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Grunge, related to human Atrophin-like proteins, has multiple functions in *Drosophila* development

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SUMMARY

We have carried out a genetic screen designed to isolate regulators of *teashirt* expression. One of these regulators is the *Grunge* gene, which encodes a protein with motifs found in human arginine-glutamic acid dipeptide repeat, Metastasis-associated-like and Atrophin-1 proteins. Grunge is the only Atrophin-like protein in *Drosophila*, whereas several exist in humans. We provide evidence that *Grunge* is required for the proper regulation of *teashirt* but also has multiple activities in fly development. First, Grunge is crucial for correct segmentation during embryogenesis via a failure in the repression of at least four segmentation genes known to regulate *teashirt*. Second, *Grunge* acts positively to regulate *teashirt* expression in proximoventral parts of the leg. Grunge has other regulatory functions in the leg, including the patterning of ventral parts along the entire proximodistal axis and the proper spacing of bristles in all regions.

Key words: *Drosophila*, Grunge, Teashirt, Legs, Atrophin-1-like proteins, Metastasis-associated proteins, Segmentation

INTRODUCTION

Embryonic segmentation and formation of the adult body are under the control of homeotic genes and signalling cascades. In *Drosophila*, the zinc-finger protein Teashirt (Tsh) is expressed from early embryogenesis to adulthood in specific domains where it acts both with Hox proteins and the Wingless signalling pathway for patterning (de Zulueta et al., 1994; Gallet et al., 1999; Gallet et al., 1998; Röder, 1992). Tsh is involved in the specification of the embryonic trunk (Fasano et al., 1991), parts of the intestine (Mathies et al., 1994) and the proximal part of the adult appendages (Erkner et al., 1999; Wu and Cohen, 1999). Expression pattern analysis of two putative murine Tsh orthologues suggest that Tsh function may have been conserved for patterning (Caubit et al., 2000).

During the first stages of embryonic development, the *tsh* expression pattern in the ectoderm is very dynamic and at gastrulation *tsh* mRNAs are homogeneously distributed in the presumptive trunk region. Genetic evidence suggest that *tsh* is activated and restricted in the trunk of early embryos by a combination of maternal and segmentation genes (Röder, 1992). Maternal and segmentation genes act either as repressors or activators of *tsh* transcription, in order to delimit the boundaries of *tsh* expression domains. The pair-rule gene *fushi tarazu* (*ftz*) activates *tsh* expression directly in even-numbered parasegments in the embryonic ectoderm (Coré et al., 1997). Later during embryogenesis, Tsh expression is maintained by homeotic genes (Röder, 1992) and autoregulation (Coré et al., 1997). Gallet et al. (Gallet et al., 1998) have shown that Wg signalling is necessary to accumulate a high amount of Tsh protein in the nucleus in order to give a trunk specific output for Wg signalling.

During *Drosophila* embryogenesis, a group of epithelial cells from each thoracic hemisegment will invaginate to form the primordia of the adult legs. These epithelia proliferate during larval life to give rise to the imaginal discs, which undergo morphogenesis and differentiation during metamorphosis (Bryant, 1978; Cohen, 1993). In the leg discs, *hedgehog* (*hh*) is transcribed in the posterior compartment and its protein is secreted to the anterior part to induce *wingless* (*wg*) and *decapentaplegic* (*dpp*) transcription in ventral and dorsal domains, respectively. Wg and Dpp proteins, which are homologous to Wnt1 and TGFβ in vertebrates, specify the ventral and dorsal cell fates, respectively, and via mutual repression establish the dorsoventral and the proximodistal axes of the leg. These signalling proteins impose progressively restricted patterning decisions on neighbouring cell groups, via independent transduction pathways, to give largely invariant appendages whether in *Drosophila* or vertebrates (Basler and Struhl, 1994; Brook and Cohen, 1996; Diaz-Benjumea and Cohen, 1994; Ingham and Fietz, 1995; Jiang and Struhl, 1996; Klingensmith et al., 1994; Lecuit and Cohen, 1997; Massague, 1998; Penton and Hoffmann, 1996; Wodarz and Nusse, 1998; Wolpert, 1969; Yang and Niswander, 1995).

Several genes have been isolated that exhibit differential, proximodistal patterns of expression in the imaginal discs

(reviewed by Abu-Shaar and Mann, 1998; Couso and Bishop, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999). The earliest is the *Distal-less* (*Dll*) gene product, which encodes a protein with a homeodomain and is expressed in the leg primordia of embryos before the invagination of the epithelia (Cohen et al., 1989; Cohen and Jürgens, 1989). Dll protein is crucial for the formation of specific distal parts of the legs (Cohen, 1990), as loss of function gives rise to an excess of proximal leg tissue at the expense of distal patterns. The Tsh protein is expressed in a largely complementary way to Dll, in the proximal leg, where it is required for the identity of the coxa and trochanter, and for the formation of a boundary to Dll-expressing cells. In ventral cells, this boundary formation is dependent on Wg signalling (Erkner et al., 1999).

We have carried out an in vivo screen in order to isolate regulators of *tsh*, and have identified a new gene called *Grunge* (*Gug*). The putative Gug protein shows similarities with human arginine-glutamic acid dipeptide repeat, vertebrate Atrophin-1 and Metastasis-associated-1 (Mta1)-like proteins. Mutations in *Gug* indicate that it is required for normal segmentation of embryos and patterning of the imaginal discs. In Gug^{-} embryos, the expression of segmentation genes and *tsh* expression is affected. A mosaic analysis of *Gug* mutations in the leg shows that, despite its ubiquitous pattern of expression, *Gug* is required for global ventral and proximal patterning of the leg, where it acts as a positive regulator of *tsh*.

MATERIALS AND METHODS

Fly stocks and mutagenesis

P(Lac, w$^+$)tsh^{J834} is a P-element insertion in the *tsh* region (Sun et al., 1995). In these flies, w^+, as *tsh* in the eye imaginal disc, is only expressed in the anterior half of the eye (Fig. 1A), indicating that w^+ expression is a reporter for *tsh* transcription in the eye. Male *red e* flies were mutagenised with ethyl methyl sulphonate (EMS) using standard procedures. These flies were crossed to female *w/w; P(Lac, w$^+$)tsh^{J834}/(Lac, w$^+$)tsh^{J834}* and their male progeny screened for alterations in w^+ expression. One of these was the Gug^{S2} mutation (Fig. 1B), which was localised to chromosome 3 after the analysis of segregation from different balancer chromosomes (*CyO* and *TM3*). Gug^{S2} was mapped to 26 cM on chromosome 3, by crossing Gug^{S2} /ro^1 h^1 *st th cu sr ca* females to ro^1 h^1 *st th cu sr ca* males. F2 males were selected and their genotypes recorded. These males were crossed individually to *w/w; P(Lac w$^+$)tsh^{J834}/P(Lac w$^+$)tsh^{J834}* and their male progeny examined for w^+ expression in the eye.

The P element allele, *l(3)PZGug^{3928}, ry$^+$* is localised on chromosome 3 at 66D1,2 (FlyBase, 1999). Deletions (Gug^{35}) of the gene were made by excision hopping: *l(3)PZGug^{3928},ry$^+$ ry^{506}/MKRS* males were crossed to *Dr Δ2-3/TM6B* females; male *l(3)PZGug^{3928},ry$^+$ ry^{506}/Dr Δ2-3* were crossed to $TM3ry^{RK}$ females to isolate jumps that had lost the ry^+ marker in their progeny. Precise excisions with Gug^+ activity were obtained, showing that the mutant phenotype is due to the *l(3)PZGug^{3928}* transposon. Different *Gug* alleles were recombined with *P(w$^+$)FRT2A* in order to carry out mosaic analysis.

