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The 11-aminoacid long Tarsal-less peptides trigger a cell signal in *Drosophila* leg development

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ABSTRACT

The polycistronic and non-canonical gene *tarsal-less* encodes several short peptides 11 to 32 aminoacids long. *tarsal-less* is required for embryonic and imaginal development in *Drosophila*, but the molecular and cellular bases of its function are not known. Here we show that *tarsal-less* function triggers a cell signal. This signal has a range of 2–3 cells in *Drosophila* legs and may be provided directly by the Tarsal-less peptides. During leg development, this Tarsal-less signal implements the patterning activity of a tarsal boundary and regulates the transcription of several genes in a specific manner. Thus *tarsal-less* is necessary for the intercalation of the tarsal segments two to four and for the activation of the homeobox gene *apterous*, the Zinc-finger gene *rotund* and the bHLH-PAS gene *spineless*, and for the repression of the homeobox gene *Bar* and the putative transcription factor *dacshund*. These regulatory effects complement the known genetic scenario required for distal leg development and explain the requirements for *tarsal-less* in this process.

Introduction

Our laboratory has recently characterised the non-canonical gene *tarsal-less* (*tal*) (Galindo et al., 2007). The functional products of the polycistronic *tal* gene are four related peptides of 11, 11, 12 and 32 aminoacids respectively (Galindo et al., 2007; Kondo et al., 2007). No known protein domains, or molecular roles, have been identified in these peptides and interestingly, full *tal* function can be provided by a single copy of any of the 11aa peptides. Thus *tal* is non-canonical in two aspects: firstly, it is polycistronic, and secondly, only contains short open reading frames. Indeed *tal* was initially considered a non-coding RNA gene (Inagaki et al., 2005; Tupy et al., 2005). *tal* is not an isolated gene in *Drosophila*, but a member of a family of related genes found in many other insects and arthropods (Galindo et al., 2007; Savard et al., 2006) and it may represent a whole new class of eukaryotic genes. A number of 'putative non-coding RNA genes' with unknown function have been found in the metazoan genomes (Consortium, 2004; Costa, 2007; Pauler et al., 2007), and some of these may in fact encode short open reading frames similarly to *tal*. It would seem that a limited number of developmental and signalling pathways have been repeatedly used in most of the patterning processes in metazoan development (Pires-daSilva and Sommer, 2003). However, these pathways may not account for the regulation of all the patterning and differentiation events in an organism, and therefore new developmental and signalling mechanisms may still await to be discovered.

tal is required for the development of the tarsal region and regulation of the tarsal gene *rotund* (*rn*), and also for the control of tarsal tissue folding (Galindo et al., 2007). In addition, during embryogenesis *tal* is expressed in ectodermal tissues undergoing morphological changes such as invaginated organs (mouthparts, trachea, and hindgut) and during epidermal denticle differentiation (Galindo et al., 2007; Kondo et al., 2007). An instance of non-autonomy has been reported in the function of *tal* in the differentiation of denticle belts (Kondo et al., 2007) where *tal* seems to control this process through the regulation of cytoskeleton arrangements (Galindo et al., 2007; Kondo et al., 2007). However, we do not know if non-autonomy is a general feature of *tal* function, nor which genes or proteins relate to the denticle non-autonomous effect. No downstream targets of *tal* in this function have been identified and *tal* function is not involved in the canonical denticle patterning cascade (Galindo et al., 2007; Kondo et al., 2007).

The development of the fly leg is a good system where to answer these questions. The main features of leg disc patterning are understood, and there exist a number of genes that could be related to *tal* function and whose interaction with *tal* could clarify: firstly, whether *tal* acts non-autonomously; secondly, if this non-autonomy is compatible with the behaviour of *tal* as a cell signal; thirdly, what

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range of diffusion this signalling has, and fourthly, what genes or pathway mediate it.

