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klumpfuss, a *Drosophila* gene encoding a member of the EGR family of transcription factors, is involved in bristle and leg development

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SUMMARY

The *klumpfuss* (*klu*) transcription unit in *Drosophila* gives rise to two different transcripts of 4.5 and 4.9 kb, both of which encode a putative transcription factor with four zinc-finger motifs of the C_2H_2 class. Zinc-finger 2-4 are homologous to those of the proteins of the EGR transcription factor family. As in the case of the most divergent member of the family, the *Wilms' tumor suppressor gene* (*WT-1*), *klu* contains an additional zinc finger, which is only distantly related. Loss of *klumpfuss* function is semilethal and causes a variety of defects in bristles and legs of adults, as well as in mouth hooks and brains of larvae. Analysis of the mutants indicates that *klumpfuss* is required for proper specification and differentiation of a variety of cells, including the sensory organ mother cells and those of the distal parts of tarsal segments.

Key words: *klumpfuss*, *Drosophila*, EGR family, transcription, bristle, leg, *Wilms' tumor suppressor gene* (*WT-1*)

INTRODUCTION

In *Drosophila*, selection of sensory organ progenitor cells (SOP) within proneural clusters (Ghysen and Dambly-Chaudiere, 1989; Romani et al., 1989) depends on a signal network formed by products of the proneural and neurogenic genes (see Campuzano and Modolell, 1992; Ghysen et al., 1993, for reviews). Proneural genes, especially the genes *achaete* (*ac*) and *scute* (*sc*) of the *achaete-scute* complex (AS-C), initially confer on the proneural clusters neurogenic capabilities (Cubas et al., 1991); lateral inhibition mediated by the neurogenic genes restricts proneural gene activity to one or a few cells in each cluster, thus determining which cells will eventually follow the neural pathway. Additional proneural genes act in internal sensory organs [*atonal* (*ato*; Jarman et al., 1993) and within the anterior margin of the wing *asense* (*ase*, Dominguez and Campuzano, 1993)]. The proneural genes encode bHLH proteins (Villares und Cabrera, 1987; Gonzalez et al., 1989; Jarman et al., 1993) which, as ectopic and overexpression experiments show, are functionally redundant (Rodriguez et al., 1990; Dominguez und Campuzano, 1993; Jarman et al., 1993; Hinz et al., 1994).

During the lifetime of a proneural cluster, there is a fixed order of events leading to the selection and specification of SOPs (Cubas et al., 1991). Within a cluster of about thirty cells, approximately six accumulate higher levels of ACHAETE and SCUTE proteins. One of these cells is finally selected to become the SOP and continues to accumulate proneural proteins, whereas the concentration of these proteins in the adjacent cells decreases. Apart from the participation of *achaete* and *scute*, very little is so far known about the mechanisms of SOP specification within a proneural cluster. The activity of proneural genes is followed by that of so-called neural precursor genes (Brand et al., 1993), such as *deadpan* (Bier et al., 1992), *couch potato* (Bellen et al., 1992) and *asense* (Brand et al., 1993), which are expressed in most or all neural progenitor cells and control differentiation steps of these cells.

The EGR family of transcription factors is defined by a set of three C_2H_2 zinc finger DNA-binding domains. At present the family is represented by six members, identified in various species**:** EGR1-3, hpath133 (also named EGR4), the Drosophila gene *stripe* (*sr*) (Lee et al., 1995; Frommer et al., 1996) and the Wilms' tumor-associated protein WT-1 (Mueller et al., 1991; see Madden and Rauscher, 1993, and Miyagawa et al., 1994 for reviews). WT-1 differs from the other members of the family by having an additional, rather divergent zinc finger domain. Moreover, unlike the other members of the family, WT-1 encodes four proteins by alternative splicing (Haber et al., 1991). Two of the WT-1 proteins bind to the same DNA target sequence as the EGR proteins and, unlikely these, seem to suppress transcription of target genes (Madden and Rauscher, 1993).

