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The *Drosophila* bZIP transcription factor Vrille is involved in hair and cell growth

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SUMMARY

Vri is closely related to bZIP transcription factors involved in growth or cell death. *vri* clonal and overexpression analyses revealed defects at the cellular level. *vri* clones in the adult cuticle contain smaller cells with atrophic bristles. The phenotypes are strictly cell autonomous. Clones induced in the eye precursor cells lead to individuals with smaller eyes and reduced number of ommatidia with an abnormal morphology and shorter photoreceptor cell stalks. Overexpression of *vri* is anti-proliferative in embryonic dorsal epidermis and in imaginal discs, and induces apoptosis. On the wing surface, larger cells with multiple trichomes are observed, suggesting cytoskeletal defects. In salivary glands, *vri* overexpression leads to smaller cells and organs. We also show that *vri* is involved in locomotion and flight and interacts genetically with genes encoding actin-binding proteins. The phenotypes observed are consistent with the hypothesis that *vri* is required for normal cell growth and proliferation via the regulation of the actin cytoskeleton.

Key words: *vrille*, Growth, Proliferation, Differentiation, Apoptosis, Cytoskeleton, *Drosophila melanogaster*

INTRODUCTION

The *vrille* (*vri*) gene was previously identified as an enhancer of *decapentaplegic* (*dpp*) embryonic and adult phenotypes (George and Terracol, 1997). *vri* RNA expression is dynamic throughout development. In the embryo, *vri* transcripts are maternally provided and later expressed in morphogenetically active epithelia: in foregut, pharynx, hindgut, in stripes along dorsal and lateral epidermis and in tracheal track. At third larval instar, *vri* is expressed in gut, brain and in imaginal discs. *vri* was later characterized as a clock-controlled gene expressed in circadian pacemaker cells (Blau and Young, 1999) and acting as a direct repressor of the *Clock* and *cryptochrome* genes (Glossop et al., 2003; Cyran et al., 2003).

vri encodes a bZIP transcription factor (George and Terracol, 1997) with a typical basic DNA-binding domain and a leucine zipper involved in homo- or heterodimerization (Vinson et al., 1989). Vri is closely related, in the bZIP extended domain, to proteins implicated in cell death or growth. The more closely related factors are, with 60% identity and 93% similarity with Vri, gene 9 of *Xenopus* (Brown et al., 1996) (which is induced by thyroid hormone during the tadpole tail resorption program) and E4BP4 (a transcription factor of the human placenta that acts as a repressor of the protein E4 of adenovirus, which is involved in apoptosis) (Cowell et al., 1992; Cowell and Hurst, 1994). This factor was later isolated as NFIL3A a transactivator of the interleukin 3 (IL3) promotor in human T cells (Zhang et al., 1995). In pro-B lymphocytes, the murine homolog of NFIL3A (100% identity in the bZIP domain) is a delayed anti-apoptotic early transcription factor induced by IL3 stimulation acting through the Ras/MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) pathways. The alteration of these pathways is likely to contribute to human B-lineage leukemia (Ikushima et al., 1997; Kuribara et al., 1999). Vri and NFIL3A are also related to the segmentation gap gene product of *Drosophila* Giant (Capovilla et al., 1992), to CES-2, the product of a cell death specification gene of *C. elegans* (Metzstein et al., 1996), and to the sub-family of proteins (PAR) (Haas et al., 1995). In mammals, the PAR bZIP proteins include DBP (Mueller et al., 1990), TEF (Drolet et al., 1991), VBP (Iyer et al., 1991) and HLF (hepatic leukemia factor) (Hunger et al., 1992). It has been postulated that members of the CES-2/PAR family are evolutionarily conserved regulators of programmed cell death (Metzstein et al., 1996; Seidel and Look, 2001). In other respects, it is noteworthy that the PAR family genes present, like *vri* and the chicken and mouse homologs of NFIL3A/E4BP4, circadian oscillations (Wuarin and Schibler, 1990; Falvey et al., 1995; Fonjallaz et al., 1996; Blau and Young, 1999; Mitsui et al., 2001; Doi et al., 2001).

We show that *vri* partial loss of function induces flight and other locomotory defects associated with a downward bending wing phenotype and hair defects. Furthermore, *vri* interacts genetically with genes encoding actin-binding proteins: *bent*

encoding a myosin light chain kinase (Ayme-Southgate et al., 1991; Daley et al., 1998), *karst* encoding a βHeavy-spectrin (Thomas and Kiehart, 1994; Thomas et al., 1998; Zarnescu and Thomas, 1999), and *α actinin* encoding an actin crosslinking spectrin superfamily member (Fyrberg et al., 1990; Fyrberg et al., 1998; Roulier et al., 1992; Dubreuil and Wang, 2000). Clonal analysis shows that *vri* is cell autonomous and that the absence of *vri* reduces cell and hair size in wing and to smaller eyes with a reduced number of ommatidia of abnormal morphology with shorter photoreceptor cell stalks. The overexpression of *vri* reduces salivary glands growth and nuclei size, a phenotype typical of an inhibition of endoreplication. Overexpression of *vri* in the embryo and in imaginal discs has an anti-proliferative effect. In the wing disc, increased apoptosis is observed and at the wing surface cells with multiple trichomes are formed, indicative of cell cycle arrest and defects in regulation of actin cytoskeleton. We propose that *vri* may control cell growth and proliferation *via* the regulation of the actin cytoskeleton.

MATERIALS AND METHODS

Fly stocks

Flies were raised on standard media and all crosses were performed at 25°C unless stated otherwise. *vri* alleles are described by George and Terracol (George and Terracol, 1997) and new 25D *P* insertions lines at the Berkeley *Drosophila* Genome Project (BDGP: http://flypush.imgen.bcm.tcm.edu/pscreen). The following stocks were used: *a, Actn, bt* and *kst* alleles (FlyBase, 2003); w^{1118}*, FLP/FRT* (Xu and Rubin, 1993); EGUF/hid (Stowers and Schwartz, 1999); *hs* FLP*; Sp/SM6-TM6B (Tb), GFP FRT40A, Act5C>CD2>GAL4, UAS-GFP* [III] (Neufeld et al., 1998a); *pnr-GAL4*[III] (Calleja et al., 1996); *vg-GAL4*[II] (Simmonds et al., 1995); *ey-GAL4*[III] (Hazelett et al., 1998); *en-GAL4*[II] and *hs-GAL4*[III] (Brand and Perrimon, 1993); *C765-GAL4*[X] (Guillén et al., 1995); *MS1096-GAL4*[X] (Capdevila and Guerrero, 1994); *A9-GAL4*[X] (Haerry et al., 1998); *F4-GAL4*[II] (Weiss et al., 1998); *UAS-rbf*[III] (Du et al., 1996); rbf^{11}, rbf^{14} (Du and Dyson, 1999); *UAS-dap*[III], *UAS-E2F-Dp*[III] and *UAS-CycE*[III] (Lane et al., 1996); *UAS-string*[II] (Neufeld et al., 1998a); and *UAS-dPTEN* (Gao et al., 2000). Balancer chromosomes and other stocks are described elsewhere (Lindsley and Zimm, 1992; FlyBase, 2003).