Cloning and sequencing of the *Gug* region

DNA was isolated from *l(3)PZGug^{3928}/MKRS* flies in order to construct a genomic library. Partial Sau3A genomic fragments were cloned into λ phage (Sambrook et al., 1989). *lacZ* DNA probes were employed to isolate genomic DNA from the *Gug* gene region from this library. Subsequent chromosomal walking gave overlapping phages from the region. Genomic fragments from the walk were used to probe Northern blots in order to identify putative transcription units of the *Gug* gene. Complementary DNAs were isolated from an embryonic library (Zinn et al., 1988). The EST clones LD10989 and LD15383 from the Berkeley Drosophila Genome Project (BDGP) were used. Sequencing of the *Gug* cDNAs was performed by Genome Express (Grenoble, France). The sequence is available with the GenBank Accession Number AF217844. Sequence alignments and calculations of sequence similarity were constructed using the Network Protein Sequence Analysis 'ClustalW' at the Pole Bio-informatique Lyonnais (http://pbil.ibcp.fr/cgbin/npsa_automat.plpage=/NPSA/npsa server.html) with manual editing.

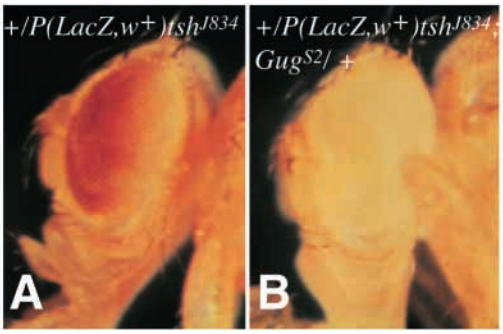

Fig. 1. *Gug* mutations act as dominant suppressors of a *tsh* reporter gene in the eye. (A,B) Heterozygotes for *P(Lac w$^+$)tsh^{J834}* (B is also heterozygous for the Gug^{S2} mutation). (A) Note that the *white* gene is active in anterior cells of the eye (Sun et al., 1995) as is the *tsh* gene; (B) expression of these genes is reduced.

Germline clones

Germline clones homozygous for *Gug* mutations were induced using the dominant autosomal germline clone technique (Chou and Perrimon, 1996). *y w hsFLP/y w hsFLP; Gug^X P(w$^+$ FRT2A)/TM6C, Sb* (where X=S2, 35, 1207D6, this work and R. Finkelstein, unpublished) females were crossed to *P{w^{+mC}=ovo^{D1-18}}3L1 P{w^{+mC}=ovo^{D1-18}}3L2 P(w$^+$ FRT2A)/TM3* males. Their progeny were heat shocked for 1-2 hours at 36°C in a water bath to induce germline clones homozygous for individual *Gug* alleles. *y w hsFLP/+; Gug^X P(w$^+$ FRT2A/ P{w^{+mC}=ovo^{D1-18}}3L1 P{w^{+mC}=ovo^{D1-18}}3L2 P(w$^+$ FRT2A)* females were then crossed to wild type, *Gug^X/TM6C, Sb* or, in the case of embryos used for in situ hybridisation, with *Gug^{35}/TM3ftzlacZ* males.

Sense and antisense injection

Sense and antisense mRNA were synthesised using T3 or T7 RNA polymerase (Sambrook et al., 1989). RNA was injected into preblastoderm embryos and the larval cuticles examined 48 hours later.

Mosaic analyses of *Gug* mutations in the adult leg

The FRT/FLP technique (Golic, 1991; Xu and Rubin, 1993) was used to produce clones of Gug^{-} (Gug^{35}, Gug^{1207D6} or Gug^{S2}) or Gug^+ cells induced by heat shock at 36°C for 1 hour in a water bath at different developmental stages from 24 to 144 hours after egg laying. Clones were induced in larvae of the genotype *y w P(hs-FLP, ry$^+$); mwh jv Gug$^-$ P(w$^+$ FRT2A)/Dp(1;3)sc^{J4}, y$^+$ M(3)i^{55} P(w$^+$ FRT2A)* (FlyBase, 1999; Lindsley and Zimm, 1992; Xu and Rubin, 1993), in order to analyse Gug^{-} clones with a growth advantage (Morata and Ripoll, 1975), and were marked by *yellow*, *multiple wing hairs* and *javelin*.

In discs, Gug^{-} cells were detected by absence of the Myc tag or green fluorescent protein (GFP), which are lost after mitotic recombination. All stocks carried balancers with the dominant *Tubby* mutation (Lindsley and Zimm, 1992), allowing larvae of the correct genotype [*y w P(hs-FLP, ry$^+$); Gug^{35} FRT80/ M(3)i^{55} P(w$^+$, hs-cMYC) FRT80 or Gug^{35} FRT2A/ubiGFP FRT2A* (Flybase, 1999)] to be selected for dissection of imaginal discs.

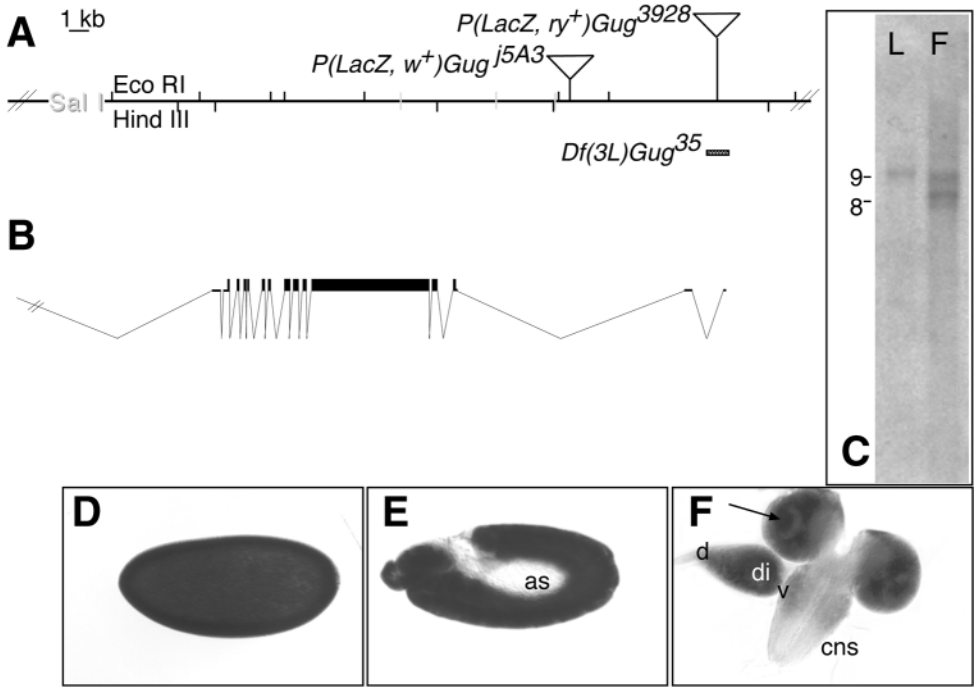

Fig. 2. Molecular analysis of the *Gug* locus. (A) The genomic region of *Gug* with the two P-element insertions and the deletion *Df(3L)Gug*^{35}. The restriction enzyme sites of *Eco*RI, *Sal*I and *Hin*dIII are shown. (B) Structure of the cDNA of *Gug*. The black boxes indicate the coding sequence. (C) Northern analysis: a 9.0 kb zygotic and a 8.0 kb maternal-specific transcript are detected. L, third instar larva; F, female. (D-F) In situ detection of *Gug* transcripts in a blastoderm (D) and germband extended (E) embryos and leg disc (F) associated with the larval central nervous system (cns, arrow). (D,E) Anterior is towards the left and ventral is towards the bottom. Note that *Gug* is not detected in the amnioserosa (as) in E and is concentrated in the hemispheres of the brain (arrow, F). In the leg disc, v, d and di indicate the ventral, dorsal and distal regions, respectively.