Here we describe a new *Drosophila* gene, which we have called *klumpfuss* (*klu*) meaning club-foot, on the basis of leg defects found in mutants. *klumpfuss* encodes a protein with four zinc finger motifs of the C_2H_2 type, three of which are homologous to those of the proteins of the EGR transcription factor family while the fourth resembles the divergent zinc finger of WT-1. Mutations in *klumpfuss* cause a variety of defects, among them loss of bristles and tarsal segment fusion. During bristle development, *klumpfuss* is required to specify an epidermal cell as an SOP, as well as for bristle differentiation. We show that the leg defects arise because the cells in the distal part of the tarsal segment enter apoptosis, probably due to a failure to

correctly specify their fate. The analysis of the bristle and leg mutant phenotypes suggests that like WT-1, *klumpfuss* also plays a role at the onset of differentation processes.

MATERIALS AND METHODS

Mutants and markers

Table 1 lists the *klu* alleles and *klumpfuss* deficiencies used in this study. In addition, the following mutations and deficiencies were used: *ase*^{1}, *sc*$^{10.1}$, *ac*^{3} and *Hw*^{49c} all maintained over an *FM7c*$^{ftz\text{-}lacZ}$ balancer. Mutations on the third chromosome were balanced over *TM6b*, which carries *Tubby*, allowing one to recognize homozygous larvae and pupae. Double mutant larvae were scored using the markers *y* and *Tubby*. P764 (kindly provided by Marc Haenlin) is a P-*lacZ* insertion in the genomic region of *klumpfuss*.

To analyse the bristle defects, we used A293.1M3 (Blair et al., 1992), an enhancer trap line that expresses *lacZ* in all cells of the sensilla; A101.F3, which drives *lacZ* expression in SOPs shortly after their generation (Boulianne et al., 1991; Huang et al., 1991); and A37, in which the socket cells show selective expression (Huang et al., 1991; Blair et al., 1992). These insertions were each placed in a *klu*$^{-}$ background carrying the amorphic allele *klu*^{212lR51C} and the deletion *klu*^{XR19}, and the wings and thorax of the mutant adults were subjected to X-gal staining. We also used A109, which carries an *achaete* promoter-*lacZ* fusion (Martinez et al., 1993). For the analysis of the leg defects, we used as markers P-(*odd*), an enhancer trap insertion in *odd-skipped*, which is expressed in the 1st and 5th tarsal segments (Cohen, 1993), and a P-lacZ insertion in the gene *disconnected (disco)*, expressed in concentric stripes in the distal region of the tarsal segments and the tibia (Cohen, 1993).

UAS-l'sc, *patched-Gal4* and *klu*^{G410}, a P-Gal4 insertion in *klu*, were kindly provided by Uwe Hinz (Hinz et al., 1994). The UAS-GFP insertion was kindly provided by G. Boulianne (Yeh et al., 1995) and recombined onto the *klu*^{G410} chromosome. Other genes used in this study are described in Lindsley and Zimm (1992). Flies were cultured, and egg and pupae collections made, under standard conditions.

Antibody and X-gal staining was done according to standard protocols. We used anti-ACHAETE (Skeath and Carroll, 1991), 22C10 (Fujita et al., 1982), 44C11 (Bier et al., 1988), BP104 (Hortsch et al., 1990) and anti-β-galactosidase (Cappel, USA) antibodies. For detection of Green Fluorescent Protein, the FITC filterset was used on the Zeiss Axiophot microscope. The FITC-conjugated secondary antibody were purchased by Jackson Immuno Research Laboratory Inc.). Acridine-orange staining was performed according to Masucci et al. (1990). In situ hybridizations were conducted on whole mounts according to the protocol of Tautz and Pfeifle (1989).

Molecular methods

Southern and northern blot analyses were conducted according to standard procedures (Sambrook et al., 1989). For isolation of the genomic clones, two EMBL-4 libraries were screened (Pirotta et al., 1983; M. Noll, for reference, see C. Klambt, 1993). For isolation of cDNA clones, we used the pNB40 embryonic library (4-8 hours) of Brown and Kafatos (1988). Colony screening followed standard protocols (Sambrook et al., 1989). For sequencing, the USB Sequenase kit was used according to the protocol (USB).