DNA preparation, *vri* deficiency mapping

Standard molecular biology experiments were performed as described by Sambrook et al. (Sambrook et al., 1989). Genomic DNA preparations and PCR amplifications were performed as described by Szuplewski and Terracol (Szuplewski and Terracol, 2001). Positions are from cDNA1 (3.8) start in the genomic region: primer 1 (forward) 5′-CGATTGTCTGCACGCTGG-3′ (322-339), primer 2 (reverse) 5′-GTTCCTTCTCCGGCGATC-3′ (2570-2553), primer 3 (reverse) 5′-CAAGGCAAGGCTGGAGAG-3′ (6083-6066) and primer 4 (reverse) 5′-AGTATCTGCAGCGCACGG-3′ (7379-7352). *P-lacW* (Bier et al., 1989) primers: 5′*P* (reverse) 5′-CCTCTCAACAGCAAACGTG-TACTG-3′ (90-67), 3′*P* (forward) 5′-TCTCTTGCCGACGGGACC-3′ (10653-10670), *lacZ* (forward) 5′-GATCATCTGGTCGCTGGG-3′ (1935-1952). vri^{5} (Török et al., 1993) was mapped by plasmid rescue (George and Terracol, 1997) and upstream sequence from the 5′*P* primer is: (1218) 5′-GATTCTTGCATCATTCGGCG-3′. The insertion site is identical to that determined from the *P-lacW* 3′ end (AG034151) by the BDGP (http://www.fruitfly.org/p_disrupt/) (Spradling et al., 1995; Spradling et al., 1999). PCR products were cloned into pGEM plasmid (Promega) and sequenced with the SP6 or T7 primers. PCR fragments and breakpoint sequences were: $vri^{5R7.2}$, 2.2 kb from primers *lacZ*-3; *P-lacW*/genomic, 5′-GCAGTGCACG-GCAGATACAC/TTGCCGCTTCGGTCACCCGT-3′; $vri^{5R1.5}$, 3.3 kb from primers *lacZ*-4; *P-lacW*/genomic, 5′-CAACATCAAATTGTCT-GCGG/CGATGATGGTGAAGTTAACG-3′; $vri^{5R5.24}$, cloned by plasmid rescue; and genomic/*P-lacW*, 5′-TGATTTAAGCAGAG-TATTTC/GCTAAATACTGGCAGGCGTT-3′. DNA was sequenced by the dideoxy-chain termination method (Sanger et al., 1977) using the US Biochemicals sequencing kit (Pharmacia) or performed on a ABI Prism™ 377 DNA sequencer (Applied Systems). DNA sequences were compiled using the Genetics Computer Group software (GCG) (Devereux et al., 1984) and compared with the *Drosophila* database using the BDGP (http://www.fruitfly.org/blast/) Blast Searches program (Altschul et al., 1990).

Clonal analysis

Mitotic clones were generated using the FLP/FRT technique (Golic and Lindquist, 1989; Xu and Rubin, 1993). *vri* alleles were recombined onto a *P[ry*$^{+}$ *hs-neo FRT]40A* second chromosome. To generate somatic clones in adult *y w/y w; vri P[ry*$^{+}$ *hs-neo-FRT]40A/CyO* females were crossed to *y P[ry*$^{+}$ *hs FLP]1/Y; P[y+] P[ry*$^{+}$ *hs-neo FRT]40A/Bc* males and *yellow* clones analyzed in *y P[ry*$^{+}$ *hs FLP]1/y w; P[y*$^{+}$*] P[ry*$^{+}$ *hs-neo FRT]40A/vri P[ry*$^{+}$ *hs-neo FRT]40A* female progeny. Clones were heat-shock induced at the third larval instar by 1 hour exposure at 38°C. To generate mitotic clones in the eye, we used the *EGUF/hid* method (Stowers and Schwarz, 1999). *y w/y w; P[ry*$^{+}$ *hs-neo FRT]40A GMR-hid l(2)CL-L1/CyO; ey-GAL4 UAS-FLP/ey-GAL4 UAS-FLP* females were crossed to *y w/Y; vri P[ry*$^{+}$ *hs-neo FRT]40A/CyO* males at 29°C. *GAL4*-expressing clones were induced by the FRT 'flip-out' method (Struhl and Basler, 1993) by crossing *P[ry*$^{+}$ *hs FLP]/Y; UAS-vri/SM6-TM6B (Tb)* males to *Act5C>CD2>GAL4, UAS-GFP/Act5C>CD2>GAL4, UAS-GFP* females. In both types of experiments, clones were heat-shock induced in the progeny 10-48 hours after egg deposition by 30 minute exposure at 37°C. *Tb*$^{+}$ female larvae were dissected at mid third larval instar.

Scanning electron microscopy, retina sections, embryonic and wing phenotypic analyses

Flies were fixed in 3% glutaraldehyde, 0.1 M PBS (2 hours at room temperature and then 24 hours at 4°C), dehydrated in ethanol series and then in amyl acetate series. Electron microscopy was performed on a JEOL JSM 6100 scanning electron microscope. To observe adult retinas, flies were fixed in carnoy, embedded in paraffin wax and 7 μm sections were cut on a Leica RM 2145 microtome. Embryonic cuticles were prepared as described by Wieschaus and Nüsslein-Volhard (Wieschaus and Nüsslein-Volhard, 1986). Wings were dissected, collected in 70% ethanol and mounted in Euparal (Labosi).

Histology

Larval tissues were dissected in 1×PBS, fixed in PBT [1×PBS, 0.1% Tween 20 (Sigma) plus 4% paraformaldehyde] for 25 minutes, washed three times for 5 minutes in PBT, stained for 10 minutes in PBT, 0.1 μg/ml DAPI (Sigma), washed overnight in PBT, incubated 24 hours in 80% glycerol and mounted in Citifluor (Kent Scientific Industry Project). Acridine Orange (Sigma) staining was performed as described in Gaumer et al. (Gaumer et al., 2000). Preparations were observed on a Leica DMR fluorescence microscope using a Micro Max (Princeton Instrument) camera and collected using the Metaview Imaging System software (Universal Imaging Corporation).

UAS constructs and transformation experiments

vri cDNAs are described by George and Terracol (George and Terracol, 1997). The 3.8 kb *Xba*I fragment from pBScDNA3.8 was cloned into the *Xba*I site of the pUAST transformation plasmid (Brand and Perrimon, 1993) and the 3.3 kb *Sal*I-*Xba*I fragment from pBScDNA3.3 was cloned into the *Xho*I and *Xba*I sites of pUAST. pUAST constructs were co-injected with the pUChΔ2-3 helper

plasmid in the pole cell region of w^{1118} preblastoderm embryos (Spradling and Rubin, 1982). 20 *UAS-vri1* (3.8 kb) and 27 *UAS-vri2* (3.3 kb) independent transformed lines were recovered. The following lines were used in this study: *UAS-vri1*^{6}[III], *UAS-vri1*^{9}[III], *UAS-vri1*^{13} [III], *UAS-vri1*^{14}[X] and *UAS-vri2*^{8}[II].

RESULTS

Characterization of *vri* 5′region and new alleles

The 5′ extension of *vri* was characterized by the recovery of new cDNAs and *P*-insertion alleles. From the nine cDNAs *vri* identified by the BDGP (http://www.fruitfly.org/EST/) (Rubin et al., 2000), six isolated from embryos (0-24h) had the same 5′ end as cDNA1 (3.8 kb) (Fig. 1). One cDNA from adult heads starts at +15 bp and two, from embryos, start at –6.061 kb from cDNA1 start. The complete sequence provided for one of them, RE29005, shows that the cDNA is 3,073 kb long with a 7814 bp first intron and is 380 bp shorter at the 3′ end (Fig. 1). This cDNA encodes the same 610 amino acid putative protein as cDNA2 (3.3 kb). Four new lethal and one viable *P*-insertion lines were mapped (BDGP, http://flypush.imgen.bcm.tcm.edu/pscreen) in the *vri* region (Fig. 1) that we identified as new *vri* alleles. *BG01569-3* (*vri*^{9}) maps at –75 bp, upstream of *vri*^{4}, *vri*^{6} (–66) and *vri*^{8} (–57), and *KG03762* (*vri*^{10}) maps in intron 2 near *vri*^{5} and *vri*^{7}. They do not complement *vri* alleles. *KG01220* (*vri*^{11}) maps at –16.7 kb and KG10174 (*vri*^{13}) at –8.6 kb. They complement partially *vri* alleles. *EY01490* (*vri*^{12}) is a *PUAS* viable line and maps at –12.6 kb.

