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Original Article

PVF1/PVR signaling and apoptosis promotes the rotation and dorsal closure of the *Drosophila* male terminalia

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ABSTRACT The *Drosophila* adult male terminalia originate from the genital disc. During the pupal stages, the external parts of terminalia evert from two ventral stalks; the everted left and right dorsal halves fuse at the dorsal midline. At the same time the male terminalia perform a 360º clockwise rotation. Several mutations are known to affect the rotation of the male terminalia, while none is known to affect dorsal closure. We show here that the *Pvf1* gene, encoding one of the three *Drosophila* homologues of the mammalian VEGF/PDGF growth factors, is required for both processes. Males either mutant for *Pvf1* or bearing a dominant negative form of *Pvr* or *stasis (stai),* the unique PVF receptor, do not complete either rotation or dorsal closure. *Pvf1* expression in the genital disc is restricted to the A8 cells. However, PVF1/PVR signaling influences A8, A9 and A10 cells, suggesting that the PVF1 protein diffuses from its source. Flies hemizygous for the apoptotic genes *hid, reaper* and *grim,* or mutant for *puckered* which encodes a phosphatase that down-regulates the n-Jun-N terminal kinase pathway, lead to the same phenotypes as mutations in PVF1/PVR. Our results indicate that PVF1/PVR signaling functions not only in apoptotic phenomena but are also required during rotation and dorsal closure of the *Drosophila* male genital disc.

KEY WORDS: *PVF1/PVR, VEGF/PDGF, apoptosis, Drosophila, male genital disc*

Introduction

The adults terminalia of *Drosophila* derive from the genital disc (reviewed in Sánchez and Guerrero, 2001). It is constituted by the fusion of three embryonic abdominal segments: A8, A9 and A10. In the male genital disc the A8 primordium gives rise to a tiny A8 tergite (T8), whereas the A9 forms the male genitalia and A10 the male analia and hindgut. The shaping process of these structures occurs during the pupal stages; among them there is the fusion in the dorsal midline of left and right halves of this bilateral symmetrical disc and a 360º clockwise rotation (Gleichauf, 1936; Adám *et al.*, 2003) (see Fig. 1 A,B) related with the maturation of the internal genitalia (Gleichauf, 1936) (see Fig. 1C). No mutations are known to affect dorsal closure, but several have been reported to affect the rotation of the male terminalia. The latter include mutations at *Abdominal-B* (*Abd-B*) (Casanova *et al.*, 1986; Sánchez-Herrero and Crosby, 1988), *head involution defective* (*hid*) (Abbott and Lengyel, 1991; Grether *et al.*, 1995), at the gene encoding transcription factor *TAF250* (Wassarman *et al.*, 2000), *spin* (Adám *et al.*, 2003) and at some unidentified genes located in the region 11A (Fahmy and Fahmy, 1958). Most of those mutants present several degrees of incomplete rotation. The involvement of *hid* is of interest, for it is a pro-apoptotic gene (Grether *et al.*, 1995) and suggests that cell death is required.

In *Drosophila*, the PVF/PVR signaling pathway has been involved in border cell migration in the oocyte (Duchek *et al.*, 2001, McDonald *et al.*, 2003) as well as in migration-survival and proliferation of the hemocytes in embryos and larvae (Heino *et al.*, 2001; Cho *et al.*, 2002, Munier *et al.*, 2002; Brückner *et al.*, 2004).

There are three *Pvfs* genes in *Drosophila*. The mammalian homologues of these ligands could form homo or heterodimers, the type of dimer formed specifies the response of the receptor (Heldin and Westermark, 1999). On the contrary, there is only one receptor for PVFs, *Pvr o stasis (stai)*, with demonstrated binding activity to PVF1 (Duchek *et al.*, 2001).

Abbreviations used in this paper: JNK, n-Jun-N terminal kinase; PDGF, platelet derived growth factor; puc, puckered gene; PVF1, platelet vascular factor1; VEGF, vascular endothelial growth factor; WT, wild-type.

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In this work we show that PVF1/PVR signaling is involved in dorsal closure and rotation of male terminalia, as mutations in genes encoding the ligand or in the receptor alter these processes. We show that *Pvf1* transcript is only expressed in part of A8 of male genital discs but is required in cells of the three segments, suggesting a diffusion of the product. We also confirm the role of *hid* in terminalia rotation (Abbott and Lengyel, 1991) and show that blocking cell death by the baculovirus caspase inhibitor p35, or altering the apoptotic n-Jun-N terminal Kinase pathway produce the same phenotype as *hid* $^{-}$ and the lack of function of PVF1/PVR.

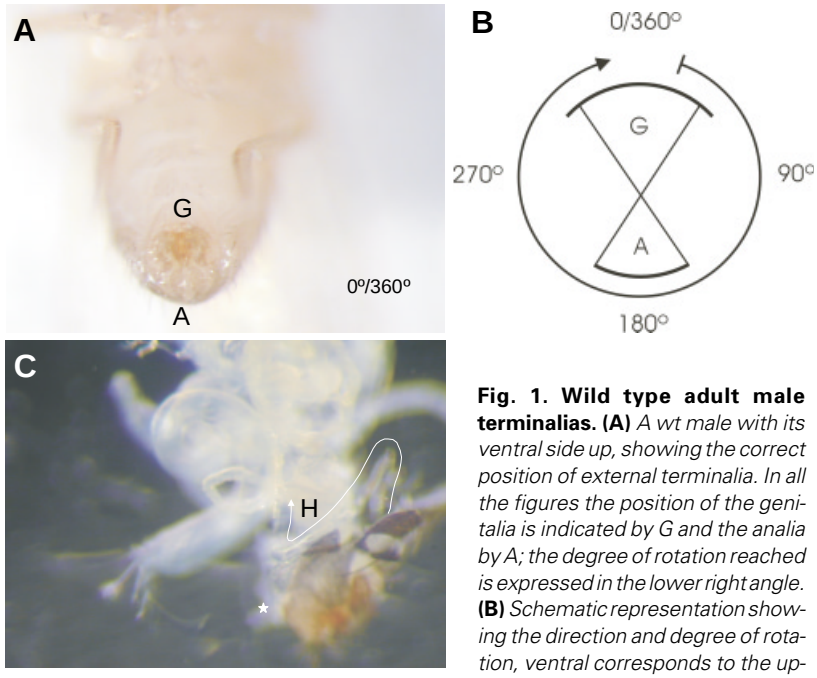

Fig. 1. Wild type adult male terminalias. (A) *A wt male with its ventral side up, showing the correct position of external terminalia. In all the figures the position of the genitalia is indicated by G and the analia by A; the degree of rotation reached is expressed in the lower right angle.* **(B)** *Schematic representation showing the direction and degree of rotation, ventral corresponds to the upper half.* **(C)** *Dissection, showing the turns of the spermiduct around the hindgut (H). The white line indicates the 360º spiral trajectory of the spermiduct; the white star indicates the sperm pump location on the right side of the organism.*