Drosophila legs develop from groups of epidermal cells that are set aside during embryogenesis. Each cluster of cells grows extensively throughout larval stages forming a sac-like structure called imaginal disc (Bryant, 1978; Cohen, 1993). During metamorphosis the imaginal discs evert and differentiate to produce the adult appendages (Fristrom and Fristrom, 1993). Proximo-distal (PD) leg fates are represented as concentric rings, with the proximal fates at the periphery and the distal ones at the centre of the disc (Couso and Bishop, 1998). During second instar two signalling proteins, Decapentaplegic (Dpp) expressed dorsally and Wingless (Wg) expressed ventrally, cooperate to subdivide the leg discs into different PD regions by activating *Distal-less* (*Dll*) and *dachshund* (*dac*) in the distal and medial presumptive regions respectively (Estella and Mann, 2008; Lecuit and Cohen, 1997). At early third instar high EGFR signalling levels at the distal-most region of the disc turns on pretarsal (PT) genes, whereas lower EGFR signalling levels around the presumptive pretarsus activate *Bar* and repress tarsal genes such as *bric-a-brac* (*bab*) (Campbell, 2002; Galindo et al., 2002). Mutual repressive interactions between these genes sharpen these PD regions and further subdivisions are achieved by activation of further genes in smaller domains, such as *apterous* (*ap*) in the fourth tarsal segment (T4) (Kojima et al., 2000; Pueyo et al., 2000). However there are still PD territories, such as the second (T2) and third (T3) tarsal segments, for which no specific genes implementing their identities have been identified. Moreover, other genes for instance *rotund* (*rn*) (Agnel et al., 1989) and *spineless* (*ss*) (Duncan et al., 1998) with a role in tarsal development await a definitive integration in this picture (Kojima, 2004).

Here we show that the Tal peptides trigger an instructive cell signal necessary for *Drosophila* leg development. This signalling occurs over a range of 2–3 cells and is most likely mediated by a novel signalling mechanism. Tal signalling fills some of the missing gaps in our understanding; in particular, a Tal-dependent cell signal leads to the specific regulation of *rn* and *ss* genes and the intercalation of a new population of cells giving rise to the second, third and fourth tarsal segments. Tal leg function mediates the effects of a patterning boundary, an important and often used strategy to deploy signals in the correct time and space and to generate new cell fates and new presumptive territories (Tabata and Takei, 2004).

Materials and methods

Fly stocks and genetic manipulations

Fly strains used in this paper are described in Flybase, except otherwise stated. *Or-R* was used as wild-type strain. The following *tal* alleles were used: tal^{1}; tal^{KG1680}; *tal-lacZ* ($l(3)S0110411$); tal^{s18}; and *tal-Gal4* (Galindo et al., 2007). Other stocks were: dac^{P}, dac^{1}, dac^{9ts} (G. Mardon); al^{ex} and al^{ice} (A. Tomlinson); rn^{16}; rn^{89}-*lacZ* (St Pierre et al 2002); ap^{UG62}; ss^{sta} $Df(1)B^{263-20}$; and *Df(3)urd*. Generation of mosaics was carried out using the FRT/FLP system (Xu and Rubin, 1993). The following alleles recombined to FRT chromosomes were used: *yw*; FRT42D *hs-myc* Dll^{SA1}/*CyO y+*; *w*; FRT82B tal^{KG} or tal^{S18}/*TM6B* and *Df(1)*B^{263-20} FRT18A/*FM7c*. Those flies were crossed to their corresponding marked FRT strains: *w hsFLP*; FRT 42D *M(2)/CyO*; f^{36a} *hsFLP*; FRT82B f^{+} *M(3)/TM6B, w hsFLP*; FRT82B *ubiGFP/TM3 Ser*; *w hsFLP*; FRT82B *ubiGFP M(3)/TM6B*, *w ubiGFP* FRT18A; and *hsFLP/TM6B*. Clones were induced by a 37 °C heat shock for 1 h from 24–72 or 48–72 h of development.

For ectopic expression experiments, the following Gal4 drivers were employed: *dpp-Gal4*; *omb-Gal4*; *rn-Gal4*; and *tal-Gal4*. Several transgenes were used: *UAS-*tal^{A1}; *UAS-*tal^{E1}; *UAS-al* (A. Tomlinson); *UAS-*Bar^{M6} (T. Kojima); *UAS-rn*; *UAS-ss* (I. Duncan) and *UAS-GFP*. The level of ectopic expression of *tal*, *rn* and *ss* was observed by *in situ* hybridisation.