A secondary *P*-induced mutagenesis was previously performed to generate *vri* deletion alleles by imprecise excision of the *vri*^{5} *P-lacW* transposon mapping in the first intron (George and Terracol, 1997) (Materials and Methods). Five independent *white* lethal lines (among 17) showing new patterns by Southern analysis and nine independent w^{+} lethal lines (among 30) were selected for amplification of genomic DNA by PCR. The nine w^{+} lines had red eyes, while *vri*^{5} eyes were orange and amplification of their DNAs from primers 1-5′*P* and 3′*P*-2 gave the same bands as *vri*^{5}. Therefore, they probably result from the insertion of a secondary transposon on the chromosome. *vri*$^{5R1.5}$, *vri*$^{5R5.7}$, *vri*$^{5R7.2}$ and *vri*$^{5R8.4}$ DNA amplified from primers 1-5′*P* and *vri*$^{5R5.7}$, *vri*$^{5R5.24}$ and *vri*$^{5R8.4}$

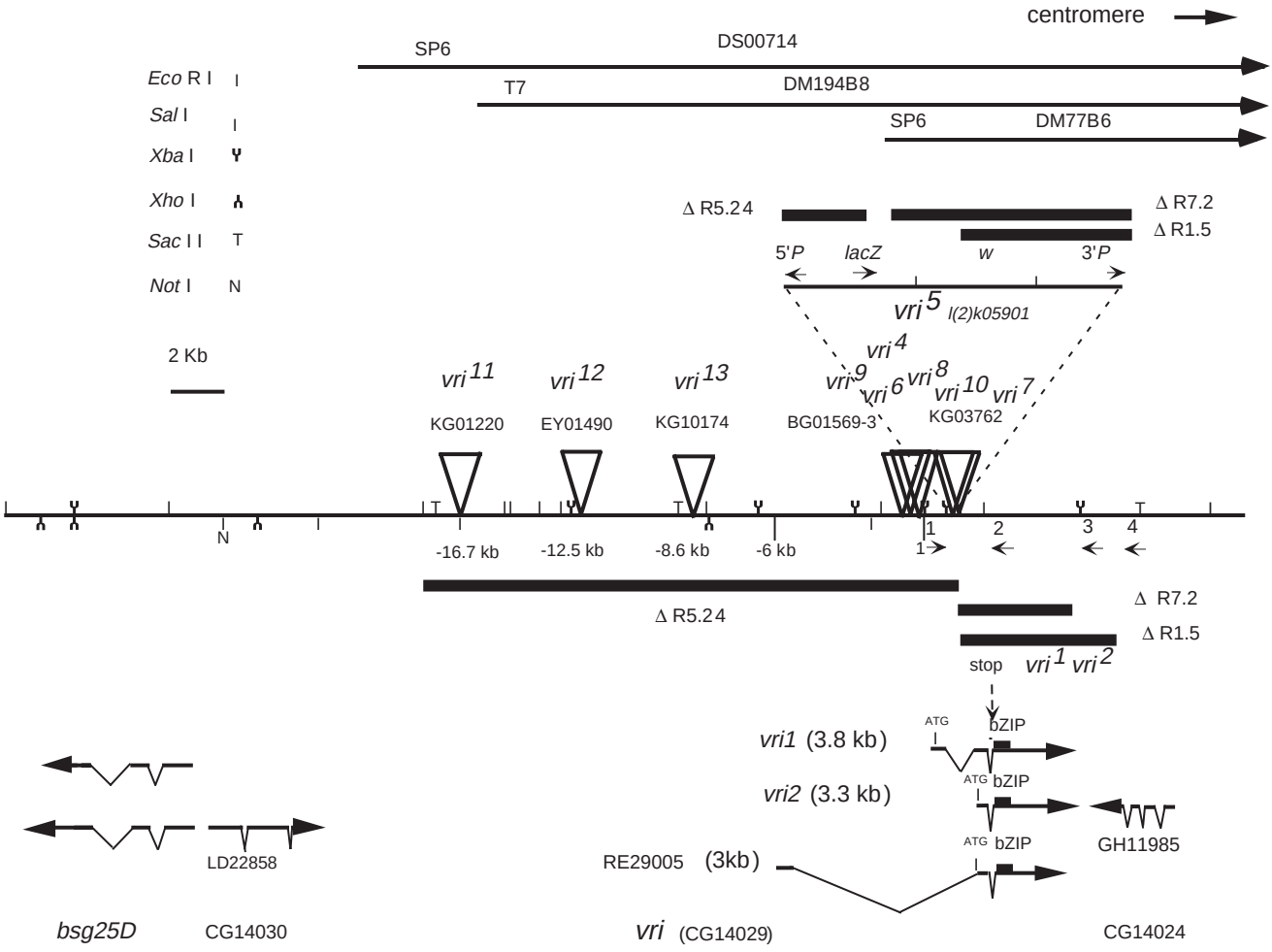

Fig. 1. Mapping of *vri* 5′ end and generation of deletion alleles by *P*-element mutagenesis. The position of the *vri* alleles and the extent of the deletions generated are shown. The localization of the *vri* cDNAs, the other described loci, the putative GadFly genes and the BDGP cDNAs are presented. The positions of the genomic (1, 2, 3, 4) and *PlacW* primers used for PCR amplification are shown (arrows). *vri1* (cDNA3.8) and *vri2* (cDNA3.3) are the two types of cDNAs used for UAS constructs. DS00714 distal end of a P1 phage isolated by the BDGP, DM194B8, T7 end and DM77B6, SP6 end of two cosmids isolated by the European Drosophila Mapping Consortium.

DNA from primers 3′*P*-2 giving the same bands as *vri*^{5}. Thus, *vri*$^{5R5.7}$ and *vri*$^{5R8.4}$ were generated by internal rearrangements in the *P* element, *vri*$^{5R5.24}$ by imperfect excision of the 5′ end and *vri*$^{5R1.5}$ and *vri*$^{5R7.2}$ by imperfect excision of the 3′ end (Fig. 1). *vri*$^{5R7.2}$ deletion eliminates 7627 bp in the transposon 3′ end and 3735 bp (1218-4952) in *vri* with a breakpoint in the 3′ non-coding end of cDNA1. The deletion eliminates most of the *vri* gene downstream from the *P-lacW* insertion, including the bZIP domain. The remaining 5′ end of cDNA1 encodes a putative 66 amino acid protein (the first 27 *vri* amino acids plus 19 amino acid from the first intron 5′ end). *vri*$^{5R7.2}$ might represent a null allele. *vri*$^{5R1.5}$ deletion eliminates 5779 bp in the 3′ end of *P-lacW* and 5771 bp (1218-6988) in genomic DNA. The 5′ end is identical to the *vri*$^{5R7.2}$ end, but the deletion extends downstream from *vri* and breaks within the GadFly gene CG14024 (BDGP, http://www.fruitfly.org/annot/) (Adams et al., 2000) corresponding to the BDGP cDNA GH11985. *vri*$^{5R5.24}$ deletion eliminates 19623 bp in the *vri* 5′ end, and 2223 bp in the transposon 5′ end. The breakpoint maps 1665 bp upstream from *KG01220* insertion site and downstream from the putative GadFly gene CG14030 corresponding to the BDGP cDNA LD22858. The short *vri* cDNA (3.3 kb) is potentially preserved. The exact sizes of the genomic deletions were deduced from our own sequences and the GadFly sequence (SEG, AE003609; http://www.fruitfly.org/annot/) (Adams et al., 2000).

vri affects wing shape and size, hair morphology and flight and interacts with genes encoding actin-binding proteins

vri^{11} and *vri*^{13} over *vri* lethal alleles and *Df(2L)tkv*^{Sz2} (25D2-4; 25D6-E1) lead to flies with pleiotropic phenotypes (100% penetrance). The wings are shorter and downward (or more rarely upward) bending, and posterior scutellar macrochaete are shorter and upturned (Fig. 2A,B). They are poorly viable and present locomotory and flight defects. *vri*^{11}/*vri*^{13} flies present the same phenotypes. Wings are notably smaller and regions of atrophic or missing hairs are present at the margin and on the surface of the wing (Fig. 2C,D). The downward bending phenotype has been described for other mutants. This is the case for mutants in the *arc* gene, which encodes an adherens junction-associated PDZ domain protein (Liu and Lengyel, 2000) and in the *karst* gene encoding β-heavy-spectrin involved in cell integrity, polarity and adhesion (Thomas and Kiehart, 1994; Thomas et al., 1998; Zarnescu and Thomas, 1999). This phenotype is also observed for mutations in *bent* encoding a myosin light chain kinase expressed in indirect flight muscle and tubular muscle (Ayme-Southgate et al., 1991; Daley et al., 1998). Viable mutants in *bent* are flightless, this is also the case for mutants in muscle actin, and in actin-binding proteins such as *flightless I* encoding a gelsolin family homolog (Davy et al., 2001) and *α actinin* encoding an actin crosslinking protein of the spectrin superfamily (Fyrberg et al., 1990; Fyrberg et al., 1998; Roulier et al., 1992; Dubreuil and Wang, 2000). We have tested for interactions in double heterozygotes between *vri* and *arc*, *karst*, *bent* and *α actn* alleles. No significant interaction was observed with *arc*, but only the hypomorphic *a*^{1} allele was tested. With the three other genes, reduced viability and wing hair phenotypes similar to those of *vri* are observed (Fig. 2E,F). With *bent (b*^{1}*)* and *Actn*^{8} (null), *Actn*^{14} and *Actn*^{G0077}, flies also present progressive locomotory defects affecting rear legs.