For the analysis of *wingless* and *decapentaplegic* expression in *Gug*$^{-}$ clones in leg discs, *CyOwgLacZ* or *dppLacZ* chromosomes were incorporated into the crosses described above.

Production of anti-Gug antibodies and immunohistochemical staining

Antibodies were raised in rabbits against the extreme C-terminal peptide (RQSLHDQYFRQRPR) of the putative Gug protein by Neosystem (Strasbourg, France). Mouse anti-Dll (from Stephen Cohen) was used at 1/1000; mouse anti-β-gal (Promega) was used at 1/500; rat anti-Tsh was used at 1/600 (Gallet et al., 1998); and anti-Gug at 1/250. Anti-Myc (9E10 mouse or rabbit; Santa Cruz Biotechnology) was used at 1/100. Secondary FITC- or TRITC-coupled antibodies (Jackson laboratories) were used at 1/100. Disc fixation and fluorescence labelling was performed as described by Gallet et al. (Gallet et al., 1998) and Xu and Rubin (Xu and Rubin, 1993). A Zeiss Confocal Microscope was used for this analysis.

In situ expression analysis

Gug, *Kr*, *hb*, *kni*, *ftz* and *lacZ* antisense RNA probes were synthesised. Homozygous *Gug* embryos were identified by the absence of expression of the *ftzlacZ* reporter gene carried by *TM3*.

RESULTS

Isolation of *Grunge*

In order to discover new genes involved in pattern formation of appendages, we have been searching for mutations affecting the expression of the region-specific patterning gene *teashirt* (*tsh*). In an EMS mutagenesis screen, we isolated mutations in a gene we call *Grunge* (*Gug*). A recessive lethal allele, *Gug*^{S2}, is a dominant suppressor of the *tsh* reporter gene *w*$^{+}$, which is expressed only in the anterior part of the eye in *P(Lac w*$^{+}$*)tsh*^{J834} flies (see Materials and Methods) (Sun et al., 1995). The P element *l(3)PZ3928* (Flybase, 1999) failed to complement *Gug*^{S2}, and thus is an allele of *Gug (P(LacZ, ry*$^{+}$*)Gug*^{3928}*)*.

We cloned the genomic DNA surrounding the insertion point of *P(LacZ, ry*$^{+}$*)Gug*^{3928} and used these genomic probes to confirm the location of the *Gug* gene to 66D1-2 on chromosome 3 (not shown). New mutations in the Gug gene were obtained by jump start mutagenesis by mobilisation of the *P(LacZ, ry*$^{+}$*)Gug*^{3928} element (Materials and Methods). Wild-type revertant chromosomes, presumably with precise excision events, suggest that the P-element is responsible for the *Gug* mutation. *Gug*^{35} corresponds to an imprecise excision of the P-element and a deletion of genomic DNA at the point of insertion (Fig. 2A). All *Gug* alleles have similar properties, dying as late embryonic zygotic lethals. One exception is *P(LacZ w*$^{+}$*)Gug*^{j5A3}, which dies at the third larval instar stage as a homozygote.

Molecular and functional analysis of the *Grunge* gene

Using northern analysis, we found a transcription unit close to the P element *P(LacZ, ry*$^{+}$*)Gug*^{3928} producing a 9.0 kb zygotic and a 8.0 kb maternal-specific transcript (Fig. 2B,C). Corresponding, overlapping cDNAs were isolated. Using in situ hybridisation, we detected transcripts in all cells (with the exception of the amnioserosa) in embryos (Fig. 2D,E) and imaginal discs (Fig. 2F).

Wild-type embryos consist of head, trunk and tail segments. In the larvae, the most obvious segments are those of the thorax and abdomen (trunk), each segment consisting of anterior denticle belts and posterior naked cuticle (Fig. 3A). Loss of zygotic *Gug* activity affects only head morphogenesis (Fig. 3B). In order to test for the maternal contribution of *Gug*, we have induced germline clones homozygous for different *Gug* alleles (Materials and Methods). All tested alleles (*Gug*^{S2}, *Gug*^{35}, *P(LacZ, ry*$^{+}$*)Gug*^{3928}) give essentially similar phenotypes. When fertilised by wild-type sperm, *Gug* germline clones give rise to embryos with severe segmentation defects

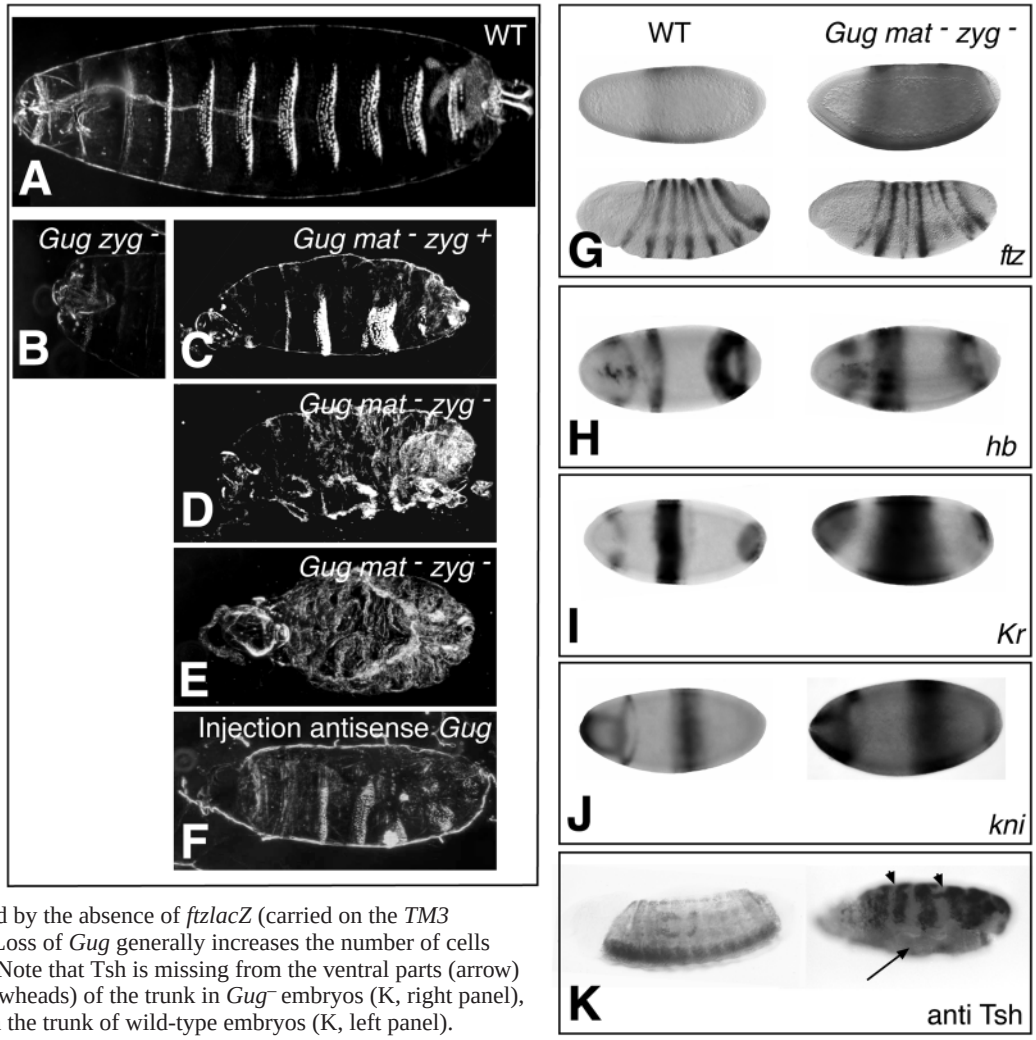

Fig. 3. Loss of maternal *Gug* activity affects embryonic segmentation and Tsh expression. (A) Ventral view of a wild-type larva showing the 11 similar trunk segments each with alternating denticled and naked cuticle. (B) Detail of the head region of an embryo homozygous for Gug^{35}. (C-E) Larvae from Gug^{35} germline clones fertilised by wild-type (C) or Gug^{35} (D,E) sperm. (C) Note the reduced number of segments compared with wild type. (D) Note the holes in the cuticle or the absence of the ventral cuticle (E). (F) Phenocopy of the *Gug* segmentation phenotype after injection of antisense *Gug* mRNA into embryos (compare with C). Expression of the segmentation genes *ftz* (G), *hb* (H), *Kr* (I), *kni* (J) and the region-specific homeotic protein Tsh (K) in wild type (left) and *Gug* mutant (right) embryos. *Gug/Gug* zygotes were distinguished by the absence of *ftzlacZ* (carried on the *TM3* balancer chromosome) expression. Loss of *Gug* generally increases the number of cells expressing the segmentation genes. Note that Tsh is missing from the ventral parts (arrow) and in stripes in dorsal regions (arrowheads) of the trunk in Gug^{-} embryos (K, right panel), where Tsh is uniformly expressed in the trunk of wild-type embryos (K, left panel).