Gain-of-function clones were generated by crossing *y w hsFLP; Act5*<FRT *y*+FRT>*Gal4*, *UAS-GFP* to the transgene strain and applying a 20 min heat shock at 37 °C between 48–72 h AEL.

In situ hybridisation

Standard procedures were followed. Detection of *tal* mRNA was carried out as described in Galindo et al. (2007) and St. Pierre et al. (2002). A *spineless* full cDNA (sscA5) (I. Duncan) was used for DIG-labelling.

Detection of β-galactosidase activity, immunocytochemistry and microscopy

X-gal staining was described previously (Pueyo et al., 2000). Antibody staining procedures were performed as described in Galindo et al. (2007). The antibodies used were: rat Anti-Ap (S. E. Lundgren); rabbit Anti-Bar (T. Kojima); mouse anti-Dll (I. Duncan); guinea pig anti-Ss (Y. N. Jan); rat anti-Al (G. Campbell), rabbit anti-β-galactosidase (Cappel) and mouse anti-β-galactosidase (Promega) and mouse anti-Dac (G. Mardon) obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa. Vector Laboratories and Jackson ImmunoResearch secondary antibodies were used. Images were acquired with a Leica DRBM microscope and a Zeiss LSM 510 confocal microscope, and processed with QWin, LMS and Photoshop software.

Results

Tal regulates tarsal development non-autonomously through the activation of rotund and spineless

We have previously reported the role of *tal* in the development of the tarsal region of the leg (Fig. 1A) (Galindo et al., 2007). *tal* is expressed transiently in the leg imaginal disc in a ring of cells in the presumptive tarsal region about 80–96 h after egg laying (AEL) (Fig. 1C). There are two regulatory mutants tal^{1} (Galindo et al., 2007), and tal^{KG} that are nulls for *tal* function in the leg, as no *tal* mRNA can be detected in the leg discs (Fig. 1D). In these mutants, most of the tarsal region fails to develop (Galindo et al., 2007) (Fig. 1B).

To explore the functional role of *tal*, we performed clonal analysis using the regulatory null tal^{KG} and the true null tal^{S18} alleles (Galindo et al., 2007). Similar results were obtained with both alleles. Clones of cells lacking *tal* in the tarsus were completely normal (Figs. 1E, F), indicating that *tal* acts non-autonomously in the legs. To confirm this, we induced larger *tal* mutant clones using the *Minute* technique (Morata and Ripoll, 1975). *Minute+ tal* mutant clones produced tarsal fusion phenotypes similar to those observed in *tal* whole mutant legs (Figs. 1B, G, H), corroborating that *tal* acts with local non-autonomy. The width of the clones with wild-type phenotype, adjusted for the time when *tal* is expressed, suggests that the range of diffusion of the putative cell signal underlying this non-autonomy is about 2–3 cells.

This non-autonomous behaviour of *tal* is also observed at the level of gene expression. *tal* expression coincides with the presumptive tarsal region as defined by the expression of the tarsal genes *rn*, *ss* and *bab* (Duncan et al., 1998; Godt et al., 1993; St Pierre et al., 2002). *tal* is required for the expression of *rn* (Galindo et al., 2007). The ring of *rn*-expressing cells (St Pierre et al., 2002) forms immediately after *tal* expression is activated, and remains until *tal* is switched off. However, *rn* and *tal* expression do not coincide fully, as there are always *rn*-expressing cells that do not express *tal* (Fig. 1I). In the oldest discs expressing *tal* and *rn*, the *rn*-expressing cells extend some two to three cells away from *tal*-expressing cells (Fig. 1J). To test this apparent non-autonomy, we induced flip-out clones to express *tal* ectopically. *tal*-expressing cells were able to activate ectopically *rn* non-autonomously in the presumptive tibia region (Figs. 1K, L). No activation of *rn* was