To obtain the UAS- *klu*^{myctag11B} constructs, a 5′ *Eco*RI site was introduced into CNB4 by PCR of the region extending from the 5′ end of *klu* up to the unique *Bgl*II site with the following primers: 5′GAATTCAAGCTTGAATTCCAATAACGATCGGCGCGT and 3′ATCGCTGCAGATCTGGCA. The amplified fragment was digested with *Eco*RI and *Bgl*II. To introduce the myc tag at the 3′ end, PCR was conducted from the 3′ end of the translated region up to the unique *Nsi*I site in CNB4, with the following primers: 5′AGCAGATGCATCATTC; 3′GAATTGCGGCCGCTTCAGGTCTTCCTCGCTGATCAGCTTCTGCTCTGTCCAGCAGCGAATGGGAAC (the sequence enconding the myc-epitope is underlined). In addition, the last primer includes a *Not*I site for convenient cloning. The amplified PCR fragments, together with the central part of CNB4 (*Bgl*II – *Nsi*I fragment), were ligated into the *Eco*RI/*Not*I-digested pUAST vector (Brand and Perrimon, 1993), the structures were verified by sequence analysis, and the constructs were injected into flies. Several transformants were recovered and tested with various Gal4 activators. Based on this characterization, UAS-*klu*^{myctag11B} lines were chosen for the rescue experiments.

RESULTS

klumpfuss was first identified by the β-galactosidase expression pattern associated with two *lacZ*-P-element insertions, *klu*^{P212} und *klu*^{P819} (A. Beermann, C. Schulz and J. A. Campos-Ortega,

Table 1.

allele	mutagen	cytology	phenotype	reference
klu^{212lR63}	transposase	n.d.	strong	a
klu^{212lR51C}	transposase	normal	strong	a
klu^{G410}	P-Gal4 Insert	n.d.	intermediate	a
klu^{819R5}	transposase	normal	intermediate	a
klu^{212lR14}	transposase	normal	intermediate	a
klu^{212lR40}	transposase	normal	intermediate	a
klu^{819lR53a}	transposase	n.d.	weak	a
klu^{212lR67b}	transposase	n.d.	weak	a
klu^{212lR13}	transposase	n.d.	weak	a
klu^{212lR13c}	transposase	n.d.	weak	a
klu^{819lR29}	transposase	n.d.	weak	a
klu^{212lR17}	transposase	n.d.	weak	a
klu^{212lR52b}	transposase	n.d.	weak	a
klu^{212lR25}	transposase	n.d.	weak	a
klu^{P212}	P-lacZ insert	normal	weak	a
klu^{P819}	P-lacZ insert	normal	weak	a
klu^{XR19}	X-rays	Df(3L)68A	strong	a
klu^{XR17}	X-rays	Df(3L)67E; 68B	strong	a
Df(3L)*vin2*	X-rays	67F2; 68D6	strong	b
Df(3L)*vin5*	X-rays	68A2-3; 68F3-6	strong	b
Df(3L)*lxd*^{2}	X-rays	68A2-3; 68C5-7	strong	b
Df(3L)*lxd*^{8}	X-rays	68A2-3; 68A5-6	strong	b

a, This study; b, Lindsley and Zimm, 1992.

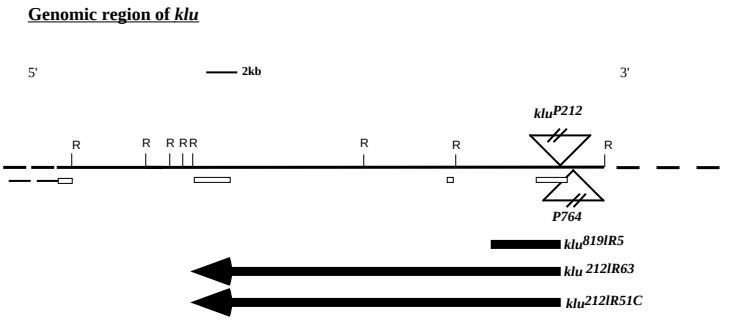
unpublished data). In these lines, β-galactosidase is strongly expressed in neuroblasts of the procephalon and of the trunk from stage 9 onwards, as well as in imaginal discs. Homozygotes for *klu*^{P212} show a characteristic fusion of tarsal segments (see below), which gives the locus its name, and various defects in the development of the imaginal sensory organs.

