of the disc (arrow) but stripes of *Dl* are observed outside the *dpp-Gal4* domain (arrowheads). L–L″ — A 5 h APF pupal leg expressing ectopically *ovo-B* using the *dpp-Gal4* driver (white outline) showing Dl protein distribution (green) and *bib-lacZ* expression (red). Arrow denotes the dorsal side of the disc where Dl is reduced and *bib-lacZ* expression is absent; the arrowhead marks the lateral side where *bib-lacZ* and Dl are present (L). Dl protein expression (L′). *bib-lacZ* expression (L″).

is involved in a negative feedback loop, by which Tal peptides activate the expression of the transcription factors, Rn and Ss, that in turn repress *B* and *dac* expression and promote tarsal development (Pueyo and Couso, 2008). Again, the non-autonomous nature of Tal signalling allows the expansion of the new intercalated territory from a single row of cells to a territory comprising three tarsal segments (Pueyo and Couso,

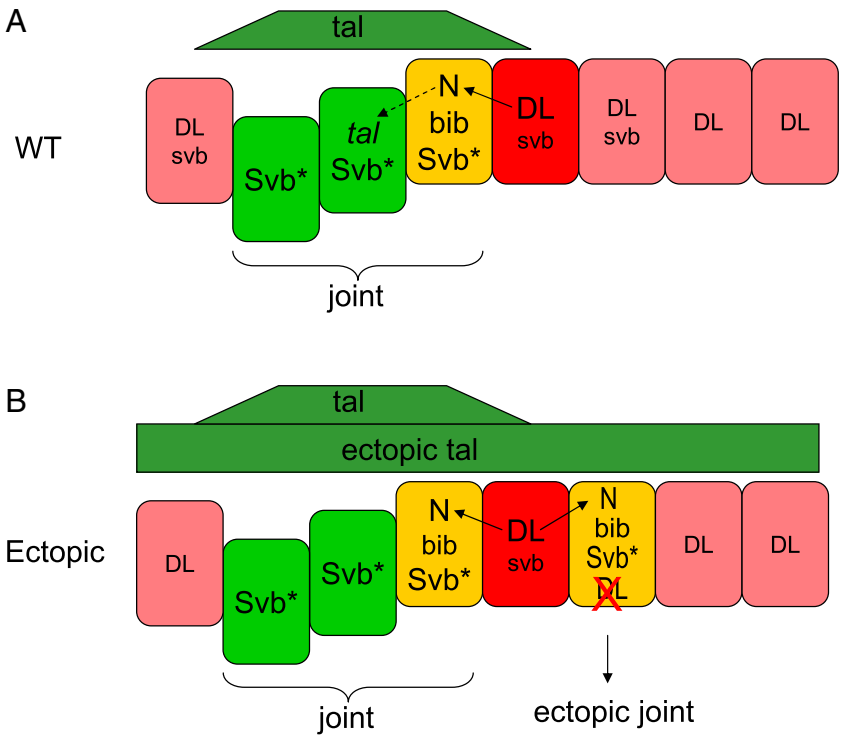

Fig. 6. Diagram depicting a model for the interactions between Tal and N during tarsal joint development. Schematic representation of the Tal-mediated mechanism controlling the formation of the N signalling boundary in the distal part of a tarsal segment in pupal legs (the orientation of the represented tarsal segments is as in other figure panels, distal to the left and proximal to the right). A negative feedback between N and Tal signalling regulates the formation and maintenance of N-signalling boundary. Joints (endogenous or ectopic) only arise if a) both *tal* and *svb* expression overlap (green) and b) this *tal-svb* overlap in turn abuts or overlaps high Dl expression (red; overlaps in yellow). These overlaps lead to the generation of a sharp Dl+/Dl− signalling boundary. A) By the end of third larval instar, Dl (red) and svb are expressed in slightly overlapping patterns in the distal part of the segment. At the onset of puparation, cells have high levels of Dl and activate N signalling in the adjacent cells (black arrow). N signalling activates (directly or indirectly) *tal* gene expression in the distalmost part of the joint region (black dashed arrow). Non-autonomous Tal signalling (green) triggers the post-transcriptional activation of the Svb transcription factor (Svb*) across the presumptive joint region (bracket). Subsequently, this Tal-mediated activation of Svb results in the direct or indirect transcriptional repression of *Dl* in the presumptive joint cells (yellow), generating a sharp Dl+/Dl− signalling boundary that leads to the activation of *bib* and other joint-promoting genes. Loss of *tal* or *svb* function results in the loss of this boundary, and hence, of joints. B) UAS-tal-mediated ectopic joints only arise in the region where endogenous Svb is present but out of reach of the endogenous Tal source, and yet overlapping or abutting the distal stripe of high Dl: these three conditions are only met in a narrow stripe proximal to the endogenous presumptive joint. Activation of Svb in this territory represses Dl and leads to the generation of a new Dl+/Dl− signalling boundary and the formation of an extra joint (arrows). Co-over-expression of UAS-*svb* plus UAS-*tal*, or expression of the Svb-activated form ovo-B throughout the segment eliminates *Dl* expression and precludes the formation of Dl+/Dl− signalling borders and joints.