Mutations in *vri* alter hair and cell size and morphology

To analyze further the effect of the absence or reduction of *vri* in the adult cuticle, we generated somatic clones with different *vri* lethal alleles. *vri*^{5}, a *P*-insertion allele, *vri*^{1} and *vri*^{2}, both with a stop codon at position 924 upstream from the bZIP domain yielding 202 and 83 amino acid putative proteins

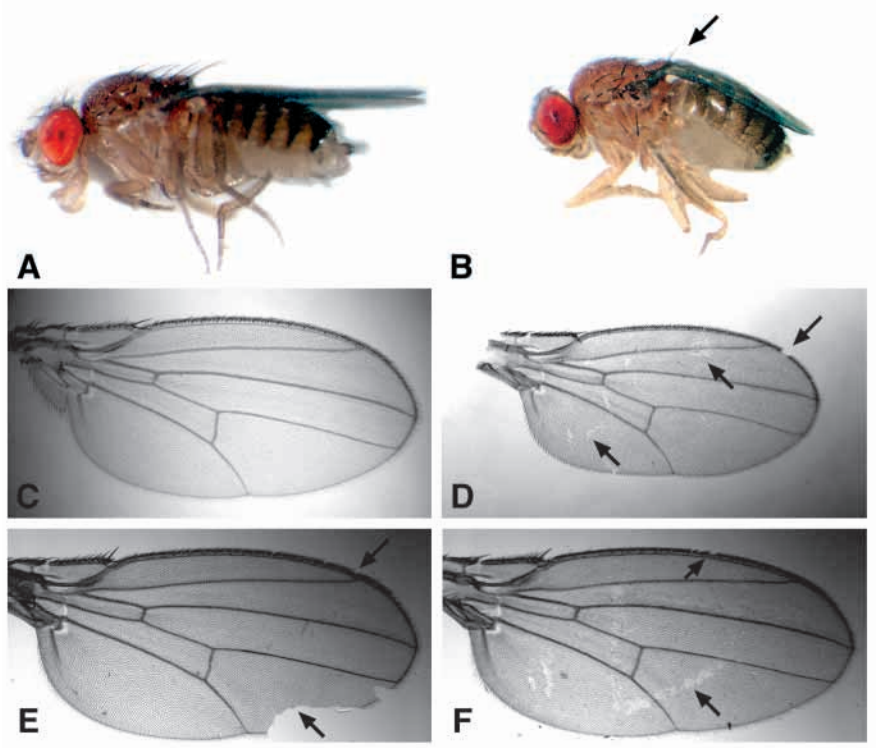

Fig. 2. *vri* affects wing shape and hair morphology and interacts with actin-binding protein encoding genes. (A) Wild-type adult female fly. (B*) vri*^{11}/*vri*^{5} adult female fly with shorter downward bending wings and shorter and upturned posterior scutellar bristles (arrow). (C) Wild-type wing. (D) *vri*^{11}/*vri*$^{5R5.24}$ wing, smaller than wild type with notching at the anterior margin (right-hand arrow) and multiple regions with atrophic or missing hairs appearing as non pigmented zones on the wing surface (arrows). (E) *vri*$^{5R7.2}$/*kst*^{01318} wing showing missing hairs at the anterior margin (top arrow) and notching at the posterior margin (bottom arrow). (F*) vri*$^{5R7.2}$/*Actn*^{G0077} wing with missing hairs at the anterior margin (top arrow) and on the wing surface (bottom arrow).

(George and Terracol, 1997), were used. We tested also $vri^{5R7.2}$, a bZIP deletion allele, and $vri^{5R5.24}$ a 5′-deletion allele (described here). In clones (marked with *yellow*) observed on the whole body, all types of bristles are thinner and reduced in size with all the alleles tested or are missing, especially with vri^{1}, vri^{2} and $vri^{5R7.2}$ alleles (Fig. 3A,B). Wing clones show pigmented regions (Fig. 3C) that could result from necrosis. The wing surface contains small holes and is wrinkled. These phenotypes could be due to cytoskeletal defects. Clones were particularly studied in the stout bristle cells of the anterior margin triple row region. In addition to the reduction in size of the bristles, the margin is often concave suggesting defects in adhesion molecules (Fig. 3D). The mutation is cell autonomous, the size of *vri* bristles is the same at the center of the clone as at the margin. Reduction in bristle size is generally an indication of a reduction in the cell size, as each bristle corresponds to a single cell. The average distance between bristles within *vri* clones was scored showing a significant reduction compared with vri^{+} clones (Fig. 3E). The *vri* cells are smaller and are about 70% of the wild-type size with vri^{1}, vri^{2}, vri^{5} and $vri^{5R7.2}$. However, $vri^{5R7.2}$ bristles are more atrophic or are missing leaving many holes in the margin. This phenotype was also observed with viable combinations. vri^{5R524} cells are 85% of wild-type size, suggesting that the allele is weaker.

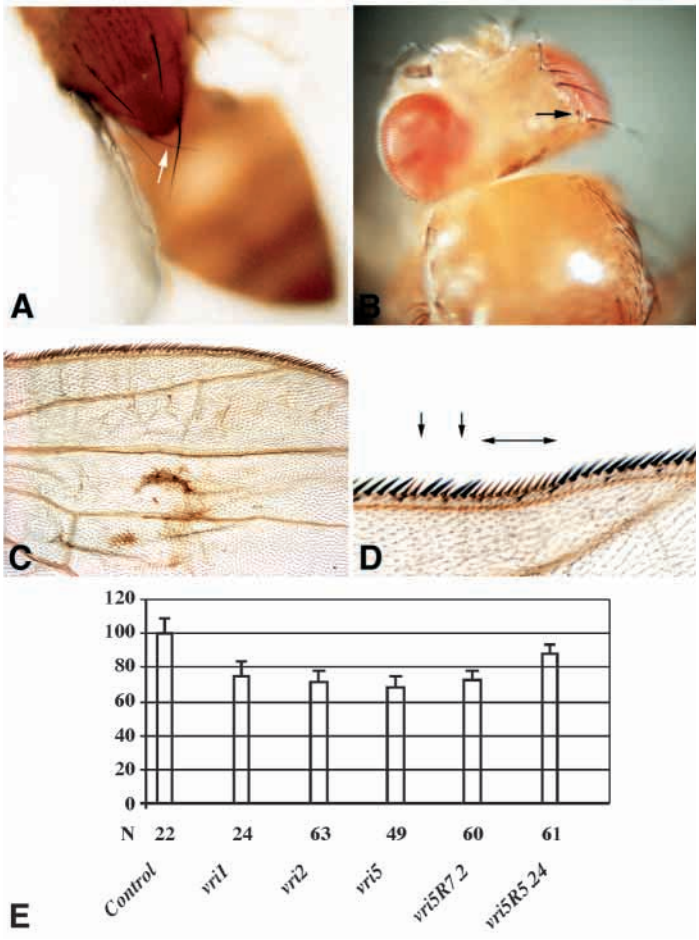

Fig. 3. Mutations in *vri* decrease hair and cell size in adult cuticle. *y/y; vri/vri* somatic clones were induced at third larval instar by the FLP/FRT technique and observed in females. (A) *y/y;* vri^{2}/vri^{2} clonal left posterior scutellar macrochaete (arrow) with a reduced size compared with the y^{+}/y; vri^{+}/vri right one. (B) vri^{1} clone showing a socket with no macrochaete on the head (arrow). (C) vri^{5} clones with pigmented cuticle. (D) *y/y;* vri^{5}/vri^{5} clones at the wing anterior margin in the triple row region with smaller and thinner stout mechanosensory bristles. The space between bristles is reduced (vertical arrows), which is characteristic of smaller cells, and the margin is concave (double-headed arrow). (E) At the anterior wing margin, the average distance between stout bristles of the triple row is significantly reduced in *y/y; vri/vri* clones (Student's test: $P<0.001$) compared with *y/y;* vri^{+}/vri^{+} clones. The values are percentages of wild type±s.e. Clones, analyzed from 12 to 31 wings, contained from two to 33 bristles.