Results

Genetic analysis of the Pvf1 *gene*

To identify genes involved in the development of adult male terminalia we analyzed P-Gal4 lines using the *yellow* method (Calleja *et al.*, 1996). The line LP23-Gal4 was selected and characterized in detail. Inverse and direct PCR, as well as Southern blot analysis, showed that the insertion is located 250 bp upstream the proposed transcription start site of the *Pvf1* gene (Fig. 2; Duchek *et al.*, 2001, Cho *et al.*, 2002). The expression pattern driven by LP23-Gal4 in embryos corresponds to a subset of *Pvf1* expressing cells previously described (Cho *et al.*, 2002; data not shown). LP23-Gal4 males have wild type terminalia. The specific *y+* adult expression in the male terminalia suggested a possible function of *Pvf1* in this region of the body. To characterize its function the LP23 insertion was mobilized to generate deficiencies after imprecise jumps (Gloor *et al.*, 1991). Six putative imprecise excisions were semilethal in males and showed adult male rotated terminalia (Fig. 3A). The *Dp (1;Y) W39* (Prado *et al.*, 1999) rescued these phenotype. Since this duplication includes the wild type allele of *Pvf1*, this result suggested that the phenotype observed in the excised LP23-Gal4 lines were probably due to a defective *Pvf1* gene. Detailed Southern blot and PCR analyses were performed for two of these mutants, *LP23* ^{semil1} and *LP23* ^{semil2} (Fig. 2). It was found that *LP23* ^{semil1} is a deletion that removes part of the 5' region upstream the transcription starts site (Duchek *et al.,* 2001; Cho *et al.,* 2002), the first exon and part of the first intron of the *Pvf1* gene, as well as most of the P-Gal4 transposon (Fig. 2). *LP23* ^{semil2} is also a deletion with a breakpoint in the first intron of *Pvf1*, extending 5' more distally than *LP23* ^{semil1}, removing the transposon and deleting the neighboring gene CG7101 (Fig. 2). We also analyzed the mutant *Pvf1* ^{1624}, carrying the transposon EP1624 (Rørth, 1996) inserted in the first intron of the *Pvf1* gene (Duchek *et al.*, 2001). This allele is homozygous viable (Duchek *et al.*, 2001), albeit there is some pupal lethality and the adults show reduced fertility (not shown). Hemizygous *Df (1)LP23* ^{semil1} and *Pvf1* ^{1624} males show a similar rotated terminalia (Fig. 3 A,B). The phenotype is not fully penetrant and the expressivity is variable, but both increase at higher temperatures (Fig. 3 D,E). Occasionally, we noticed a lack of dorsal closure in some individuals (not shown). The variability of the phenotype could be due to a partial redundancy in *Pvf* genes function, as it has been described for other *Pvf* associated phenotypes (Ducheck *et al.*, 2001; Cho *et al.*, 2002.)

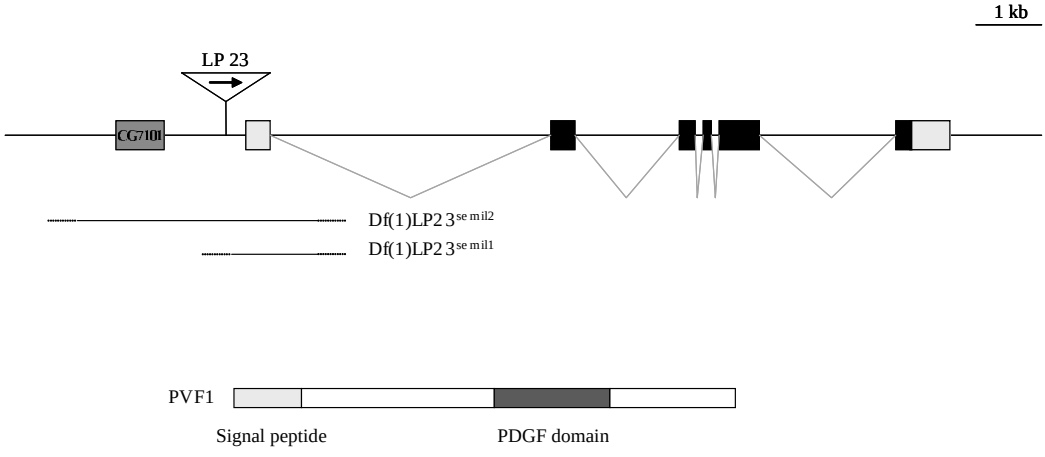

Fig. 2. Structure and product of the *Drosophila Pvf1* gene. *The* Pvf1 *locus at cytological position 17E. The open reading frame (ORF) of the transcript is indicated as black boxes. The arrow on P transposon pGawβ, called LP23 indicates the direction of* Gal4*-induced transcription. The sequences deleted in* Pvf1*, i.e.* Df(1)LP23^{semil1} *and* Df(1)LP23^{semil2} *are indicated; dotted extensions indicate the range of uncertainty in deletion endpoints.*