(Fig. 3C). In the absence of maternal and zygotic *Gug* activity, embryos lack ventral pattern elements (Fig. 3D,E) and exhibit holes in the ventral cuticle.

In order to verify if the transcribed region shown in Fig. 2 corresponds to *Gug*, we have injected sense or antisense *Gug* RNA into wild-type preblastoderm embryos. Sixty-two percent of embryos injected with antisense RNA gave rise to phenocopies of the *Gug* mutant phenotypes [32% with segmentation defects (Fig. 3F) and 30% with ventral holes in the cuticle see Fig. 3B-E; n=115]. Injection of sense *Gug* RNA produced no defects (n=135). These results suggest that *Gug* corresponds to the transcript shown in Fig. 2.

To understand how loss of Gug activity affects segmentation, we analysed the expression of *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*) and *fushi tarazu* (*ftz*) in embryos derived from Gug^{35} germline clones fertilised by Gug^{35} sperm. In wild-type embryos, the expression of these segmentation gene products localise to discrete domains in the early embryo (Fig. 3G-J left) (reviewed by Rivera-Pomar and Jackle, 1996). In almost all of the expression domains, loss of Gug activity increases the number of cells expressing these segmentation genes (Fig. 3G-J right) suggesting that Gug plays a role in their repression. Later the expression of *ftz* displays a more complex defective pattern with some stripes being broader, and others narrower, than wild type (Fig. 3G).

Loss of Gug activity also affects the distribution of the Tsh protein (Fig. 3K). In wild-type embryos at the germ band retraction stage, Tsh is expressed evenly in trunk segments (left) and not the head or tail (Alexandre et al., 1996). In Gug^{-} embryos (right), Tsh is expressed in the trunk but is lost from ventral regions (arrow) and is expressed in a striped pattern in the dorsal part of the embryo (arrowheads). These results suggest that Gug is a regulator of the *tsh* gene during embryogenesis.

Grunge encodes for a protein similar to human RERE

Sequence analysis of two overlapping cDNAs reveals an open reading frame encoding for a putative protein of 1966 amino acids (Fig. 4A; GenBank Accession Number, AF217844). The putative Gug protein has closest similarity to human arginine-glutamic acid dipeptide repeat (RERE) protein, an Atrophin-1-related protein (Seki et al., 1997; Yanagisawa et al., 2000) (Fig. 4B-D). Distinct domains of this protein also show similarity with vertebrate Atrophin-1-related and with the Metastasis-associated (Mta)-like proteins (Fig. 4C,D). Atrophin-1 and Atrophin-1-related proteins are found in mice, rats and humans. Human Atrophin-1 contains a poly-glutamine repeat, which is expanded in individuals with a dentatorubral-

pallidoluysian atrophy (DRPLA), resulting in neuronal apoptosis (reviewed by Kanazawa, 1998). The normal function of Atrophin-1 is not known. Atrophin-1 and the human Atrophin-1-related (RERE) protein are similar in the C-terminal half of each protein (60% identity), but RERE has no poly-glutamine stretch. Gug contains two poly-glutamine stretches (grey in Fig. 4A) and has a conserved C-terminal box found in Atrophin-1 and Atrophin-1-like proteins (Fig. 4D; orange in Fig. 4A). Human RERE exhibits weaker identity in a second region of Gug, extending from amino acid 334-513 (green box Fig. 4A,B). This domain is also conserved in vertebrate Atrophin-1 proteins but is less extensive (not shown). Another weak region of homology is found between Gug and mouse Atrophin-1 (30% identity, 43% homology) (purple box Fig. 4A,B) and rat Atrophin-1-related (22% identity; 30% similarity); this domain is not found in human RERE.

An N-terminal region of Gug shows homology with the *C. elegans* protein EGL-27, which is similar to vertebrate Mta1 (Ch'ng and Kenyon, 1999; Herman et al., 1999; Solari et al., 1999) and to human RERE protein (Fig. 4B,C) (Seki et al., 1997; Yanagisawa et al., 2000). This domain (blue box in Fig. 4A,B) includes a putative DNA-binding domain called SANT (or Myb) preceded by an ELM2 homology region (Ch'ng and Kenyon, 1999; Solari et al., 1999). RERE, EGL-27 and Mta1 possess a GATA-like domain, which Gug does not (black box Fig. 4B), and RERE has a BAH (bromo adjacent homology) domain, unlike Gug, at the extreme N-terminal end. Mta1 is thought to be required for normal chromatin structure as it associates with components possessing histone deacetylase and nucleosome remodelling activities (Xue et al., 1998). EGL-27 is a nuclear protein and is required with Hox and Wnt signalling components for normal cell migration and polarity. EGL-27, like Gug, has polyglutamine repeat regions (Ch'ng and Kenyon, 1999; Herman et al., 1999; Solari et al., 1999). Finally, Gug possesses three putative nuclear localisation signals, one of which overlaps the SANT domain (yellow, Fig. 4A). These observations suggest that Gug plays a role in the nucleus.

Grunge protein is localised in the nucleus

To analyse the cellular localisation of Gug, we raised an antibody to the putative Gug product (see Materials and Methods). During embryogenesis, this antibody recognises epitopes localised in the nucleus in all cells (Fig. 5A,B), although not within the putative mitotic domains (asterisks in Fig. 5B). To verify that the protein corresponds to Gug, we analysed the distribution of this antibody in embryos and in tissues mutant for different *Gug* alleles. In embryos derived from *Gug* germline clones, nuclear staining was not detected, as in wild type (Fig. 5C,D). We also induced Gug^{35} clones (marked by loss of GFP) in the imaginal discs and analysed the expression of Gug. Staining was significantly reduced in the clones (Fig. 5E,F) compared with wild-type GFP^{-} control clones (not shown). At present, we do not know if the antibody is specific to Gug but our results show that *Gug* mutations affect the distribution of a protein detected by the antiserum. Taken as a whole, these results suggest that the *Gug* alleles correspond to loss-of-function alleles that affect the function of the protein depicted in Fig. 4.

Grunge is required for the patterning and morphogenesis of the ventral parts of legs

To analyse the function of the *Gug* locus in the leg, clones of cells homozygous for Gug^{35} were induced at different stages of development. Mutant *Gug* clones were found in all parts of the leg with a frequency similar to that of control Gug^{+} clones showing that Gug^{+} function is not required for cell viability. Mutant and control clones were always restricted to the anterior or posterior compartment (Steiner, 1976), and never changed the overall segmental identity of the legs.

Differential behaviour of Gug^{-} clones is observed along the dorsoventral axis of the legs. Mutant cells located in dorsal or lateral parts of the leg give rise to essentially wild-type patterns (Fig. 6A,C), although they exhibit a slight cell autonomous increase in bristle density, compared with wild type (Fig. 6B).