We induced *vri* clones in the eye using the *EGUF/hid* method (Stowers and Schwarz, 1999), which generates eyes that are composed exclusively of clones because all other genotypes are eliminated. This is done by using the dominant photoreceptor cell lethal *GMR-hid* and a recessive cell death mutation. The *vri* eyes are smaller with a rough aspect (Fig. 4A,B), and ommatidia are disorganized with abnormal morphology and duplicated or missing bristles (Fig. 4C,D). The total number of ommatidia is significantly reduced by 10% in vri^{5}, 17% in vri^{1} and 19% in $vri^{5R7.2}$ eye clones compared with control eyes (Fig. 4E). The mean size of ommatidia, measured from at least 60 ommatidia from several independent heads, was significantly reduced (Student's *t*-test, $P<0.001$) in $vri^{5R7.2}$ eyes only and was 80±9% of wild-type size. In $vri^{5R7.2}$ retina, depth is markedly reduced and the photoreceptor stalks are distorted and their length is shortened by half (Fig. 4F,G). The analysis of wing and eye clones shows that $vri^{5R7.2}$ is the stronger allele and that, as expected, it might be a null allele.

vri overexpression induces lethality and inhibits proliferation

We used the GAL4-UAS system (Brand and Perrimon, 1993) to assess the effect of overexpressing *vri* in different types of cells. Two types of UAS transgenes containing the two major cDNA forms were constructed (Materials and Methods). *UAS-vri1* (3.8 kb) and *UAS-vri2* (3.3 kb) encoding, respectively, 729 and 610 amino acid putative proteins (George and Terracol, 1997) (Fig. 1). The results are similar with the two types of transgenes. Overexpression of *vri1* and *vri2* is lethal with most of the GAL4 drivers or leads to severe alterations in the targeted tissues.

Overexpression of *vri* using the driver *pannier*, the expression of which is restricted to dorsal tissues throughout development (Calleja et al., 1996; Heitzler et al., 1996), leads to embryonic lethality due to defects in the dorsal epidermis and dorsal closure (Fig. 5B). Use of the *vg-GAL4* driver (Simmonds et al., 1995), which directs GAL4 expression in the wing pouch, leads to notching at the wing margin (Fig. 5E). Overexpressing *vri* with the leaky *hsp70-GAL4* (Brand and Perrimon, 1993) driver results in atrophic or missing bristles on the adult cuticle (Fig. 5H). Similar phenotypes are observed by overexpressing with the same drivers the inhibitor of proliferation Rbf (Datar et al., 2000) (Fig. 5C,F,I) or Rho1, a small GTPase involved in actin cytoskeleton regulation (Hariharan et al., 1995). Overexpression of *vri* driven with *ey-GAL4* (Hazelett et al., 1998), the expression of which is specific to the eye disc and starts in the embryonic eye disc primordia, results in atrophy of the eye (Fig. 6). We observed

similar phenotypes (not shown) by overexpressing Rbf (Datar et al., 2000) or Rho1 (Hariharan et al., 1995). According to the strength of the *UAS-vri* transgene and the temperature, a progressive reduction in the size of the eye and a rough aspect are observed (Fig. 6A-D). The ommatidia are disorganized and bristles are either duplicated or missing. In some flies, the eyes are totally absent (Fig. 6E) and dissection of third instar larvae revealed no eye disc and atrophic brain (not shown). The stronger phenotype observed at 29°C leads to an absence of head (Fig. 6F). These phenotypes are compatible with an inhibition of proliferation.

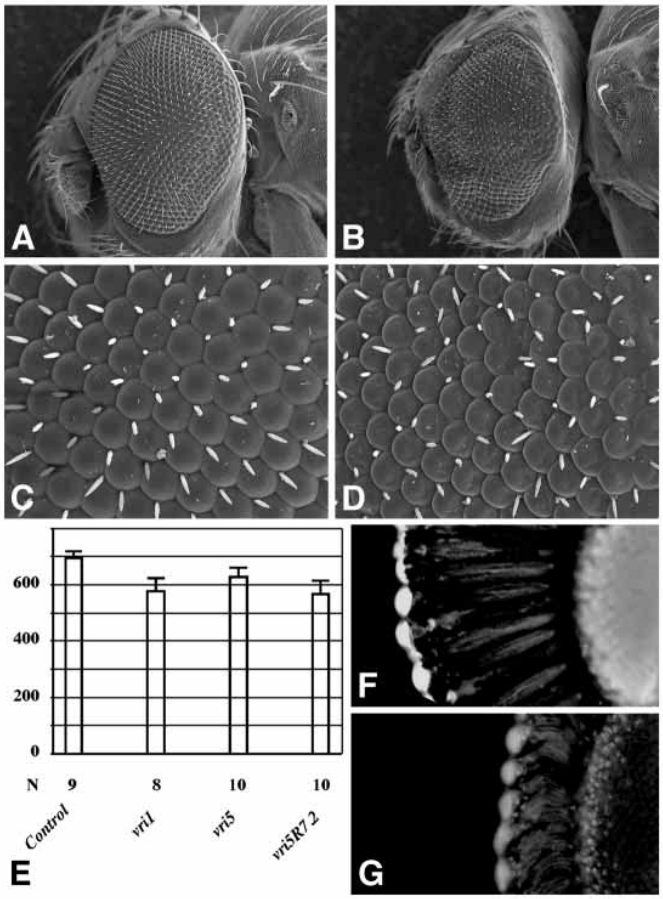

Fig. 4. *vri* loss of function allele reduces eye size, alters morphology and size of ommatidia and photoreceptors. Scanning electron micrographs of *vri* clonal eye were generated by the EGUF/hid method. Photoreceptor cells bearing the dominant photoreceptor cell lethal transgene *GMR-hid* die during metamorphosis. The *l(2)CL-L1* recessive cell lethal mutation leads to lethality at an earlier stage of eye development. The crosses were performed at 29°C and the eyes were observed in males. (A,C,F) vri^{+} *FRT40A/* vri^{+} *FRT40A* control eyes from *[ry*$^{+}$ *hs-neo FRT]40A/[ry hs-neo FRT]40A GMR-hid l(2)CL-L1; ey-GAL4 UAS-FLP/+* flies. (B,D,G) $vri^{5R7.2}/vri^{5R7.2}$ clonal eyes from $vri^{5R7.2}$*[ry*$^{+}$ *hs-neo FRT]40A/[ry hs-neo FRT]40A GMR-hid l(2)CL-L1; ey-GAL4 UAS-FLP/+* flies. (B) Clonal eye smaller than control (A) and with a rough aspect. (C,D) The ommatidia are disorganized and present an abnormal morphology. They are spherical rather than hexagonal and bristles are missing or atrophic. (E) The number of ommatidia is reduced in *vri* clonal eyes. Values are mean numbers of ommatidia (±s.e.) observed in male eyes. The mean number of ommatidia is reduced by 10% in vri^{5}, 17% in vri^{1} and 19% in $vri^{5R7.2}$ eyes compared with the vri^{+} control eyes (Student's test: $P<0.001$). (F,G) Longitudinal sections through adult retinas. (G) Clonal retina with photoreceptor stalks twisted and shorter than control (F).

vri overexpression alters cell growth, cell cycle and trichome differentiation in wing

vri overexpression driven in the wing with dpp^{blk}*-GAL4*, *ap-GAL4* or *en-GAL4* is lethal therefore we used the ubiquitous drivers, *A9-GAL4* (Haerry et al., 1998), *C765-GAL4* (Guillén et al., 1995) and *MS1096-GAL4* (Capdevilla and Guerrero, 1994) expressed predominantly in the dorsal surface. With weaker *UAS-vri* transgenes, results are similar with the three drivers (Fig. 7; *C765-GAL4* not shown). The L5 vein is often shortened and ectopic bristles are found with the *A9-GAL4* driver (Fig. 7C). The cells are larger than normal, as shown by the more widely spaced wing bristles, and possess multiple, abnormally oriented trichomes (Fig. 7C,D,G,H). A stronger viable phenotype is observed in flies with two copies of *UAS-*$vri2^{8}$. The wings are severely reduced in size and possess cells with multiple trichomes of abnormal morphology (Fig. 7I,J). We observed the same phenotype when overexpressing *rbf* or the cyclin dependant kinase inhibitor, *dacapo* (De Nooij et al., 1996) (not shown).