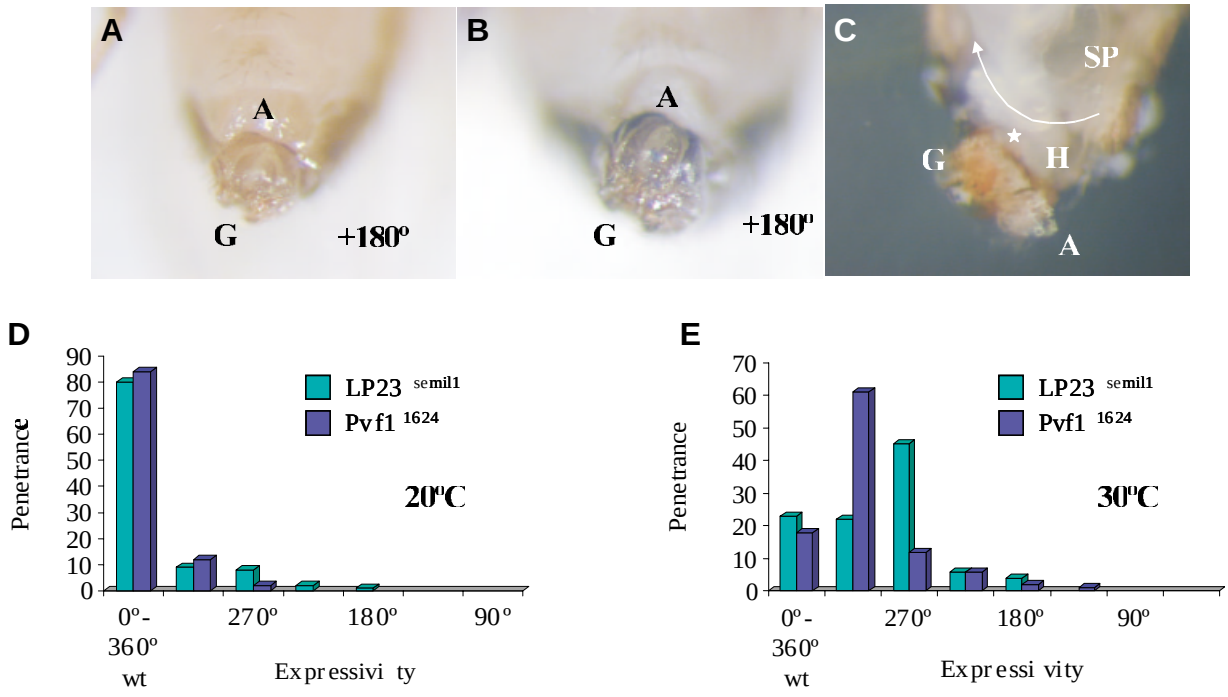

Fig. 3. Adult male terminalias, Pvf1 lack of function. *In (A,B) the males are with their ventral side up.* **(A)** *Df(1)* LP23^{semil1} *grown at 30ºC.* **(B)** Pvf1^{1624} *grown at RT.* **(C)** *Dissected* Df (1) LP23 ^{semil1} *with rotated external terminalia at position 180º. The line in white shows the trajectory of the spermiduct around the hindgut. The failure in rotation is shown by the single bend of the spermiduct around the hindgut, and the sperm pump (SP) which remains on the left side, H, hindgut.* **(D,E)** *Graphics showing penetrance (% of individuals which manifest the phenotype) in relation to expressivity (degree of rotation) of* Pvf1 *mutants at different temperatures; the rotated phenotype is temperature sensitive. (D) Grown at 20ºC. (E) Grown at 30ºC.*

The analyses of terminalia phenotypes are easily performed by microscope observation of the spermiduct trajectory and the sperm pump position (Fig. 1C). In wild type males, the spermiduct bends twice around the hindgut and the sperm pump is located in the right side of the abdomen (Fig. 1C). The internal analyses of *Pvf1* mutant flies, with various degrees of rotated terminalia, showed that the direction of rotation is not altered in the mutants (Figs. 3C, 1C). The spermiduct normally initiated a clockwise- turn but stops prematurely, while the sperm pump that is on the right side, is on the left in the mutants. All the external structures of the terminalia appear to move coordinately with the internal spermiduct and sperm pump during rotation, because all of them are miss-positioned in a equivalent rotated stage when the movement fail to be completed.

Pvf1 *expression in the male genital disc*

Pvf1 transcripts are first detected in late third instar larvae, in cells that belong to A8 segment (Fig. 4 A,B). We delimited *Pvf1* expression using as a reference the *engrailed (en)*, *decapentaplegic* (*dpp)* and *patched (ptc*) (Fig. 4 C,D) genes. *ptc* expression is closely associated to that of *en* in the genital disc (Fig. 4D), while *dpp* is more restricted and co-expresses with *en* only in few cells (Fig. 4C). In A8, *Pvf1* expression may overlap that of *en*, *dpp* and *ptc* only in few cells indicated in Fig. 4 C,D. The *Pvf1* expression domain extends to a group of anterior cells, outside the A8 *ptc*/*dpp* domain and corresponds to two bands of cells -left and right- that occupy the central region of the segment A8 separated by an intermediate zone that does not express the gene. Since PVF1/PVR activates the RAS-MAPK pathway (Duchek *et al.*, 2001, Cho *et al.*, 2002, Brückner *et al.*, 2004)), PVF1/PVR activity can be detected with an antibody that reveals active ERK, (anti- dpERK*)* (Gabay *et al.*, 1997). In the wild type male genital disc dpERK could be detected only in scattered cells of A8 (Fig. 4E). However, increasing the amount of the receptor PVR (e.g. using *ptc*-Gal4,*;* UAS-*Pvr*) dpERK activity was detected in the periphery of *Pvf1* transcript expression, in the *ptc* domain of A8 and a subset of *ptc* A9 cells in the vicinity (Fig. 4F). This observation indicates that PVF1 activity extends further to its expression domain.

Inactivation of the Pvf receptor

We extended the study of the role of *Pvr* function by expressing in restricted domains a dominant negative form, *Pvr* ^{DN} (Duchek *et al.*, 2001), using the Gal4/UAS system (Brand and Perrimon, 1993). In these studies we used drivers expressed in the posterior (*hh*-Gal4, *en*-Gal4) or in the anterior compartment (*ptc*-Gal4, *dpp*-Gal4) (Fig. 4 C,D). The activity of *Pvr*^{DN} results in a phenotype of rotated terminalia (Fig. 5 A,B), which is stronger with *en*-Gal4 (Fig. 5D) and similar to that observed in flies carrying mutations in *Pvf1* (Fig. 3 A,B). The penetrance and expressivity of this phenotype was variable, suggesting that some residual *Pvr* function was present; sometimes, as observed with *Pvf1* mutants, the dorsal closure was not completed.