Molecular analysis of *klumpfuss*

The P-element insertion site in *klu*^{P212} was cloned by standard plasmid rescue of the P-lacW plasmid (Bier et al., 1989). The 2.8 kb of flanking DNA obtained hybridised to the 68A region and was used to screen two genomic EMBL-4 phage libraries (see materials and methods). A total of 52 kb of genomic DNA around the insertion sites was cloned (Fig. 1A). Southern-blot analysis of genomic DNA from *klu*^{P212} and *klu*^{P819} suggests that the P-element inserted at the same site in both cases. An 9.6 kb genomic *Eco*RI fragment including the insertion site was labelled with digoxigenin and used for in situ hybridization to embryonic wholemounts. Since the observed pattern corresponds to the β-galactosidase pattern seen in the insertion lines (see below), this fragment was used to screen the pNB40 cDNA library (Brown and Kafatos, 1988). Three overlapping cDNAs (CNB4, of about 3.8 kb, CNB8, of 2.7 kb and CNB2, of 1.7 kb) were obtained from a total of 400 000 clones screened. The largest, CNB4, hybridised at four different sites distributed over about 40 kb of the genomic walk and revealed that the 5′ part of the transcription unit is missing. The smaller cDNAs (CNB2 and CNB8) have an additional *Sst*1 site, relative to CNB4. The significance of this polymorphism is not clear at the moment. Sequencing showed that CNB4 includes a large open reading frame (see below). Various genomic fragments and the CNB4 cDNA were radioactively labelled and hybridized to a northern blot prepared with total RNA from the various embryonic stages. Two major transcripts, of 4.5 and 4.9 kb, were detected from early stages on, peaking at about 10-12 hours.

Attempts were made to map restriction enzyme polymorphisms in genomic DNA of *klu*^{P212} and *klu*^{212lR25}, *klu*^{819lR5}, *klu*^{212lR14} and *klu*^{212lR40}, *klu*^{212lR63}, *klu*^{212lR51C} and the cytologically visible deficiency *klu*^{XR19}. In addition, we also mapped P764, a P-insertion in the *klumpfuss* region that has no obvious abnormal phenotype when homozygous. The strongest hypomorph *klu*^{819lR5} carries a 4.5 kb deletion, which removes the region containing the last, non-coding exon. In situ hybridisations with CNB4 revealed that, while the deletion does not affect the translated region of the transcript, the expression of the gene is strongly reduced (Fig. 1A). This suggests that, in addition to the one exon, regulatory regions are also affected. The amorphic alleles *klu*^{212lR63} and *klu*^{212lR51C} carry deletions with one breakpoint at the insertion site; the position of the second breakpoint could not be determined owing to the presence of repetitive sequences within the cloned genomic region (Fig. 1A). Since no transcripts could be detected with the longest cDNA in hybridizations to *klu*^{212lR51C} mutant discs, the complete transcription unit must be deleted