By contrast, *Gug* clones, which occupy any ventral part of the leg, delete specific pattern elements and replace them with patterns that resemble those formed in more lateral distal regions of the leg. Gug^{-} clones delete ventral-specific patterns in both the anterior and the posterior compartments. For example, the large ventral bristles of the posterior compartment in the femur of the first leg are not produced (compare Fig. 6D with Fig. 6C). The apical bristle at the distal tip of the anterior tibia (not shown), the spur bristles at the tip of each tarsal segment (Fig. 6E), and the transverse row and sex comb bristles of leg 1 (Fig. 6F; compare with Fig. 6G) never develop in such clones. Ventrally located *Gug* clones in posterior or anterior compartments fuse the femur to the tibia (Fig. 6D,H), which reflects a defect in the leg-specific morphogenetic process that separates these segments during pupation (Fristrom and Fristrom, 1993).

Grunge specifies the proximal identity of legs

Large Gug^{-} clones located in the coxa, trochanter or proximal femur, irrespective of their provenance in the anterior or posterior compartment, lead to fusion of these segments. Pattern elements, which are associated with clones and in neighbouring cells, were replaced with those found in more distal parts of the legs. That is Gug^{-} clones in these proximal parts generally bear bracts (Fig. 7B), as do bristles located more distally (Fig. 7A). Clones situated in dorsal regions do not affect proximal identity (Fig. 6A). However, proximal clones, which occupy a large region of both the dorsal and ventral domains, replace all patterns with more distal identities and cause a reversal of the polarity of bristles (Fig. 7B). These *Gug* clones have a non-autonomous effect on the polarity of more distally located, ventral bristles (Fig. 7B, arrowheads). Smaller clones affect patterning if they are located ventrally. Such clones lead to outgrowths forming a partial new axis (Fig. 7C). Although bristles in these outgrowths show a distal (bracted) identity, they never form a complete new leg. Outgrowths consist of Gug^{-} and Gug^{+} tissue, suggesting that *Gug* activity is crucial for normal cell communication.

Grunge is required for the expression of Tsh in the proximal-ventral leg

As Gug^{+} activity is required for the identity of proximal cells of the leg, we tested whether the expression of Tsh and Dll was affected in Gug^{-} clones. Tsh and Dll are expressed respectively in proximal and distal domains of the wild-type

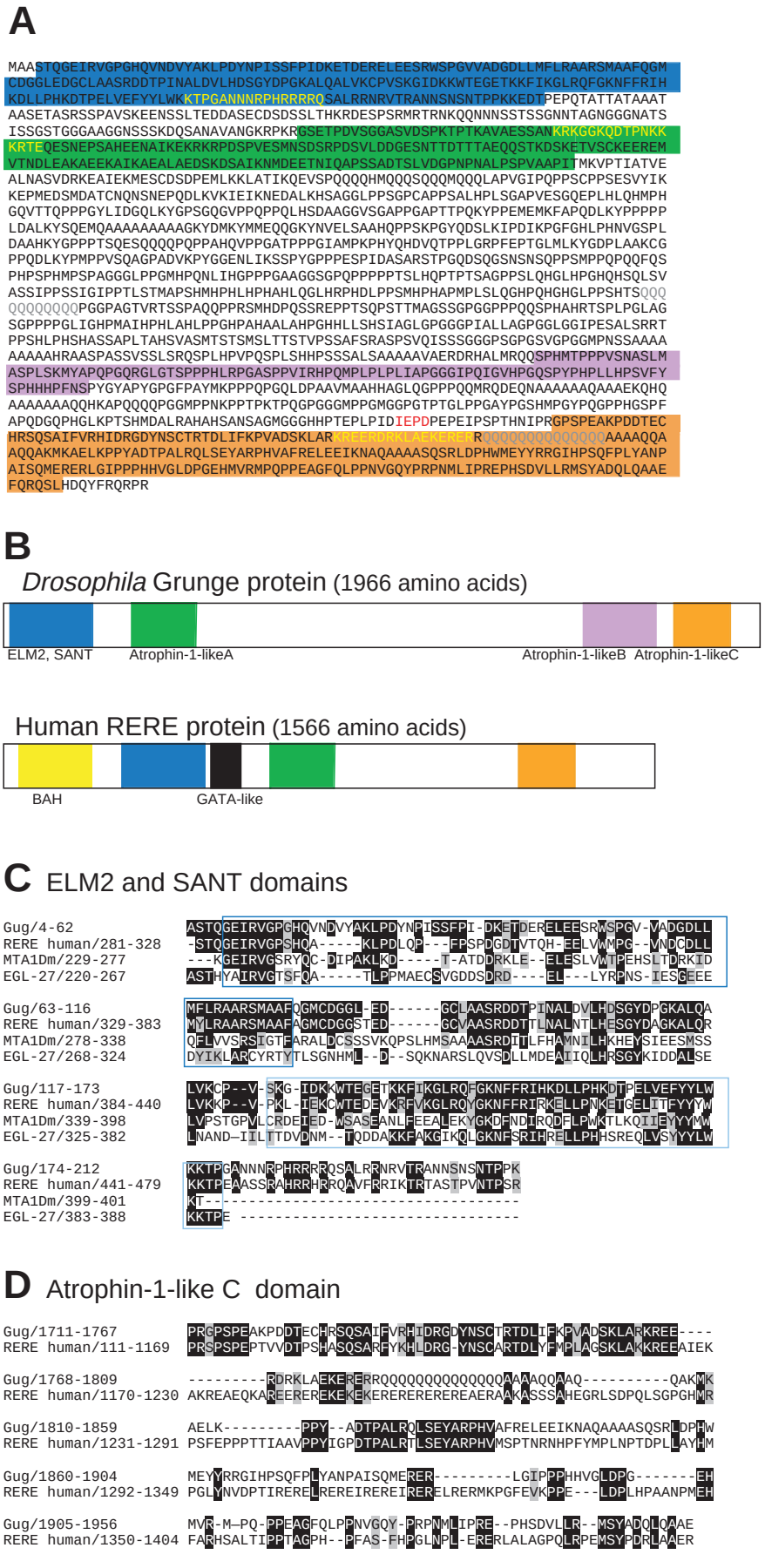

Fig. 4. The Grunge protein. (A) The predicted amino acid sequence of Grunge protein. The blue box highlights a domain similar to that found in human arginine-glutamic acid dipeptide repeat (RERE) protein (Seki et al., 1997; Yanagisawa et al., 2000) (63% identity, 66% similarity), human Mta1 (25% identity, 33% similarity), *Drosophila* Mta-1 (27% identity, 36% similarity) and EGL-27 (29% identity, 35% similarity) (see Fig. 4C). The green box corresponds to a domain with weak similarity to human RERE (20% identity, 41% similarity), human Atrophin-related (20% identity, 40% similarity) and mouse atrophin 1 (23% identity, 44% similarity). The purple box is a domain of weak homology found in mouse atrophin 1 (30% identity, 45% similarity) and rat atrophin 1 related (22% identity; 30% similarity). Finally, the orange box shows homology to human RERE (40% identity, 44% similarity), human atrophin-related (28% identity, 45% similarity) and human atrophin 1 (30% identity, 45% similarity; see Fig. 4D). The location of the two poly-glutamine (Q) stretches is indicated in grey and three putative nuclear localisation signal motifs are indicated in yellow. A putative Caspase protease site is present (IEPD, red letters). (B-D) Conserved domains in the Gug protein. Comparison of the structure of Gug and the human arginine-glutamic acid dipeptide repeat (RERE) (Seki et al., 1997; Yanagisawa et al., 2000), Atrophin-1-like, protein. Colour codes are as in A. The black box indicates a GATA-like domain and the yellow box to a brahma adjacent homology (BAH) domain present in RERE but not in Gug. The purple box is a weak domain of homology found in mouse and human atrophin 1 but not human RERE. (C) Alignment of the amino acids from the N-terminal domain of Gug compared with *Drosophila* Metastasis-associated factor (MTA1), the *C. elegans* EGL-27 protein and the human RERE, Atrophin-1-like, protein. This domain contains an ELM2 region (dark-blue outline) and a SANT DNA-binding domain (light blue outline) (Solari et al., 1999). (D) Sequence comparison of the C-terminal domains of Gug and the human RERE protein. This region has comparable levels of similarity to vertebrate DRPLA proteins (not shown). Identities are indicated by white letters on black, similarities are indicated by black letters on grey.