We tested if there is a more extreme phenotype in males *Pvf1*$^{-}$*;* *en*-Gal4; UAS- *Pvr*^{DN}. All these males have the terminalia rotated between 180º and 270º and show the dorsal split phenotype (not shown). This confirms the specificity of PVF1 for PVR (Duchek *et al.*, 2001). The observation of the internal spermiduct and sperm pump structures in these flies is similar to that observed in the *Pvf1* mutants (Fig. 5C).

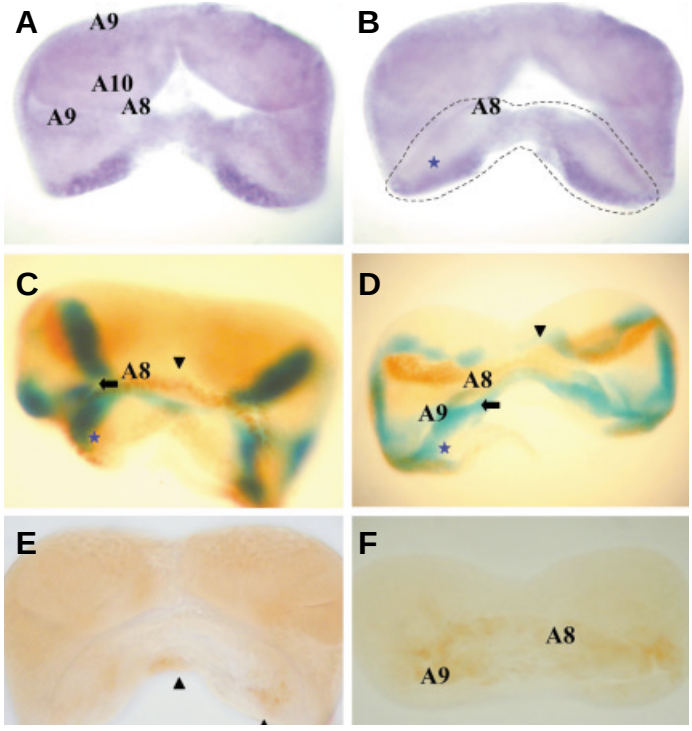

Fig. 4. Pvf1 expression in wild type male genital discs. *(A,B)* Pvf1 *transcript expression.* **(A)** *The dorsal side is in focus; the regions corresponding to the different abdominal segment cells are indicated.* **(B)** *The ventral side is in focus; the dotted line border outlines the A8 segment; the blue star indicates the region of probable overlap between* Pvf1, en, dpp *and* ptc *expression. (C,D) Domains of expression of the pattern genes* en, dpp *and* ptc. *Gal 4 insertions in these genes were used to direct targeted gene expression. The ventral side is in focus, revealing the position of the A8 segment. The blue stars indicate the same region as in (B). The arrows indicate* dpp *or* ptc *expression, while the arrowhead indicates* en *expression.* **(C)** *The expression of* dpp *(blue) is revealed using the* dpp-lacZ *transgene (arrow) and the expression of EN (brown-orange) is revealed using an anti-EN antibody (arrowhead). In some areas,* dpp *follows EN expression.* **(D)** ptc *expression in blue revealed by the* ptc-lacZ *transgene (arrow);* EN *expression in brown-orange, was revealed with an anti-EN antibody. Note that* ptc *expression is parallel to that of* en (arrowhead). **(E)** *A wt disc showing the presence of activated ERK (brown) by means of an anti-dpERK antibody (arrowheads).* **(F)** ptc-*Gal4; UAS*-Pvr, *the cells in brown are positive for dpERK; these cells correspond to the* ptc *domain of A8 and part of A9 cells. See* ptc *expression in (C).*

Taken together these results show that lack of *Pvf1* or inactivation of its receptor causes similar phenotypes, so the PVF1/PVR signaling is required. Remarkably, blockage of the signaling mechanism is most sensitive in the *engrailed* domain (Fig. 5D), where *Pvf1* transcript was not detected.

Pvf1 *gain of function*

The EP11235 line (Rørth, 1998) has a gain of function allele for *Pvf1* as the EP element is inserted upstream of the *Pvf1* transcription start site (Duchek *et al.*, 2001). With the *dpp*-Gal4 or *ptc*-Gal4 drivers EP11235 overexpression has no phenotypic effects, but with *en*-Gal4 or *hh*-Gal4 it does (Fig. 5E). Increasing PVF1 levels in males EP11235;*en*-Gal4.:2xUAS-*Pvf1* augmented the percentage of miss-rotations and the split phenotype (Fig. 5F). As the *wt* expression of *Pvf1* does not overlap that of *engrailed* in the genital disc, this result supports the idea that the diffusion of PVF1 is instructive in terminalia rotation and dorsal closure.

Apoptosis involved in dorsal closure and rotation

hid is a pro-apoptotic gene (Grether *et al.*, 1995). *hid* alleles are embryonic lethal and heterozygous individuals are wild type. Males escapers hid^{A22} (Grether *et al.*, 1995) over *Df(3L)H99* (a deficiency that removes *hid* and the other pro-apoptotic genes *reaper* and *grim* (Grether *et al.*, 1995; Chen *et al.*, 1996; Chao and Nagoshi, 1999) reared at 25ºC have the terminalia rotated between 90º and 270º (Abbott and Lengyel, 1991) (data not shown). Interestingly, 50% of heterozygous *Df(3L) H99/+* males have a dominant phenotype of rotated terminalia when grown at 30ºC. To analyze whether *Pvf1* and *Df(3L)H99* interact in eliciting this phenotype we constructed males of genotypes $Df(1)LP23^{semi1}$; *Df (3L)H99/+* and $Pvf1^{1624}$; *Df(3L)H99/+*. They showed a notable increase in penetrance, from 20% of $Df(1)LP23^{semi1}$ or $Pvf1^{1624}$ alone to 80% with the deficiency (Fig. 6 A,B). This increment suggests an interaction between the pro-apoptotic genes included in *Df(3L)H99* and *Pvf* in the process of terminalia rotation. Since *hid* is a pro-apoptotic gene (Grether *et al.*, 1995), we hypothesized that the rotated phenotype may be caused by and inhibition or reduction of apoptosis. To test this possibility, the gene encoding the caspase inhibitor *p35* (Hay *et al.*, 1994) was expressed in the male genital disc. We find that males of genotype *en*-Gal4; UAS-*p35* and *dpp*-Gal4; UAS-*p35* possess rotated terminalia and a gap in the dorsal midline (Fig. 6C). Driving the expression of *p35* to the posterior cells led to a stronger phenotypes than using anterior Gal4 drivers (not shown).