A



B

MTMAEGTDPMGHGHHTNPHNPPQQPPQHHPHHLPLPHPPPAPPPQLLNFNHHQQQYLPHMYPAAAA

AAQSHSHYGGLQYPHPHALQHQHHPHQHQHHLPLALSLHQQAAAAAAVAAAVATNHSNNNNQASVA

NNNIVVPWKQ*RKRKR*LSAVLDKLHHNNNNNNNNNNETHSNGMACKAEPMEMKGDLAEDAEQDEDHD

GSVPDEEDDSVDHDGDAGKYIKSEQPVSEDEENEQEMDQDIEDISGEDLELSHSDDNDQDDSPRIS

MSPLQLQAAQSNLPQDQNQPMNKENPLHVDIKTEIPSPYDRYFPIPSPLFGYYLHTKYLNEVF*RRR*

*H*DLYPSPLQHTPSSIASETETSPNAQERKSSSNHVLPHALLANNSPPPSLPSPPRSESSVTNNVAT

TTTSSTTKKRTSPKPKGKKGDKKPMPPPQERPLDLCMRNEVEPKKYKKSGSKSSLESRSAGMMPPP

PALSAASSLESMSALSPASSSHSGHMPITSAATPNHQPPPNSPYANAMNAAHGGTAAAAAMIKMEM

PLHPLHHQQMHHSQVPTTTVGVPVIKGDVASPTTKETVAWRYNLDVSPVVEEMPPGSDVAYV**CPTC**

GQMFSLHDRLAKHMASRHKSRNPANDIAKAYS**CDVCRRSFARSDMLTRHMRLH**TGVKPYT**CKVCGQ**

VFSRSDHLSTHQRTHTGEKPYK**CPQCPYAACRRDMITRHMRTH**TRYDSRGGSRE**GREGREGREGKE**

SAIRAGATSESRSPTRPVPIRCWT

Fig. 1. (A) The genomic region of *klumpfuss* was assembled from genomic phage clones. Regions of cDNA hybridization are indicated by open bars, the dashed lines indicate that the 5′ end of *klumpfuss* is still missing. The locations of various disruptions of the locus are indicated on the map as solid bars, including the insertions of *klu*^{P212} and P764. *klu* ^{212lR5} is a 4.5 kb deletion removing the last exon-containing region; *klu*^{212lR63} and *klu*^{212lR51C} each delete at least three exon-containing regions. The 5′ breakpoint has not been mapped, as indicated by the arrow. R, *Eco*RI. (B) The protein sequence predicted from the longest cDNA clone, CNB4. The three putative nuclear localisation signals are written in italics. The first two overlap. The four zinc-finger in the N-terminal region are indicated with bold underlined letters; C terminal to the zinc finger domains the four collagen-like repeats are highlighted in bold letters. The EMBL/GenBank sequence accession number for *klumpfuss* is Y11066.

in this allele, the deletion extending beyond the limits of the cloned region. All the other alleles tested exhibit polymorphisms in the region of the P-element insertion similar to those associated with the the P-insertions in *klu*^{P212} and *klu*^{P819}. However, the size of the fragments used for Southern blot analysis did not allow us precisely to determine whether genomic deletions were produced by the reversion event.

klumpfuss encodes a member of the *EGR* family of transcription factors

Sequence analysis of the longest cDNA, CNB4, predicts a single open reading frame encoding a protein of 750 amino acids (Fig. 1B). The KLUMPFUSS sequence is characterized by four zinc-finger domains of the C2H2 class; part of the N terminus is negatively charged; the C terminus, including the zinc-fingers, is positively charged. Moreover, the N-terminal region contains glutamine-, histidine- and proline-rich stretches, features found in transcriptional activation and repression domains (Mitchell and Tjian, 1989; Han and Manley, 1993). There are three poly-alanine stretches, two in the N- and one in the C-terminal region. Such stretches are implicated in transcriptional repression (Licht et al., 1990). In addition, there are three poly-asparagine stretches in the N-terminal half and, at its very end, four repeats of a collagen-like triplet GXY, where X is arginine or lysine and Y is always glutamine. The significance of these repeats is unknown. Three putative nuclear localisation sites are found in the protein.

A comparison with the proteins in the SWISS-PROT, PIR(R) and GenPept databases shows that the zinc fingers 2-4 of KLUMPFUSS have a high degree of similarity to the zinc-finger domains of the members of the EGR family (Fig. 2A). As in WT-1, KLUMPFUSS contains an additional zinc finger 1 which is only distantly related to those of the EGR proteins. Besides WT-1, KLU is the only other member of the Family with four zinc fingers. Closer comparison of fingers 2-4 of KLUMPFUSS with those of the other EGR-like proteins revealed complete conservation of the amino acids that contact the DNA-binding consensus sequence (arrows in Fig. 2A; Pelletier et al., 1991; Pavletich and Pabo, 1991; Nardelli et al., 1992). Furthermore, the aspartic acid that follows the first arginine contacting the target sequence, which is conserved among EGR proteins, is also conserved in KLUMPFUSS (squares in Fig. 2B). This aspartic acid in the third finger of WT-1 is crucial for its binding capacity; it is thought to stabilize the binding of the preceding arginine to a guanine base in the target sequence (Pelletier et al., 1991).