We tested the rescue of *vri MS1096-GAL4/+; UAS-*$vri2^{8}$*/+* phenotype by co-overexpressing the cell cycle activators, dE2F (Duronio et al., 1995; Duronio and O'Farrell, 1995) and two of its targets, Cyclin E, which promotes S phase initiation, and the *string*-encoded phosphatase that regulates the G2/M phase transition (Neufeld et al., 1998a). No rescue was observed with any of the transgenes neither when co-expressing the growth inhibitor, *dPTEN* of the PI3K pathway (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000). We also performed tests in double heterozygotes with *vri* and the null alleles rbf^{11} and rbf^{14} (Du and Dyson, 1999) and with available members of the Ras/MAPK and PI3K pathways regulating cell growth but we did not recover any genetic interaction.

vri overexpression induces apoptosis and reduces cell size and endoreplication

To observe the effect of *vri* overexpression at earlier stages, larval tissues were studied. With the strong *UAS-vri* transgenes such as *UAS-*$vri1^{9}$, their combination with *MS1096-GAL4* is pupal lethal and leads to increased apoptosis in the undifferentiated proliferating imaginal wing disc (Fig. 8A,B). Overexpression of *vri* was driven in the salivary gland using the *F4-GAL4* transgene whose expression starts in late stage 13 of embryogenesis, once cell proliferation in the salivary primordium is complete, between the first and second rounds of endoreplication. The driver stays active throughout larval stages. *F4-GAL4/+; UAS-*$vri1^{9}$*/+* glands grow to about one half the size of the control gland (Fig. 8C,D). Nuclei are smaller indicating an inhibition of endoreplication and often present a degenerative aspect characterized by a condensation of the chromatin (pyknosis), suggestive of high levels of apoptosis. Overexpression in the fat body, another polytenic tissue, using the 'flip out' technique, results in small clones with smaller cells, indicating an inhibition of endoreplication (Fig. 8E,F).

Overexpression phenotypes are often difficult to interpret, for example, it has been shown that ectopic expression of other transcription factors is able to generate headless flies by

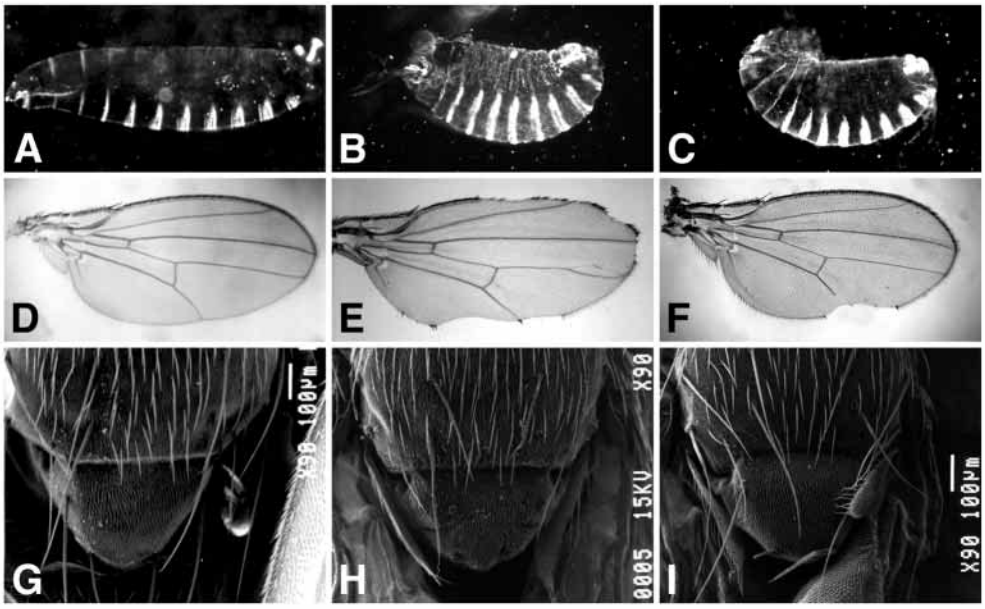

Fig. 5. *vri* overexpression induces atrophy of embryonic epidermis, adult wing and thoracic hairs. (A) Wild-type embryo. (B) *UAS-vri1^{6}/pnr-GAL4* dead embryo showing atrophic dorsal epidermis and anterior hole and head defects. (C) *pnr-GAL4/UAS-rbf* dead embryo with a similar phenotype. (D) Wild-type wing. (E) *vg-GAL4/+; UAS-vri1^{13}/+* wing at 29°C showing notching at the margin. (F) *vg-GAL4/+; UAS-rbf/+* wing at 25°C with notching of the posterior margin. (G) Scanning electron micrographs of wild-type thorax. (H) *hsp70-GAL4/UAS-vri1^{9}* thorax at 29°C showing atrophic macrochaete. (I) *hsp70-GAL4/UAS-rbf* thorax at 29°C with a similar phenotype.

inhibition of cell proliferation (Jiao et al., 2001). However, we have observed consistent phenotypes with different drivers and even if overexpression has not a consequence opposite to loss-of-function mutations, which is not rare, both phenotypes are compatible.

DISCUSSION

vri is required for normal hair and cell growth

The functional analysis of *vri* performed by mutant clone induction observed in adult indicates that *vri* is cell autonomous and involved in hair and cell growth. The fact that *vri* acts in a strict cell-autonomous manner suggests that it does not regulate the expression of a diffusive molecule such as a growth factor or a hormone. Smaller cells are recovered on the whole cuticle with shorter thinner or atrophic bristles. In the wing, clones have an abnormal shape and degenerative tissues are observed. These defects could be due to cytoskeleton defects. Clones induced in the eye precursor cells result in smaller eyes with a significantly reduced number of ommatidia with an atrophic morphology and a reduced size with the stronger allele. The photoreceptor cell stalks are shorter and atrophic. These results suggest that *vri* cells grow more slowly and are less viable than *vri^{+}* cells, even when they are not surrounded by wild-type cells

Smaller cells and bristles can result from metabolic defects, for example, mutations in tRNAs, ribosomal proteins (*Minute*) or rRNAs. This is the case for mutations in the *S6 kinase* gene, a regulator of ribosomal protein production (Montagne et al., 1999) and in *Drosophila myc*, *diminutive*, which is involved in growth and metabolism (Johnston et al., 1999). They are also found with genes regulating the cell cycle, such as *rbf* (Datar et al., 2000), and with genes of the Ras/MAPK and Insulin/PI3K pathways controlling growth (Stocker and Hafen, 2000; Oldham et al., 2000; Prober and Edgar, 2001). Hypomorphic mutations in the *rbf* gene, which encodes a homologue of the retinoblastoma protein, Rb, an important regulator of cell proliferation and differentiation leads to atrophic bristles and rough eyes (Du, 2000). Null-clone cells

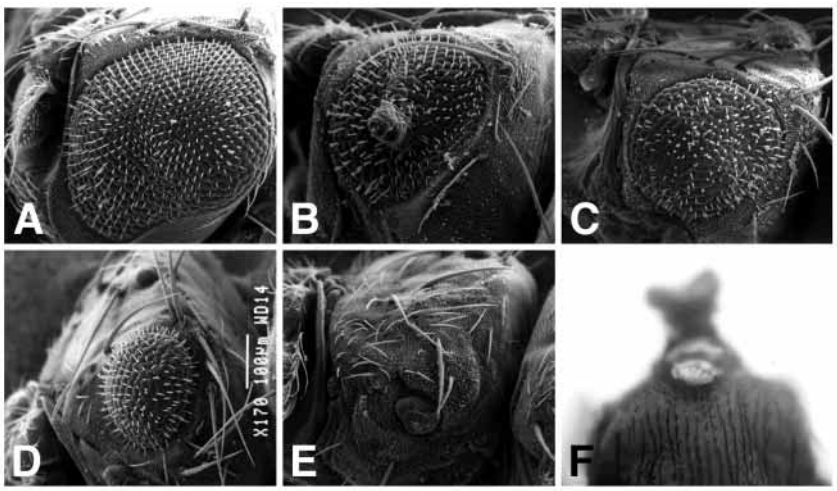

Fig. 6. *vri* overexpression induces atrophy of the eye. Scanning electron micrographs *of ey-GAL4/UAS-vri1^{9}* eye series with progressive reduction of the eye at 25°C (A,E), and extreme phenotype with *UAS-vri1^{14}/+; ey-GAL4/ UAS-vri1^{13}* at 29°C (F). (A) Rough eye with a moderate reduction in size. (B,C) Ommatidia are disorganized with a globular aspect, bristles are duplicated or missing and regions of apparent irregular growth are observed (B). (D) Very reduced eye. (E) Eye totally absent. (F) At 29°C the *UAS-vri1^{14}/+; ey-GAL4/ UAS-vri1^{13}* flies lack all head structures derived from eye-antennal disc. The proboscis, which is derived from the labial disc, is present.