It has been shown that the activity of RAS-MAPK, one of the mayor signal transduction players of RTK (reviewed in Seger and Krebs, 1995), produces a decreased activity of *hid* (Kurada and White, 1998; Bergmann *et al.*, 1998). Accordingly, we analyzed the consequence of *Ras* overexpression in the rotation of the male terminalia. A low percentage (4%) of males carrying the UAS-Ras^{AV12} transgene, a constitutive form of the RAS (Fontini *et al.*, 1992), show at RT, a dominant phenotype of rotated terminalia (not shown). This effect may be based on a basal expression of the construct. However, expression of Ras^{AV12} at 30ºC using the driver LP23-Gal4 led to a much higher percentage (55%) of adult males with rotated terminalia (Fig. 6D). Therefore, overexpressing *Ras* in the male genital disc causes the same phenotype observed in the eye-antenna disc, as it is reduces *hid* activity (Kurada and White, 1998; Bergmann *et al.*, 1998).

Role of the JNK signalling pathway

The JNK pathway is involved in apoptosis in the imaginal discs (Moreno *et al.*, 2002 a). Furthermore, JNK, together with Eiger -the Drosophila TNF homologous- up-regulates *hid* (Moreno *et al.*, 2002 b). Therefore, we hypothesized that alterations in this pathway could also affect terminalia rotation and dorsal closure.

JNK activity can be down regulated in *Drosophila* by increasing the expression of the gene *puckered* (*puc*), an element of the pathway encoding a dual-specificity phosphatase that generates a negative feedback loop (Martín-Blanco *et al.*, 1998). Targeted expression of UAS-*puc* in the genital disc using *en*-Gal4, *dpp*-Gal4, *or ptc*-Gal4 causes rotation of the male terminalia and dorsal split, remarkably observed in the *ptc-en* domains (Fig. 7 B-E). In these