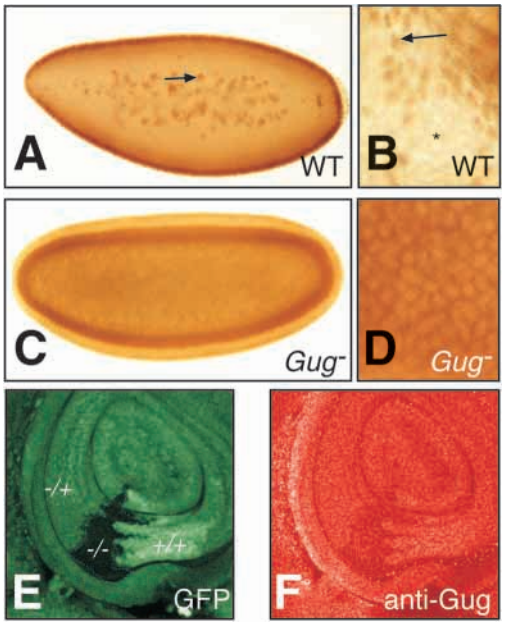

Fig. 5. *Gug*$^{-}$ tissues affect the protein distribution detected by antisera raised against the Gug protein. Anti-Gug antibody stains to embryos at blastoderm (A,C) and early gastrula stages (B,D). Wild-type (A,B) and embryos derived from *Gug*^{35} germline clones (C,D). Note that for the lower panels, staining is absent from the nuclei unlike in wild type (arrow, A). The asterisk in B indicates one of several mitotic domains where Gug is lost. Note that staining of embryos with the relevant pre-immune serum when overstained gives similar results to that shown in D. Clones of cells homozygous for *Gug*^{S2} in imaginal leg discs, detected by the absence of GFP (E), show reduced levels of staining with anti-Gug antibody (F). Clones of cells that lack *Gug*$^{+}$ products were induced at 48-72 or 96-120 hours after egg laying. Ventral is towards the bottom.

leg (Erkner et al., 1999). *Gug*$^{-}$ clones were identified by the absence of Myc epitope tag (Xu and Rubin, 1993) and Tsh expression was simultaneously monitored in third instar leg imaginal discs. In proximoventral positions, *Gug* activity is required autonomously and non-autonomously for the expression of Tsh in the leg imaginal disc (Fig. 8A, white and red lines). In dorsal or lateral positions, Tsh expression is not affected by loss of Gug activity in clones (Fig. 8A green line). In the peripodial membrane, which corresponds to the future body wall, Tsh does not depend on *Gug*$^{+}$ activity, even ventrally (not shown). In late third instar discs, Dll is expressed ectopically in such outgrowths (Fig. 8B, arrow), consistent with the observation that lack of *Gug*$^{+}$ function replaces proximal with distal cellular identities (Fig. 7B-C). Abnormal patterns of Dll expression were not observed in other parts of the legs (Fig. 8C). These experiments show that *tsh* and *Dll* expression depends directly or indirectly on *Gug*$^{+}$ activity in the proximoventral leg, confirming the crucial role of *Gug* in ventroproximal patterning of the leg.

Grunge does not affect the expression of *wg* and *dpp* except in proximal-ventral outgrowths

Gug^{-} clones lead to outgrowths in the ventroproximal region in a non-autonomous manner (Fig. 7C). Wg is known to act in the patterning of ventral cells and Dpp acts in the patterning of dorsal positions (see Introduction) (Lecuit and Cohen, 1997). Loss of Wg and gain of Dpp signalling in any part of the ventral leg produces outgrowths (Brook and Cohen, 1996; Jiang and Struhl, 1996) similar to those we describe for Gug, specifically in the proximal ventral leg (Fig. 7C). We examined *wg-lacZ* and *dpp-lacZ* expression in *Gug*$^{-}$ clones in the leg discs. When *Gug*$^{-}$ clones produce outgrowths in proximal ventral positions, *wg-lacZ* expression is diminished

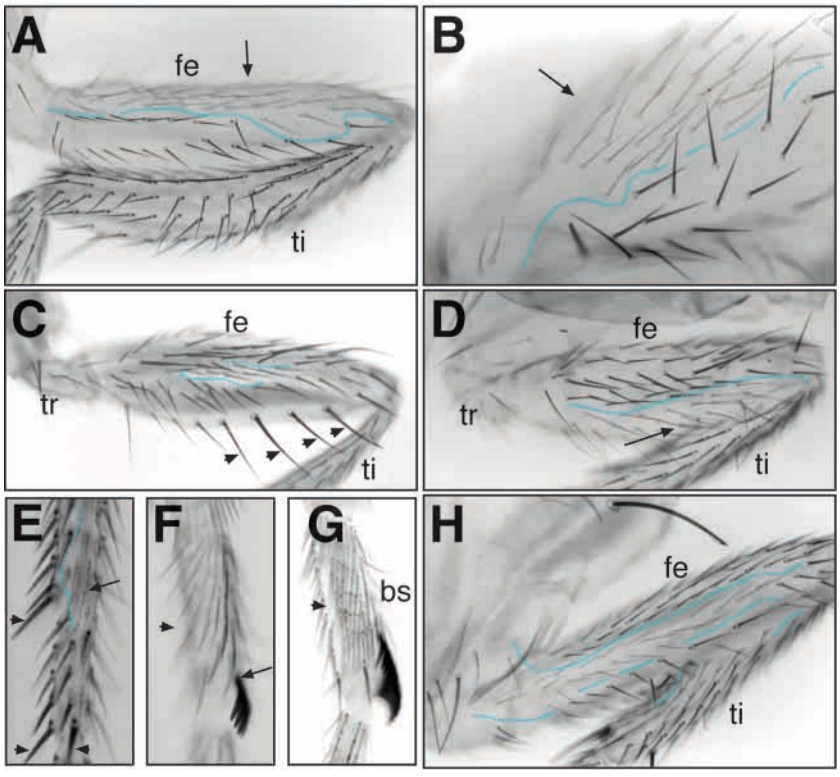

Fig. 6. *Gug*$^{-}$ clones affect ventral-specific patterning and morphogenetic events in legs. Clones were induced at 48-72 or 72-96 hours after egg laying and are marked by the *yellow* bristle marker (highlighted by the broken blue lines). (A,B) Anterodorsal clones in the femur (fe) showing normal morphology (A) and higher density of bristles (B) compared with a wild-type leg. (C) A first leg with a small posterolateral *Gug*$^{-}$ clone with little effect on leg patterning, although showing a higher density of bristles than normal. Arrowheads indicate the large bristles formed in a wild-type first leg femur, in a ventral position in the posterior compartment. (D) A posterior *Gug* clone in the ventral region of the femur. The large bristles do not form (arrow; compare with C). (E) Ventral clone in the second tarsal segment of a second leg shows the deletion of a spur bristle (arrow). In wild type, each tarsal segment has two spur bristles at the distal end (arrowheads). (F) First leg carrying a *Gug*$^{-}$ clone. The transverse rows (arrowhead; compare with the wild type in G) and the sex comb (arrow) in the basitarsus are not formed in the clone. In wild type (G), the sex comb carries 10-12 specific bristles. Here, only four bristles are made, deriving from wild-type tissue. (G) Wild-type basitarsus of a first leg, showing the ventral transverse rows. (H) Antero- and posteroventral *Gug*$^{-}$ clones in the femur-tibia region lead to fusion of these leg segments.