2008). Interestingly, several pieces of evidence suggest that *tal* function in tarsal intercalation is independent of Svb. Firstly, *svb* is not expressed in the presumptive tarsus in mid third instar leg discs (not shown). Secondly, *svb* loss of function precludes the development of tarsal joints but not of tarsal segments themselves (see below). Thus, Tal peptides are involved in distinct negative feedback loops in the formation of patterning and signalling boundaries.

Regulation of N signalling during tarsal joint development

By late third larval instar, the different presumptive tarsal segment regions have been specified by the gene regulatory network of *PD* genes, including *tal* (Campbell, 2005; De Celis Ibeas and Bray, 2003; Galindo and Couso, 2000; Kojima et al., 2000; Pueyo and Couso, 2008; Pueyo et al., 2000). A concentric ring of *Dl*-expressing cells appears in the distal part of each tarsal segment (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). In addition, other factors such as Fringe, the Ras and PCP pathways and the Dve transcription factor counteract N activity in the cells proximal to the *Ser*/*Dl* rings of expression and restrict the ability to respond to N signalling to the cells distal to the *Ser*/*Dl* expression domains (Bishop et al., 1999; Ciechanska et al., 2007; de Celis et al., 1998; Galindo et al., 2005; Shirai et al., 2007). However, a sharp Dl+/Dl− signalling boundary must be generated for downstream gene activation and joint determination to occur. The AP-2 transcription factor and other unknown factors are involved in repressing *Ser* and *Dl* in the tarsal joint region (Ciechanska et al., 2007). During puparium formation, this signalling boundary must be maintained as the leg expands and undergoes morphogenetic changes (Fristrom and Fristrom, 1993; Greenberg and Hatini, 2011; Mirth and Akam, 2002). *tal* expression is precisely activated by N signalling in the presumptive joint region at the onset of puparation (Figs. 1 and 3). Although it is not known whether N mediated activation of *tal* expression is direct or indirect, we have found two putative Su(H) binding sites (Bailey and Posakony, 1995), one at 0.6 Kb upstream of the start of *tal* transcription, and the other at 1.5 Kb downstream of the *tal* transcript, suggesting that the regulation of *tal* by N could be direct (unpubl. obs.). Clonal analysis and ectopic expression studies show that *tal* is required for joint development (Figs. 1 and 2), and that *tal* is involved in a negative feedback loop with N signalling by which Tal peptides repress *Dl* expression in the joint region (Fig. 4). This repression is implemented by Svb, either directly acting as a transcriptional repressor on the *Dl* gene, or indirectly by activating another repressor, such as the *AP-2* gene. Therefore, Tal is a factor that maintains the Dl+/Dl− signalling boundary in tarsal joints.

Denticle formation in embryogenesis relies on Tal triggering a post-translational modification of the Svb transcription factor (Kondo et al., 2010). Upon this modification Svb switches from a repressor, which is localized in nuclear foci, to an activator, which is evenly distributed in the nucleus (Kondo et al., 2010). Similarly, *svb* is

expressed in the joint region in every segment from third larval instar and our results indicate that *svb* is required for tarsal joint formation (Fig. 5). Our functional analyses suggest that Tal-mediated activation of Svb regulates the N signalling border in the tarsal segments in correlation with Svb-GFP being evenly distributed in the nuclei of the distalmost cells of each segment where *tal* is functional. However, there exist some differences between the role of Svb in denticles and in tarsal joints. For instance, ectopic expression of Svb does not affect tarsal segmentation. This supports the view that endogenous *tal* expression controls the activation of Svb only in the joint region, and Svb does not play a role in joint formation in the absence of Tal.