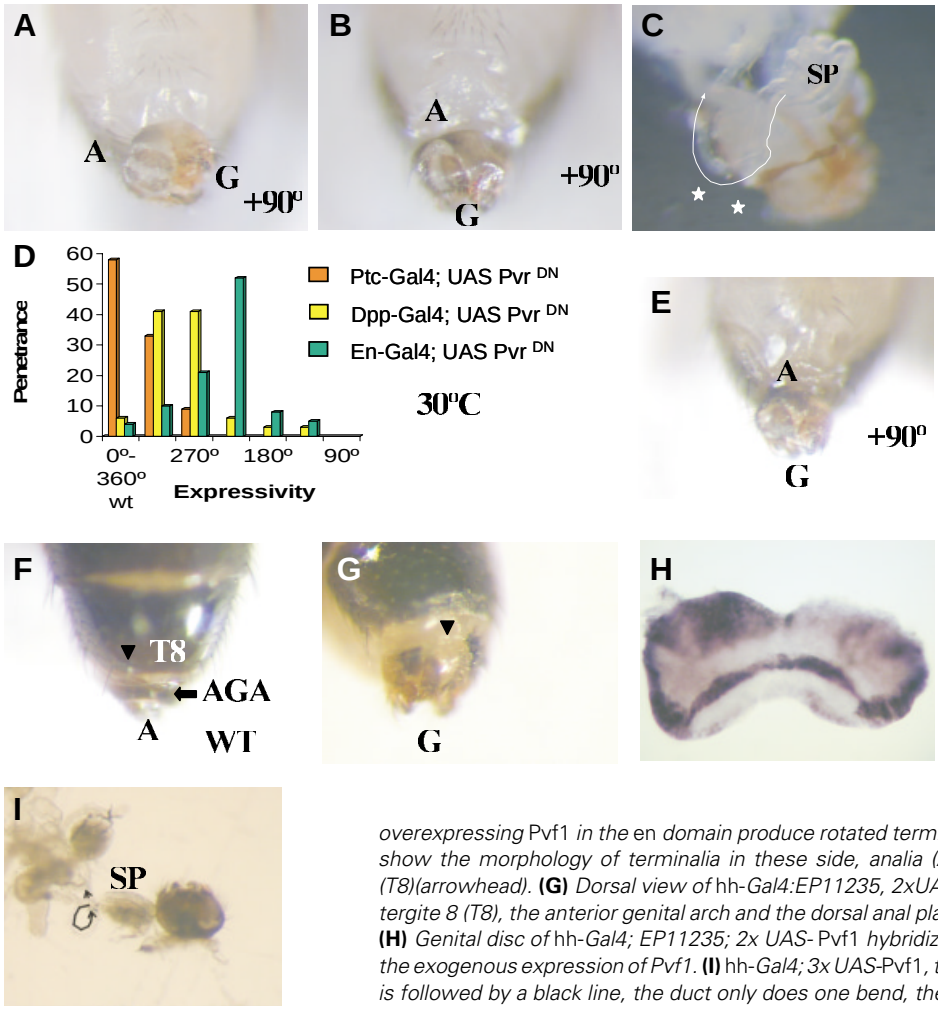

Fig. 5. Adult male terminalia, Pvr lack of function and Pvf1 gain of function. *The flies were grown at 30ºC. The males A, B and E are with its ventral is side up.* **(A)** dpp-GAL4; UAS-Pvr^{DN}. **(B)** en-Gal4; UAS-Pvr^{DN}. **(C)** *Dissected* en-Gal4;UAS-Pvr^{DN} *male, the external terminalia was at +270º, the white line show the trajectory of spermiduct, the duct could do two bends (white stars) but the sperm pump is found on the left.* **(D)** *Graphic penetrance/expressivity of* PvrDN *expression, the response to the receptor lack of function is stronger in the* en *domain.* **(E)** en-*Gal4:2x UAS*-Pvf1, *overexpressing* Pvf1 *in the* en *domain produce rotated terminalia.* **(F)** *Wild type male with its dorsal side up to show the morphology of terminalia in these side, analia (A), anterior genital arch (AGA) (arrow), tergite 8 (T8)(arrowhead).* **(G)** *Dorsal view of* hh-*Gal4:EP11235, 2xUAS*-Pvf1, *to show the failure in dorsal closure. The tergite 8 (T8), the anterior genital arch and the dorsal anal plates do not fuse in the dorsal midline (arrowhead).* **(H)** *Genital disc of* hh-*Gal4; EP11235; 2x UAS*- Pvf1 *hybridized with* Pvf1 *RNA, showing the endogenous and the exogenous expression of Pvf1.* **(I)** hh-*Gal4; 3x UAS*-Pvf1, *the external terminalia was at 180º, the spermiduct is followed by a black line, the duct only does one bend, the sperm pump (SP) is on the left.*

flies, the spermiduct and sperm pump are miss-rotated (Fig. 7D), as in *Pvf1*| *Pvr.*

To further study the association between the JNK and the PVF1/PVR pathways we examined JNK activity by checking the expression of *puc-lacZ* transgene in males either *puc^{E69}* a lethal allele with the *puc-lacZ* insertion, or *hh*-Gal4; UAS-*Pvf1/puc^{E69}*. It was observed that the expression domain of *puc* surrounds that of *Pvf1* (Fig. 7F) and extends to A9 cells. Targeted expression of *Pvf1* can ectopically activate *puc* (Fig. 7G). We also analyzed whether increasing the activity of JNK by lowering *puc* may rescue the dominant phenotype of *Df (3L) H99/+* males. We found that 100% of the males double heterozygous for *Df(3L)H99* and *puc^{E69}* are wild type at 30ºC, whereas 50% of *Df(3L)H99/+* males have a mutant phenotype. This result strongly supports the idea that in the genital disc the JNK pathway is also activating *hid*, as it has already been demonstrated in other genetic context (Moreno *et al.*, 2002 b).

Discussion

The PVF1/PVR pathway and the rotation and dorsal closure of male terminalia

In this work we demonstrate that either mutations at *Pvf1* or the expression of a dominant negative form of its unique receptor, *Pvr^{DN}*, result in various degrees of male rotated terminalia and failure of dorsal closure. These observations indicate that the PVF1/PVR pathway is relevant in these morphogenetic processes. Although the *Pvf1* gene is only expressed in a subset of cells from the segment A8, reduction or abolition of PVF1/PVR signaling affects the normal development of all terminalia precursors (A8, A9 and A10). Interestingly, mutations in the *Abd-B m* function, which affect only the A8 segment (Casanova *et al.*, 1986) have a phenotype of rotated terminalia. Thus, these results highlight the importance of the A8 segment in this process. We propose that A8 cells affect the development of structures originated from A9 and A10 through the activity of the PVF1 protein diffusing from A8. Although our data concern transcript expression, Rosin *et al.*, 2004, demonstrated that PVF1 is capable of extensive lateral diffusion, so it has the properties of a long range signaling molecule.

PVFs could form homo and heterodimers, what opens the possibility of different effects in the binding responses of the receptor. McDonald *et al.*, 2003, observed that homodimers are not equivalent, because PVF1 seems to be the relevant signal for the migration of border cells and Brüncker *et al.*, 2004, describe two function for PVR in the embryonic hemocytes, suggesting a diversity of functions. We have not analyzed other PVFs and although we observed some partial redundancy, it will be necessary to separate PVF individual or associated functions.