in wing discs are smaller than wild-type cells and exhibit many pyknotic nuclei, suggesting elevated levels of apoptosis. *rbf*-null clones induced in the eye exhibit slight to moderate hypoplasia, missing or duplicated bristles and fused ommatidia. However, RBF inhibits cell cycle progression, rather than cellular growth directly (Datar et al., 2000). Cells homozygous for partial loss-of-function mutations in components of the Ras/MAPK pathway such as *Ras1* grow slowly and remain small. *Ras1* controls growth, survival and differentiation in the eye (Wassarman and Therrien, 1997; Halfar et al., 2001) and coordinates cellular growth and cell cycle progression in the wing (Diaz-Benjumea and Hafen, 1994; Karim and Rubin, 1998; Prober and Edgar, 2000). Cells devoid of the lipid kinase PI3K (Leevers et al., 1996; Weinkove et al., 1999) have a reduced growth and size, while loss of its antagonist dPTEN results in bigger cells than wild type (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000).

Smaller cells and bristles can also result from cytoskeletal defects. For example, the *miniature* and *dusky* genes encoding ZP proteins required for cytoskeletal reorganization are

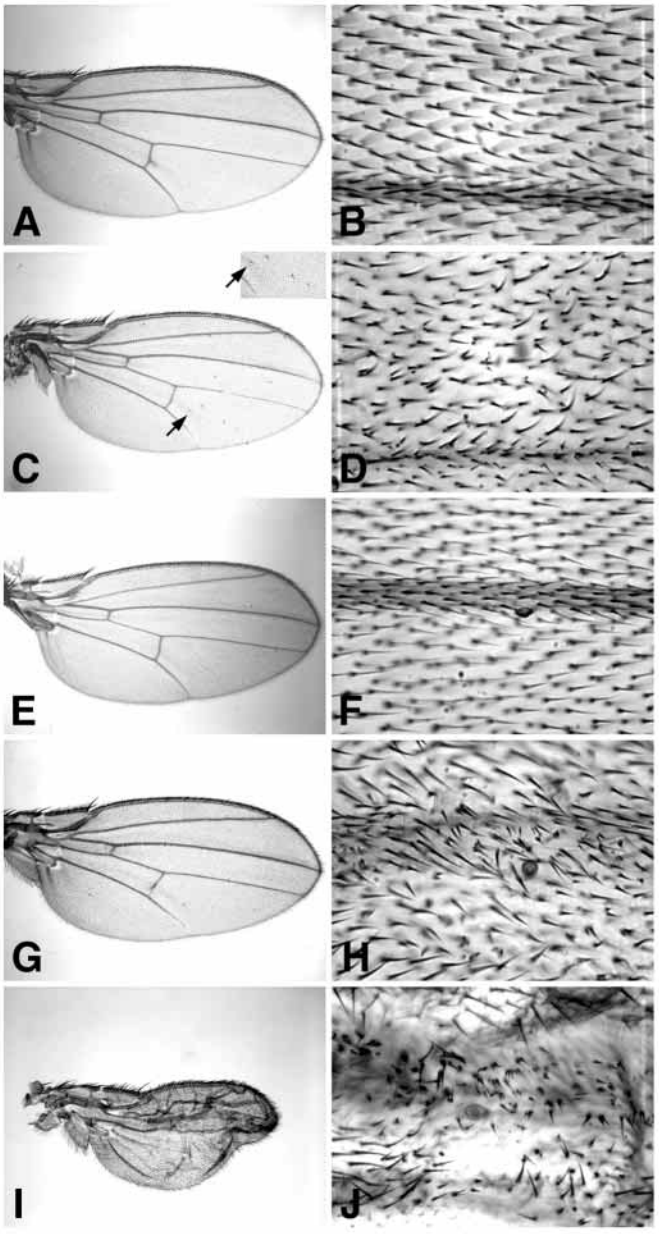

Fig. 7. *vri* overexpression alters trichome number, size and morphology in wing. All wings are from females. (A) *A9-GAL4/+* control wing. (B) Magnified view of *A9-GAL4/+* control wing around L4 vein. (C) *A9-GAL4/UAS-vri1*^{14}*; UAS-vri1*^{13}*/+* wing with a slightly reduced size, enlarged cells and reduced L5 vein. Long ectopic bristles are observed (magnification in the top right-hand corner). (D) Magnified *A9-GAL4/UAS-vri1*^{14}*; UAS-vri1*^{13}*/+* wing surface, around L4 vein. Larger cells are observed with multiple trichomes. (E) *MS1096-GAL4/+* control wing. (F) Magnified view of *MS1096-GAL4/+* control wing around L3 vein. (G) *MS1096-GAL4/+; UAS-vri2*^{8}*/+ wing*. (H) Magnified *MS1096-GAL4/+; UAS-vri2*^{8}*/+* wing showing enlarged L3 vein, enlarged cell size with multiple trichomes, some of them with an abnormal morphology. (I) *MS1096-GAL4/+; UAS-vri2*^{8}*/UAS-vri2*^{8} wing with a greatly reduced size and an abnormal morphology. (J) Magnified *MS1096-GAL4/+; UAS-vri2*^{8}*/UAS-vri2*^{8} vein around L3 vein showing multiple trichomes with a reduced size and an abnormal differentiation.

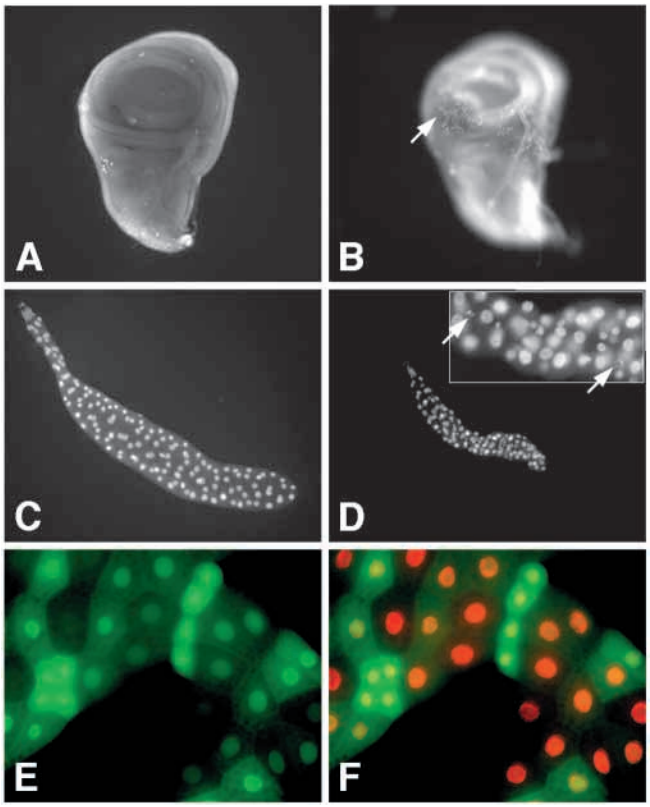

Fig. 8. *vri* overexpression induces apoptosis and inhibits cell growth in larval tissues. Tissues were dissected at third larval instar. (A,B) Wing imaginal discs stained with Acridine Orange. (A) *MS1096-GAL4/+* control disc with low level of apoptosis. (B) *MS1096-GAL4/+; MS1096-GAL4/+; UAS-vri1*^{9}*/+* with increased number of apoptotic cells (arrow) in the wing pouch region. (C,D) DAPI staining of male salivary gland. (C) *F4-GAL4/+* control gland. (D) *F4-GAL4/+; UAS-vri1*^{9}*/+* gland (same magnification as control) with a size reduced by about one half and with pyknotic nuclei (arrows). (E,F) Fat body clones co-expressing *vri* and *GFP* induced by the 'flip out' technique in *hsp FLP; UAS-vri1*^{9}*/Act5C<CD2<GAL4, UAS-GFP* females. (E) GFP detection (green) with *vri* overexpressing clones of greater green intensity. (F) GFP and DAPI (red) stainings showing clones (yellow) with smaller cells.