Expression pattern of *klumpfuss* during imaginal disc development

Hybridization of *klumpfuss* cDNAs to *Drosophila* embryos and imaginal discs reveals a complex and dynamic expression pattern throughout much of larval and imaginal development. Here, we focus solely on the *klumpfuss* expression pattern in the imaginal discs, since it is relevant to the phenotypic defects described further below. We found *klumpfuss* transcripts in every disc examined in a pattern that is identical to that of β-galactosidase expression of *klu*^{P212} and *klu*^{P819} and that produced by *klu*^{G410}-driven expression of an UAS-GFP fusion (Fig. 3).

Expression starts in the wing imaginal disc within the prospective wing area early in the third larval instar. Shortly

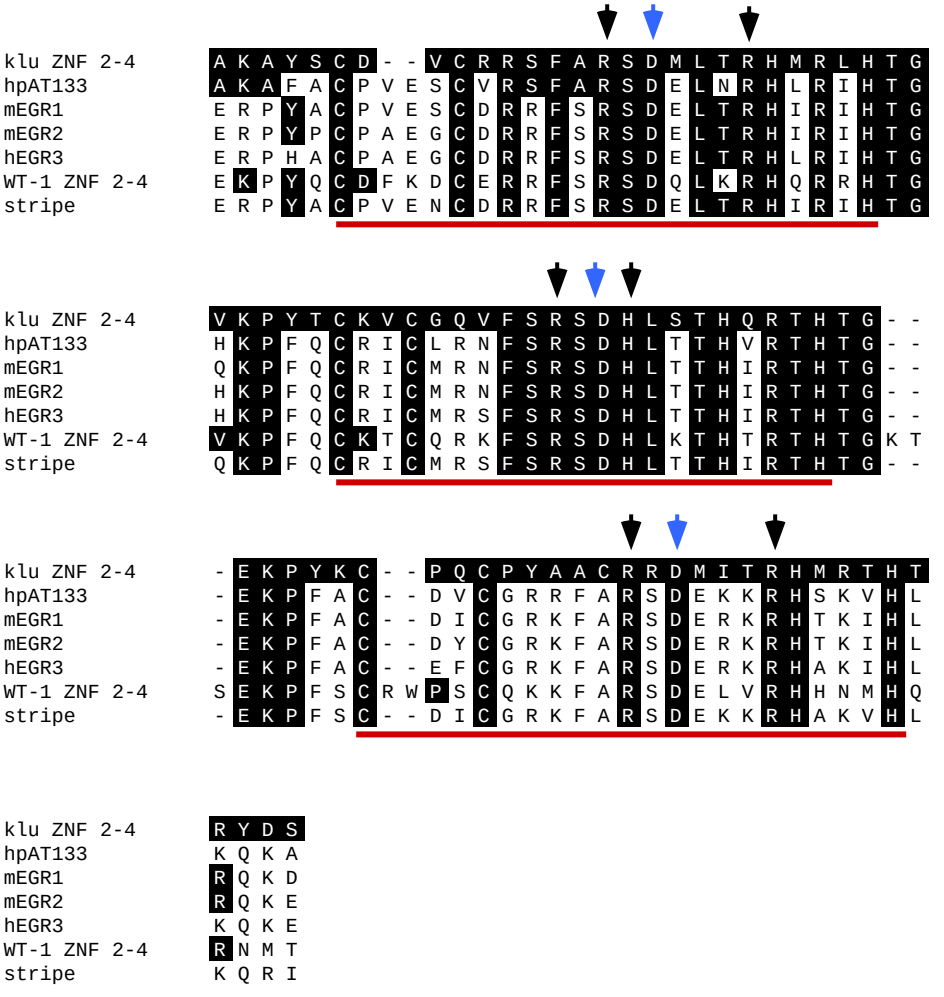


Fig. 2. Comparison of the region including zinc fingers 2-4 of KLUMPFUSS with the corresponding region in other members of the EGR family and zinc fingers 2-4 of human WT-1. The black arrows indicate amino acids which contact the DNA target sequence (Pavletich and Pabo, 1991; Nardelli et al., 1992). The blue arrows indicate the aspartic acid required for stabilization of the contact made by the preceding arginine with guanine 4 in the target DNA sequence (Pelletier et al., 1991; Pavletich and Pabo, 1991). The red bars underline the region of the actual zinc fingers. Abbreviations: h, human; m, mouse