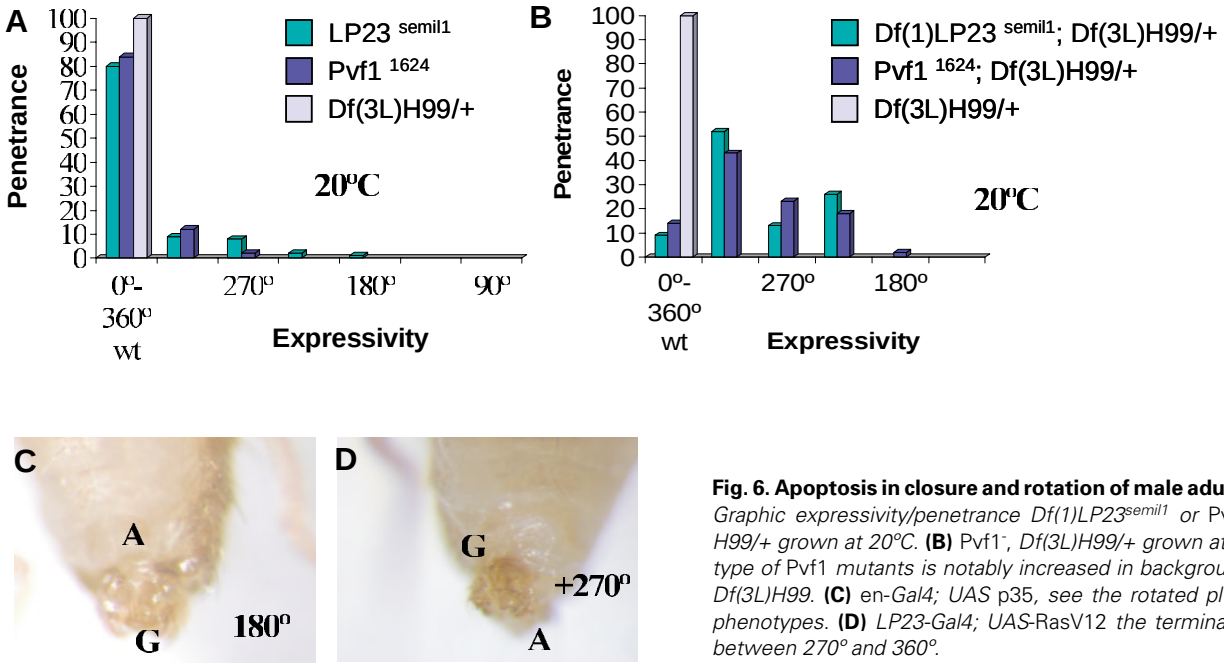

Fig. 6. Apoptosis in closure and rotation of male adult terminalias. (A) *Graphic expressivity/penetrance Df(1)LP23^{semil1} or* Pvf1^{1624} *and Df(3L)H99/+ grown at 20ºC.* **(B)** Pvf1^{-}, *Df(3L)H99/+ grown at 20ºC. The phenotype of* Pvf1 *mutants is notably increased in background hemizygous to Df(3L)H99.* **(C)** en-*Gal4; UAS* p35, *see the rotated plus the split dorsal phenotypes.* **(D)** *LP23-Gal4; UAS-*RasV12 *the terminalia is miss-rotated between 270º and 360º.*

We obtained indirect evidence about where the PVR receptor is activated or expressed. First, by recognition of factors that mediate the activity of the PVF1/PVR signaling mechanism (i.e. dpERK), whose expression was located at the periphery of the group of cells expressing *Pvf1*. Second, blocking PVF1 activity using *Pvr^{DN}* and overexpressing *Pvf1* the effects are stronger in the *engrailed* domain where *Pvf1* is not expressed. These findings provide additional evidence that there are specific domains for ligand expression and for responsive cells. In the ovary *Pvf1* is expressed in the ovule while *Pvr* is expressed in the follicle cells, the importance of this non overlapping domains is reflected by the fact that overexpression of a constitutive active form of the receptor *(λ Pvr)* produces the same phenotype of its lack of function (Duchek *et al.*, 2001). In the wing disc Rosin *et al.*, 2004 observed that the restrictions in the activity are regulated by a polarized secretion of the ligand in the apical membrane.

Apoptotic genes and the JNK pathway

Mutations in the pro-apoptotic gene *hid* have been shown to affect male terminalia rotation (Abbot and Lengyel, 1991), although this phenotype was observed in trans heterozygotes for *Df(3L)H99,* which includes the three pro-apoptotic genes *hid*, *rpr* and *grim* (Grether *et al.*, 1995). Trans heterozygotes for *hid* mutations are of wildtype phenotype, indicating that the rotated phenotype over deficiency is not only due to *hid* but to the haploinsufficiency of one or the two other genes. Our result that preventing cell death with p35 leads to miss rotation and split dorsal is also consistent with an involvement of apoptosis in these processes.

Additionally, we show that overexpressing *puc* results in the same phenotypes as PVF1/PVR and reduction of apoptosis, lowering *puc* rescues the rotated terminalia defects observed in *DfH99/+* males. The level of *puc* is considered as indicative of the JNK pathway activity (Martín-Blanco *et al.*, 1998; Moreno *et al.*, 2002 a-b) so these experiments suggests that JNK promotes apoptosis, probably by up-regulating *hid*.

PVF1/PVR, JNK and apoptosis

The fact that alterations in the PVF1/PVR pathway and in JNK/apoptosis give rise to similar phenotypes suggests a functional link between these two pathways.

The penetrance of the phenotypes of *Pvf1* mutations in the terminalia increases when they are additionally heterozygous for *Df(3L)H99.* This increase is non additive, suggesting PVF1/PVR and the apoptotic machinery affect the same aspect of the process. The overexpression of *Pvf1* ectopically activates *puc* and impedes the normal rotation and closure. This activation would down-regulate the JNK apoptotic pathway (Martín-Blanco *et al.*, 1998), thus reducing apoptosis and giving rise to the terminalia phenotype, but since the JNK pathway is a transcriptional activator of *puc* (Martín-Blanco *et al.,* 1998), this result opens up the possibility that *Pvf1* ectopically activates JNK rather than *puc*.

Apoptosis is necessary for terminalia rotation and dorsal closure and our results and those of others indicate that it is mediated by JNK activity. PVF1/PVR is also affecting these processes and our data suggest that PVF1/PVR may also affect JNK-mediated apoptosis. It is not clear however, whether all these elements act on the same developmental cascade.

Materials and Methods

Fly stocks

Mutant *Pvf1* alleles used in this study are: *Pvf1^{1624}* and EP11235, corresponding to EP insertions in the *Pvf1* gene (Rørth, 1998; Duckek *et al.*, 2001). Two deficiencies in *Pvf1*, *Df(1)LP23^{semil1}* and *LP23^{semil2}*, were isolated in this work and are described in the text. Dp(1;Y)W39 (Prado *et al.*, 1999) is a duplication covering the *Pvf1* gene. The Gal4/UAS system (Brand and Perrimon, 1993) was used to drive the expression of several gene constructs: *UAS-Pvf1* expressing the long spliced form of the *Pvf1* gene (Duchek *et al.*, 2001); UAS-*Pvr* and UAS-*Pvr^{DN}* express the native and a dominant negative form of the receptor for the PVF proteins, respectively (Duchek *et al.*, 2001); UAS-*Ras^{V12}* (Fontini *et al.*, 1992)