involved in growth and morphogenesis of cells and hairs in the wing (Roch et al., 2003). Mutant wings are smaller, phenotype also observed in *vri* viable combinations. *Drosophila* bristles are single cells with very long extensions supported by actin bundles of crosslinked actin filaments (Tilney et al., 2000a; Tilney et al., 2000b). The assembly of actin filaments is under the control of the Rho GTPases, including Rho1, Drac1 and DCdc4*2*. Rho GTPases act as molecular switches involved in many processes such as morphogenesis, chemotaxis, axonal guidance and cell cycle progression (Hall, 1998; Van Aelst and Symons, 2002). They regulate actin cytoskeleton and are involved in planar polarization, hair morphology and outgrowth processes (Eaton et al., 1995; Eaton et al., 1996; Guichard et al., 1997; Hall, 1998; Adler et al., 2000), and photoreceptor morphogenesis. The dominant-negative form of Drac1, N17Drac1, leads to reduced and disordered rhabdomeres (Chang and Ready, 2000; Colley, 2000), while overexpression of *Rho1* induces rough eyes and causes atrophy of the retina (Hariharan et al., 1995). Regulation of actin cytoskeleton is coupled with other pathways, including MAPK and PI3K pathways (Hall, 1998). For example Drac1 acts upstream of JNK cascade and DCdc42 downstream of Dpp pathway in dorsal and thorax closure processes (Ricos et al., 1999). *lilli* acts in cytoskeleton regulation, control of cell identity and cell growth, in parallel with the Ras/MAPK and PI3K/PKB pathways. It is noteworthy that retinal cells and wing margin bristles lacking *lilly* are significantly smaller than wild type (Wittwer et al., 2001; Tang et al., 2001). The flightless I-mediated cytoskeletal regulation involves PI3K, and the small GTPases Ras, RhoA and Cdc42 (Davy et al., 2001). Viable mutations in *flightless I* cause also ultrastructural defects in the indirect flight muscles.

vri overexpression inhibits proliferation and alters cytoskeleton regulation and endoreplication

Overexpression of *vri* in the embryo and imaginal discs induces an atrophy of the resulting tissues. With strong transgenes, the discs do not proliferate and the adult structures are totally absent. Similar phenotypes are observed when the inhibitor of proliferation Rbf or the regulator of actin cytoskeleton Rho1 are overexpressed. In wing discs, strong transgenes lead to lethality and increased apoptosis is observed. On the wing surface larger cells with multiple trichomes are observed. With strong viable transgenes, the wing is reduced and trichomes are disoriented with abnormal morphology. This phenotype has been attributed to cell cycle arrest in G1 phase. Inactivation of Cdc2 kinase which regulates entry into mitosis or of the *myb* gene required for G2/M transition and maintenance of diploidy leads to a similar phenotype (Weigmann et al., 1997; Katzen et al., 1998). This phenotype is observed when the human cyclin-dependant kinase inhibitor P21CIP/WAF1 is overexpressed with *UAS-P21* driven by the *dpp-GAL4* transgene (Karim and Rubin, 1998). We observed the same phenotype when overexpressing *dacapo*, the *Drosophila* P21 homolog (De Nooij et al., 1996), under the control of *MS1096-GAL4*. DAP binds to Cyclin E/Cdk2 complexes inducing cell cycle arrest in G1 phase and epidermal cell proliferation inhibition (Lane et al., 1996). The same phenotype is also observed when disrupting cytoskeleton regulation by overexpressing the activated form of Drac1, DRac1N17 (Eaton et al., 1995) or a dominant-negative form of DRacGAP, DRacGAP$^{\Delta EIE}$. DRacGAP is a negative regulator of the Rho-family GTPases, Drac1 and DCdc42, regulating actin cytoskeleton via EGF/Ras signaling pathway in the developing wing. The P21 overexpression phenotype is suppressed by *UAS-Ras1^{V12}*, an activated form of Ras1, or *UAS-DRacGAP* (Sotillos and Campuzano, 2000).

In salivary glands, *vri* overexpression leads to glands whose size is reduced by about one half with numerous pyknotic nuclei, suggesting inhibition of endoreplication and elevated levels of apoptosis. A reduction in the size of the gland and an inhibition of the level of endoreplication is observed when the genes encoding cell cycle regulators *cyclin E, dacapo* (Follette et al., 1998; Weiss et al., 1998) and *rbf* (Datar et al., 2000) are overexpressed. Pulses of Cyclin E are required to drive endocycle S phase, and continuous expression inhibits endoreplication cycles. In the fat body (polytenic tissue), *vri*-overexpressing clones are small with smaller cells often presenting a degenerative aspect.

Possible level of *vri* function

vri was previously identified as an enhancer of *dpp* phenotypes both in embryo and adult (George and Terracol, 1997). *dpp* encodes a TGFβ homolog closely related to BMP4 (bone morphogenetic protein 4), which acts as a morphogen at different stages of development (reviewed by Podos and Ferguson, 1999). Dpp plays a proliferative role in all imaginal discs at larval stages but induces a cell cycle arrest in G1 phase in the eye-antennal disc during third larval instar (Horsfield et al., 1998). Dpp might promote cell growth and/or proliferation directly, in a cell-autonomous manner (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002). Other genes have been identified or described as enhancers of *dpp* phenotypes. Some of them are integral members of the *dpp* pathway, which is the case for *tkv* (Terracol and Lengyel, 1994), *Mad* and *Med* (Raftery et al., 1995), but others [such as *lilliputian* (Su et al., 2001) and *cyclope* (Szuplewski and Terracol, 2001)] have been shown to act in different pathways. Interestingly, *lilli* has been also identified in other screens as a dominant suppressor of activated MAP kinase pathway phenotypes (Dickson et al., 1996; Neufeld et al., 1998b; Rebay et al., 2000). *lilli* acts in parallel with the Ras/MAPK and PI3K/PKB pathways in the control of cell identity, cell growth and/or cytoskeletal arrangement (Wittwer et al., 2001; Tang et al., 2001). *vri* mutations do not alter *dpp* pathway target gene expression and probably act in a parallel pathway.

vri overexpression phenotypes suggest a role in cell cycle and proliferation. However, these phenotypes are not rescued by simultaneous overexpression of the genes encoding activators of proliferation, *Drosophila E2F*, *cyclin E* or *string*. Therefore, it is unlikely that Vri is either a direct repressor of genes that activate proliferation or an activator of those acting as inhibitors of proliferation like *rbf* or *dacapo*. It could act upstream in the Ras/MAPK or PI3K pathways regulating growth and involved in the regulation of the mammalian homolog of Vri, NFIL3A, acting mostly as a repressor. Genetic interactions have been tested in double-heterozygotes with available members of these pathways and *vri*, but no reduction in viability nor any strong phenotypes was recovered. This could result from genes with non-limiting products and/or be due to the functional redundancy of *vri*. Alternatively, *vri* may control cell size independently of growth signals.

vri loss-of-function and overexpression phenotypes, more probably, could result from primary defects in cytoskeletal actin network. Although cytoskeletal integrity and adhesion are altered in mutants of regulators of cell growth and proliferation, these effects are indirect. Wing hair atrophy phenotypes were observed in interaction with the *karst* gene involved in cytoskeleton arrangement. The downward-bending wing and the reduction of the photoreceptor stalk size (Pellikka et al., 2002) are two other phenotypes observed in *vri* and *kst*. We observed new *vri* phenotypes affecting wing shape flight and locomotion. Locomotory defects could result from neurological or muscular alteration (or both). We found interaction with the α *actn* and *bent* genes involved in muscle actin function, which suggests that the effect is rather at the muscular level, although we have not observed gross defect in indirect flight muscle. However these defects appear degenerative and must be studied in more detail. We also observe hair atrophic phenotypes in interaction with these two genes, suggesting an effect in other cell types. Although the locomotory and hair defects are not necessarily related, it is notable that the genes interacting with *vri* affect different types of actin, muscle and non-muscle actin. It will be interesting to search for the direct targets of Vri to understand its implication in locomotion and cytoskeletal integrity.

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