thereafter, expression becomes restricted to the prospective margin and the hinge of the wing and, at about the same time, transcripts appear in the anlagen of notum and scutellum (Figs 3A, 4B). Double staining with ACHAETE antibody reveals that *klumpfuss* is expressed in most proneural clusters at, or shortly after, the onset of ACHAETE expression (Fig. 4A-C); furthermore, simultaneous staining of SOPs with A101 (Boulianne et al., 1991) reveals that *klumpfuss* expression in these regions precedes the appearance of SOPs (Fig 3H; Fig. 4E,F). *lacZ* expression in klu^{P212} is spotty in the wing (and leg, see below) imaginal discs, with zones of higher density surrounding areas lacking expression (Fig. 3G,I). These areas correspond to the SOPs as shown by double stainings with A101 (Fig. 3H), and the spotty pattern is not seen in imaginal discs of $In(1)sc^{10\text{-}1}$ flies, which lack SOPs (Fig. 4G). We also do not detect expression of *lacZ* until the onset of the differentiation of the sensillum cells (Fig. 4I-J). Thus, while cells of the proneural clusters express *klumpfuss*, SOPs themselves do not. Since *klumpfuss* expression is rather uniform before the appearance of SOPs, the gene must be switched off in the cells that initiate neural development.

Expression in the leg discs starts early during the third larval instar. At this time, the *klumpfuss* expression domain occupies a wedge-like sector encompassing roughly one third of the circumference of the leg disc. Rings of expressing cells successively become visible underneath a knob-like central structure during the third larval stage (data not shown). The rings correspond to the anlagen of the leg segments and the order of their appearence reflects the developmental pattern of the leg disc (Schubiger, 1974; Fristrom and Fristrom, 1975; Norbeck and Denburg, 1991). Around the time of puparium formation, *klumpfuss* expression seems to be restricted to the distal half of each leg segment in concentric domains (Figs 3D, 4D),