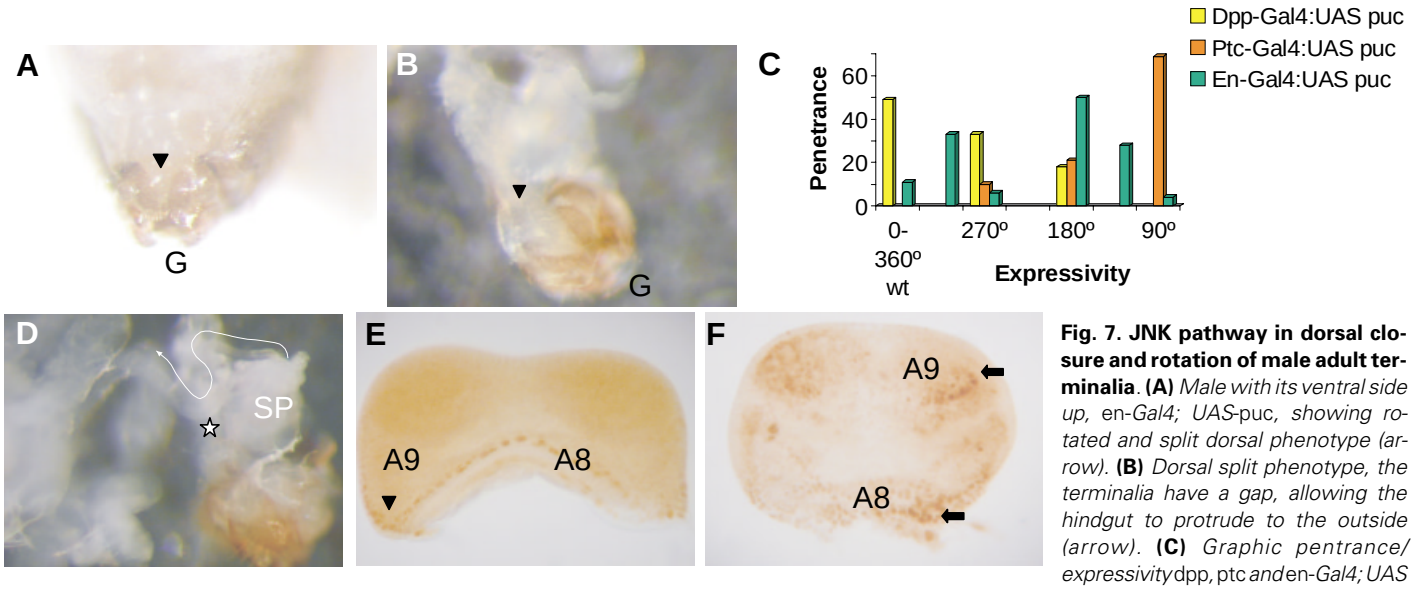

Fig. 7. JNK pathway in dorsal closure and rotation of male adult terminalia. **(A)** *Male with its ventral side up,* en-*Gal4; UAS*-puc, *showing rotated and split dorsal phenotype (arrow).* **(B)** *Dorsal split phenotype, the terminalia have a gap, allowing the hindgut to protrude to the outside (arrow).* **(C)** *Graphic pentrance/expressivity* dpp, ptc *and* en-*Gal4; UAS* puc. *These experiments were performed at 24ºC because* dpp *and* ptc *driven expression at 30ºC is a lethal condition. Note the mayor incidence of the phenotype with the targeted expression in the* en-ptc *domains.* **(D)** *Dissected* en-Gal4; UAS-puc *male. The external terminalia was at 180º, the white line follows the spermiduct, the duct does one bend (white star) and the sperm pump is on the left side of the fly.* **(E,F)** *Male genital discs. Expression of* puc *by* puc-lacZ *transgene revealed by anti-βgal stain. The insertion of P-lacZ in puc generates a recessive lethal mutation called* puc^{E69}. *In males,* puc^{E69}. puc^{E69} *+, wt puc expression is in A8 cells which surround the segment and extend to A9.* **(F)** hh-*Gal4; UAS*-Pvf1/puc^{E69}, *stained with anti-βgal to reveal puc expression, the ectopic expression of* Pvf1 *activates the gene* puckered *outside its own domain, the arrows point the normal and the ectopic expression.*

expresses a constitutive form of the RAS protein, while UAS-*puc* expresses the wild type allele of the *puckered* (*puc*) gene (Martín-Blanco *et al*., 1998). The driver's *dpp*-Gal4, *en*-Gal4, *ptc*-Gal4 and *hh*-Gal4 (Staehling-Hampton, K. *et al*., 1994; Hinz, U. *et al*., 1994; Tanimoto, H. *et al*., 2000).), as well as the *hid* allele hid^{A22} and Df(3L)H99 (Abbott and Lengyel, 1991; Grether *et al*., 1995), have previously been described. puc^{E69} is a null allele for the gene *puc* carrying an insertion of a *lacZ* gene (Martín- Blanco *et al*., 1998).

Immunostaining, X-Gal staining and in situ *hybridization*

Immunostaining and X-Gal staining were performed as described (Macías *et al*., 1990). The anti dpERK used is a monoclonal anti-Map Kinase, Activated antibody (SIGMA #M8159). The detection system used for the immunoreactions was ELITE of Vector Lab. The RNAs probes were transcribed from: *Pvf1*: EST 30334; CG7101: EST 44815. For *in situ* hybridization we followed the protocol adapted from Lehner and O'Farrell (1990). Stained embryos or discs were soaked in 87% glycerol, mounted onto cover slips and photographed with a NIKON digital camera Coolpix 950. The images were obtained with a stereomicroscope LEICA MZ 9.5 and a NIKON LABOPHOT light microscope. Given the low amount of products to be analyzed in these studies, incubation and developing times in our protocols were adjusted to optimize signal/background ratios.

PCR and Southern analysis

The LP23-Gal4 insertion was located by inverse PCR (Rehm, J.E; BDGP Resources). The primers used to map the LP23-Gal4 site of insertion are: 5'-CTG AGC GAC ATC TCA CGT CC-3' and 5'-TAG TCA GCG GAG ACC TTT TGG-3' for the 5' end and 5'-TGA CCA TGA TTA CGC CAA GCG-3' and 5'-CCA AGC ACC AGC AAG TTC CG-3' for the 3' end. For Southern analyses, genomic DNA from FM7 (*wt* control), $Pvf1^{1624}$ and EP11235 (insertions into *Pvf1*), Df(1)$LP23^{semi1}$, Df(1)$LP23^{semi2}$ and LP23-Gal4 were digested with the restriction enzymes *Pvu*II, *Hind* III, *Bgl*II, *Xho*I and *Eco*RI. Blots were hybridized with α-(^{32}P)-dATP (Amersham) oligolabeled probes (Feinberg and Vogelstein, 1984) prepared from template DNA corresponding to EST 30334, EST 44815, pBluescript KS and the *Gal4* gene. EST 30334 is a near full-length cDNA of *Pvf1* (Cho *et al*., 2002).

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