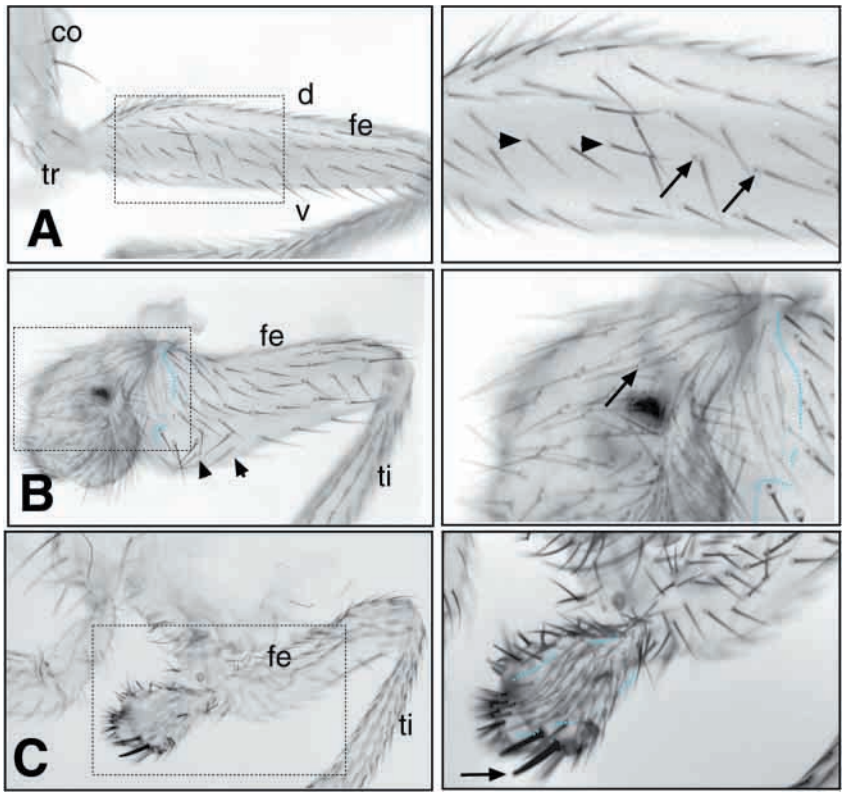

Fig. 7. Cell autonomous and nonautonomous effects of *Gug* mosaic clones for proximal identity in the leg. The right panels show higher magnifications of the panels on the left. (A) The proximal region of a second leg showing the region between bracted (arrow) and non-bracted (arrowhead) bristles in the femur. Distal to this border, almost all bristles have bracts. (B) A proximal $Gug^{-}M^{+}$ clone filling a large region of the anteroproximal part of a second leg. Coxa, trochanter and proximal femur are replaced with unpatterned leg tissue. Bristles show more distal (arrows) identity. Note that bristles change their polarity in a autonomous and non-autonomous (arrowhead) manner. (C) A clone, induced at 120-144 hours, where a secondary leg axis protrudes from the ventral region of the proximal femur and trochanter region. The ectopic leg is incomplete, consisting of mosaic *y* Gug^{-} and Gug^{+} cells. Gug^{+} cells in the ectopic leg have formed large bristles (arrow) typically found in the distal leg. We believe that these bristles have dorsal identity that represents the preapical bristle of the tibia.

(Fig. 8D) and *dpp-lacZ* (Fig. 8F) is expressed ectopically. In more distal leg parts or in proximal clones that lack outgrowths, Gug^{-} clones had no effect on the expression of *wg-lacZ* (Fig. 8E) or *dpp-lacZ*. Similarly, no effect of loss of Gug activity was observed on the expression of Wg signalling target genes H15 (not shown) (Brook and Cohen, 1996) and *Dll* (Fig. 8C) or on the expression of the Dpp signalling target gene *omb* (not shown) (Grimm and Pflugfelder, 1996). We conclude that even though Gug^{+} activity acts in the patterning of ventral cells of the leg, this effect is not due to a deregulation of the *wg* or *dpp* genes, except in a proximoventral position.

DISCUSSION

We describe the characterisation of a regulator of *tsh*, called *Gug*, which is produced in all cells and codes for a protein with similarities to vertebrate Atrophin-1-like and Metastasis-associated proteins. The *tsh* gene is transcribed specifically in the cells of the proximal part of the leg imaginal discs (Erkner et al., 1999), where it is necessary for normal proximal development. *Gug* determines the global identity of the proximal leg and acts as a positive regulator of *tsh* specifically in ventroproximal cells. Additionally, we show that Gug activity is required along the entire proximodistal leg axis especially in ventral leg cells. Tsh also acts in the trunk segments of the embryo (Fasano et al., 1991). Gug activity is required for the normal repression of four segmentation genes known to be required for regulation of *tsh* during embryogenesis (Coré et al., 1997; Röder, 1992).

Grunge has motifs with similarity to Atrophin-like and Metastasis-associated protein families

Atrophin-1, which shares homology with Gug, has been implicated in the neurodegenerative disease dentarubral-pallidoluysian atrophy (DRPLA). This pathology results from expansion of glutamine repeats (reviewed in Kanazawa, 1998). Whereas the Gug protein is the only known *Drosophila* member of the Atrophin family of proteins, vertebrates have at least two Atrophin proteins and several Atrophin-like members. The closest relative to Gug is the Atrophin-1-related protein called human arginine-glutamic acid dipeptide repeat (RERE) protein (Seki et al., 1997; Yanagisawa et al., 2000), which has similarity to both the Atrophin-1 and Metastasis-associated (Mta) families of proteins (Fig. 4). Two glutamine repeat regions are found in Gug (Fig. 4A) that are not found in human RERE. Gug, human RERE, human Mta1 and *C. elegans* EGL-27 proteins possess a homologous ELM2 SANT domain located at the N terminus (Fig. 4B,C). Mta1 is thought to be required for normal chromatin structure, as it associates with histone deacetylase and has nucleosome remodelling activities (Xue et al., 1998). Additionally, Mta1 is upregulated in metastatic carcinomas. Interestingly, analysis of *egl-27* mutations in *C. elegans* reveals that EGL-27 has a function in common with the Wnt pathway (Herman et al., 1999), as we describe for Gug. The presence of a SANT-like DNA-binding domain (Fig. 4B,C), three putative nuclear localisation motifs (Fig. 4A) and its nuclear localisation (Fig. 5A,B) suggest that Gug acts as a transcriptional regulator.

Grunge is required for embryonic segmentation

Loss of Gug activity severely affects the process of segmentation and the expression of segmentation genes when