These findings together with the similarities found in the *tal* and *svb* mutant phenotypes in other developmental contexts (Delon et al., 2003); and unpubl. obs.) suggest that most, if not all, of the Svb functions, in which Svb post-transcriptional activation is required, maybe regulated by Tal peptides. Conversely, there exist roles of Tal that are independent of Svb, such as in the trachea and tarsal intercalation (Kondo et al., 2010; Pueyo and Couso, 2008), indicating that Tal peptides have an alternative and yet unknown mode of action.

Two distinct negative feedback mechanisms are involved in the segmentation of the *Drosophila* leg. In the true joints, a negative feedback cascade involving the regulation of the degradation of Bowl and N signalling permits the formation and maintenance of the Dl+/Dl− signalling boundary (Greenberg and Hatini, 2009). In the tarsal joints, activation of *tal* expression by N signalling in the joint region promotes Svb activation and repression of *Dl*. However, there exist other regulators, such as *AP-2* and other *odd*-related genes (*sister of odd and bowl* (*sob*) and *odd*) that may also be involved in either of these two mechanisms refining the Dl+/Dl− signalling boundaries (Ciechanska et al., 2007; Greenberg and Hatini, 2009; Hao et al., 2003).

Conservation of a tal-Svb-Notch regulatory pathway

The available comparative evidence suggests that Tal regulation of N signalling is ancestral for arthropods. Expression and functional analyses of *N*, *Ser* and *Dl* have shown that N is involved in the segmentation of legs of spiders (Prpic and Damen, 2009) and basal insects such as the cockroach *Periplaneta americana* (Chesebro and Couso pers. comm.). In addition, the N target genes such as *AP-2*, and *odd*-related genes are also expressed in all the segments in spiders indicating conservation in the segmentation mechanisms across arthropods (Prpic and Damen, 2009). In the beetle *Tribolium castaneum*, the *tal* homologue, *mille-pates(mlpt)*, is expressed in three stripes in the developing leg, one in each of the three larval leg segment, all of which will give rise to true joints (Savard et al., 2006). *mlpt* RNAi embryos seem to display slightly shorter and deformed legs, possibly as a result of fusion of leg segments (Savard et al., 2006). Furthermore, stripes of *tal* expression can be observed near the leg joints in *Periplaneta* and in the cricket *Grillus bimaculatus* (Chesebro and Couso, 2009). Although further work into the role of *tal* and *odd*-related genes in leg segmentation in basal insects and other arthropods is needed, these comparative data support the hypothesis that both the Odd-related gene cassette and Tal signalling regulate the formation of N signalling boundaries in the leg joints in most basal arthropods. In more derived insects, such as *Drosophila* the Odd-related gene cassette and Tal mechanisms may have specialized into the formation of joints of either true or tarsal segments respectively.

The *tal* expression observed in leg joints in Coleoptera (*Tribolium*), Orthoptera (*Gryllus*) and Dyctioptera (*Periplaneta*) suggests that *tal* joint function and its relationship with *svb* predate the association of *tal* and *svb* in the development of denticle patterns in Dipterans, which must have been co-opted a posteriori. This conclusion would also push the functional link between *tal* and *svb* in regulating N signalling back almost 400 Myr. However, this functional connection may extend back even further in time. The vertebrate Svb homologues MOVO1 and 2 share some functional similarities with their *Drosophila* counterpart. They are expressed in epidermal hair cells and in reproductive systems and their knockout mutants fail to form these structures properly (Dai et al., 1998; Li et al., 2002a, 2002b). In addition, N signalling has two distinct roles during epidermal hair development, an early role acting as a switch between different epidermal cell lineages and a later one in the terminal differentiation of the hair cells (Blanpain et al., 2006; Pan et al., 2004; Vauclair et al., 2005). Interestingly, the MOVO2 transcription factor regulates terminal differentiation of keratinocytes by repressing directly the expression of the Notch 1 receptor (Wells et al., 2009). These findings reveal a functionally analogous feedback loop involving Svb/Ovo and N in *Drosophila* and vertebrates. Finding the *tal* homologue in vertebrates and exploring its role in hair development and N regulation would therefore seem a worthwhile quest. Reducing N signalling using small molecules has been shown as a promising avenue of research for treating diseases where mis-regulation of N is involved, such as in leukaemias and breast cancer (Moellering et al., 2009; Rizzo et al., 2008; Weng et al., 2004). Testing a putative role for small peptides in regulating N signalling in human cells could also open new therapeutic avenues for these diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.03.033.

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