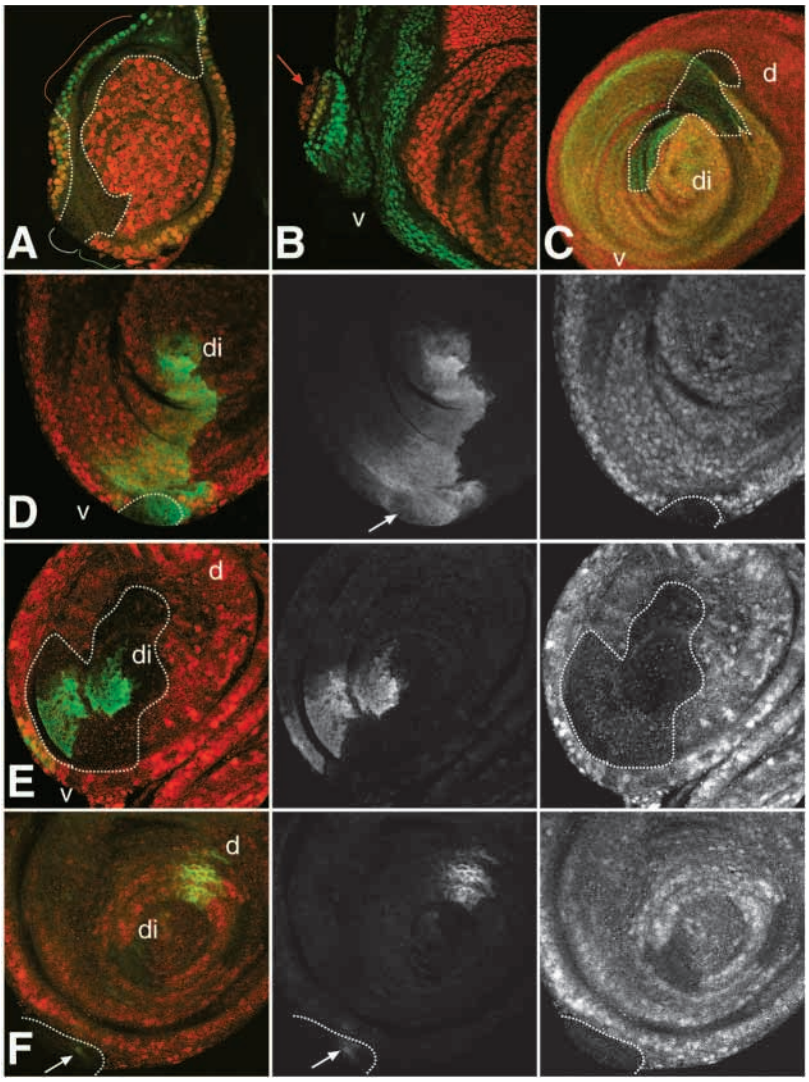

Fig. 8. Gug activity is required for regulation of *teashirt* (*tsh*). Clones of *Gug*$^-$ cells in imaginal leg discs, detected by the absence of the Myc epitope. (A) Clone, showing a ventral outgrowth in the proximal, ventral region. Overlapping expression of Tsh and Myc is shown in yellow. Tsh is absent in ventral *Gug*$^-$ cells (white line) and in wild-type cells (red line) adjacent to the clones. In dorsal clones, Tsh is expressed normally as shown by the green staining (green line). (B) Tsh (green) and Dll (red) expression in a late third instar disc showing a small secondary axis. Distalisation is indicated by the ectopic Dll expression in this new axis (arrow). Note that this disc is shown at twice the magnification. (C) Distal (ventrally and dorsally) clone marked by the absence of Myc (red); Dll (green) is expressed normally in the clone. (D) Wg-*lacZ* expression (green and middle panel) is reduced in a small ventral outgrowth (arrow) induced by a *Gug*$^-$ clone (absence of the red staining; right panel). (E) Wg-*lacZ* expression (green and middle panel) is normal in a distal ventral clone (absence of the red staining and right panel). Note that there must be at least two clones, one in the anterior and another in the posterior compartment. (F) Dpp-*lacZ* (green and middle panel) is expressed weakly in some *Gug*$^-$ cells in a ventral outgrowth (arrow; right panel).

missing from the female germ line (Fig. 3). At the blastoderm stage, most of the expression domains of *hb*, *Kr*, *kni* and *ftz* genes are expanded compared with wild type. These observations indicate that maternal production of Gug is crucial for the repression of these genes to precise domains in the early embryo. Gap proteins, including Hb, Kr and Kni are known to be required to restrict each others domains of expression (Rivera-Pomar and Jackle, 1996). It will be interesting to test if Gug acts with these proteins for these repression activities.

Loss of gap gene products and especially the pair rule product *ftz* affects the normal regulation of *tsh* (Coré et al., 1997; Röder, 1992). Ftz acts as a positive and probably direct regulator of *tsh*. Loss of Gug activity does not effect the location of Tsh to the trunk segments of the embryo but Tsh expression is affected (Fig. 3K).

Grunge and patterning of the proximal parts of the legs

One striking feature of *Gug*$^+$ function is its role in the formation of proximal specific patterns of the leg (Fig. 7). Loss of *Gug*$^+$ activity in proximal ventral cells changes bristle polarity and replaces proximal with more distal cellular identities. Thus, patterns typical of the coxa, trochanter and proximal femur are replaced with leg tissue that partially resembles that found in more distal femur or tibia. These effects resemble those seen in clones of cells lacking Extradenticle or Homothorax activities (Gonzalez-Crespo and Morata, 1996). As *Gug*$^+$ activity is also crucial for ventral patterning of the leg, the proximal-to-distal change is never complete. *Gug* mutant clones also affect cell communication in the proximal leg, as they exhibit cellular non autonomy causing neighbouring wild-type tissue to differentiate distal patterns in proximal positions (Fig. 7C-D, Fig. 8B).

The role of *Gug* in patterning the proximal leg is shown at the molecular level, where *tsh* requires positive input from the *Gug* gene specifically in ventral proximal parts of the leg imaginal disc (Fig. 8A). Loss of *Gug* results in ectopic expression of Dll in this position (Fig. 8B). As Gug is ubiquitously produced in the leg (Fig. 2F, Fig. 5F), proximodistal specificity of Gug function presumably derives from other proteins located in proximoventral parts. Recently, we showed that Dll and possibly Tsh act as mutual repressors only in cells where Wg is signalling (Erkner et al., 1999). *Gug* may normally be required for this process (Fig. 6, Fig. 7A-D).

Grunge is required for ventral-specific patterning and morphogenesis of the leg

Gug activity is essential for patterning the ventral parts of the leg along the entire proximodistal leg axis (Fig. 6). Loss of Gug in dorsal or lateral parts has no drastic effect on patterning (Fig. 6A-C), although the number of bristles is augmented in *Gug* mutant cells irrespective of dorsoventral position (Fig. 6B,C).

Ventrally in the femur-tibia region, loss of ventral Gug activity causes the fusion of these leg segments (Fig. 6H). During early pupariation, a sack of cells is known to give rise

to the femur and tibia. Ventrally situated cells then migrate to meet and separate the femur and tibia (Fristrom and Fristrom, 1993). If Gug is missing in these migrating groups of cells, the femur and tibia remain attached (Fig. 6D,H). *Gug* mutant clones also affect the process of segmentation of the tarsus (Fig. 6E-G). Similar defects on the morphogenesis of the femur-tibia and tarsus have been observed in clones lacking components of the Notch signalling pathway (de Celis et al., 1998). The relationship between Gug and Notch signalling activities will be reported elsewhere.

The normal ventral patterning of the legs is specifically under the control of the Wg signalling cascade of molecules (reviewed by Wodarz and Nusse, 1998); thus, there is a correlation between the domains where Wg signalling occurs and where Gug is active. Furthermore, both Gug and Wg signalling act in domains where *wg* is transcribed and where Wg is secreted (for example, in the posterior ventral part of the leg) (Fig. 6D-G).

Although Wg and Gug act in the same domains of the leg with similar roles, they exhibit distinct functions. Gug seems to act in a fraction of Wg-dependent developmental events. First loss of Wg signalling induces a novel axis in ventral leg parts, irrespective of proximodistal position. *Gug*$^{-}$, however, induces bifurcated legs only if its activity is removed from proximal ventral parts of the leg. Contrary to the loss of Wg signalling, *Gug* mosaics do not distalise bifurcated legs properly, presumably because Gug activity is required for this process (Fig. 6, Fig. 7). Finally, Gug replaces proximal tissue with distal patterns (Fig. 8B, Fig. 7B,C); loss of Wg signalling never produces such homeosis (Brook and Cohen, 1996; Jiang and Struhl, 1996). These observations suggest that Gug functions are related to those controlled by Wg signalling but are more specialised. This specialisation may reflect the fact that Gug controls the expression of *tsh*, which is required to modulate Wg signalling activity (Gallet et al., 1998; Waltzer et al., 2001).

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