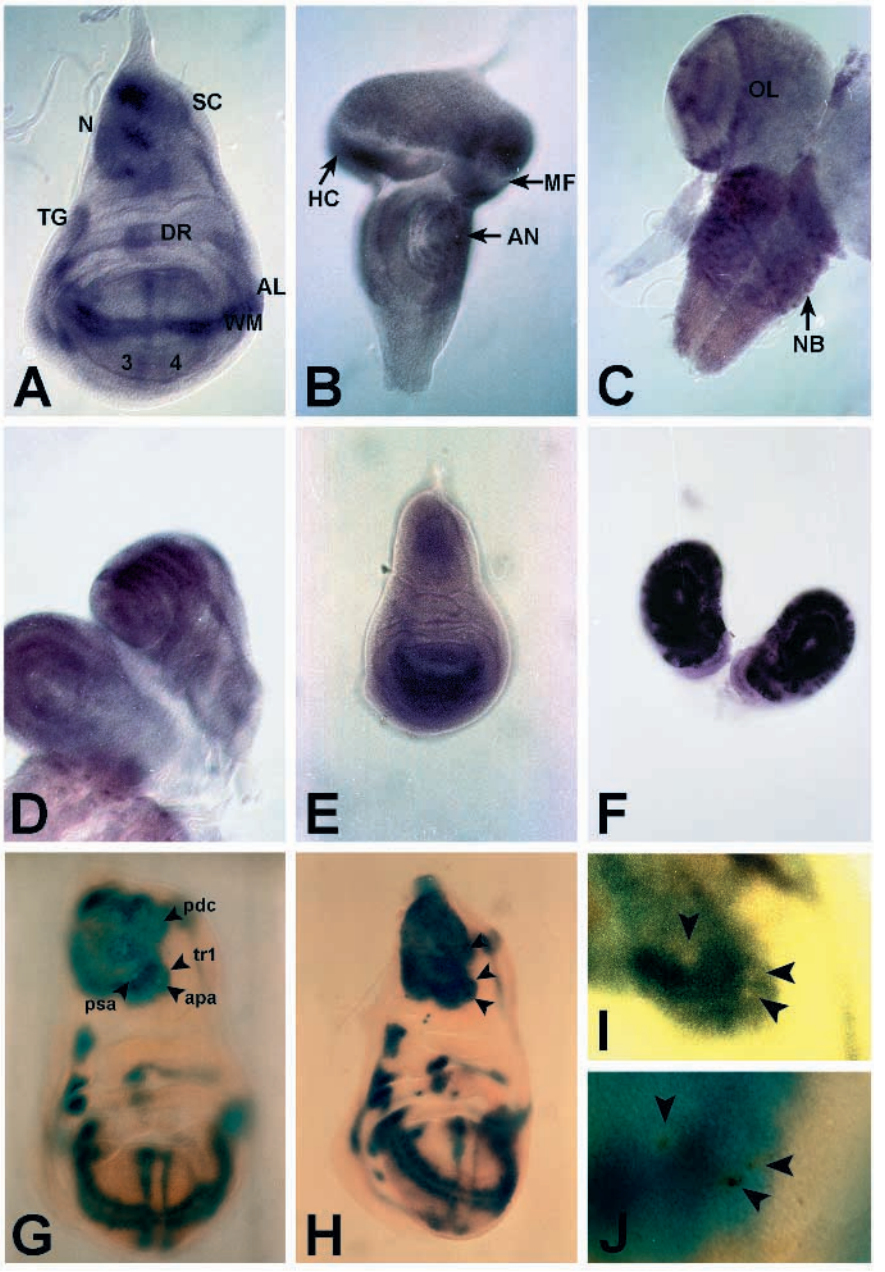


Fig. 3. The expression pattern of *klumpfuss* in late third instar imaginal discs detected by in situ hybridization. (A) Wing disc, anterior to the left, ventral to the bottom. *klumpfuss* is expressed in a dorsal and ventral stripe along the wing margin (WM), in the anlagen of wing vein three and four (3,4), in the region of the alula and tegula (TG), in broad domains of the notum (N) and in the scutellum (SC); DR, dorsal radius. (B) Eye-antennal disc. Expression in the antenna (AN) is restricted to concentric rings. In the eye region, expression is found behind the morphogenetic furrow (MF) and in the region that forms the head capsule (HC). (C) Expression in the larval brain is restricted to the proliferation zone of the optic lobes (OL) and the neuroblasts (NB). (D) In the leg disc *klu* is expressed in concentric rings, corresponding to the leg segments. (E) Wing disc of a $klu^{212lR51C}$ homozygote. No transcript is detectable. The disc was overstained to allow detection of possible residual expression. All other discs and the brain are also devoid of any specific signal. (F) Detection of transcript in leg discs in which UAS-*klumyc11B* is activated by the P-Gal4 insertion klu^{G410}. Note the irregular expression of *klu*. (G) X-Gal staining of a klu^{P212} wing disc. Arrowheads indicate spots of non-expression of β-galactosidase. The SOPs are located within these spot as shown by the A101-klu^{P212} double staining in H. (I) The spots that show no expression are also present 4 hours apf, as revealed at a higher magnification of the region where the apa /tr1/ psa sensilla are located. At this time the sensillum cells are already undergoing cytodifferentiation, as indicated by 22C10/Xgal double stainings of this region in a slightly younger disc (3 hours apf) in J. Labelling of the recently generated neuron of the apa macrochaete by the 22C10 antibody shows that the sensillum cells are present and already undergoing cytodifferentiation. Still no expression of β-galactosidase is detectable, indicating that the cells of the sensillum are devoid of *klumpfuss* activity.

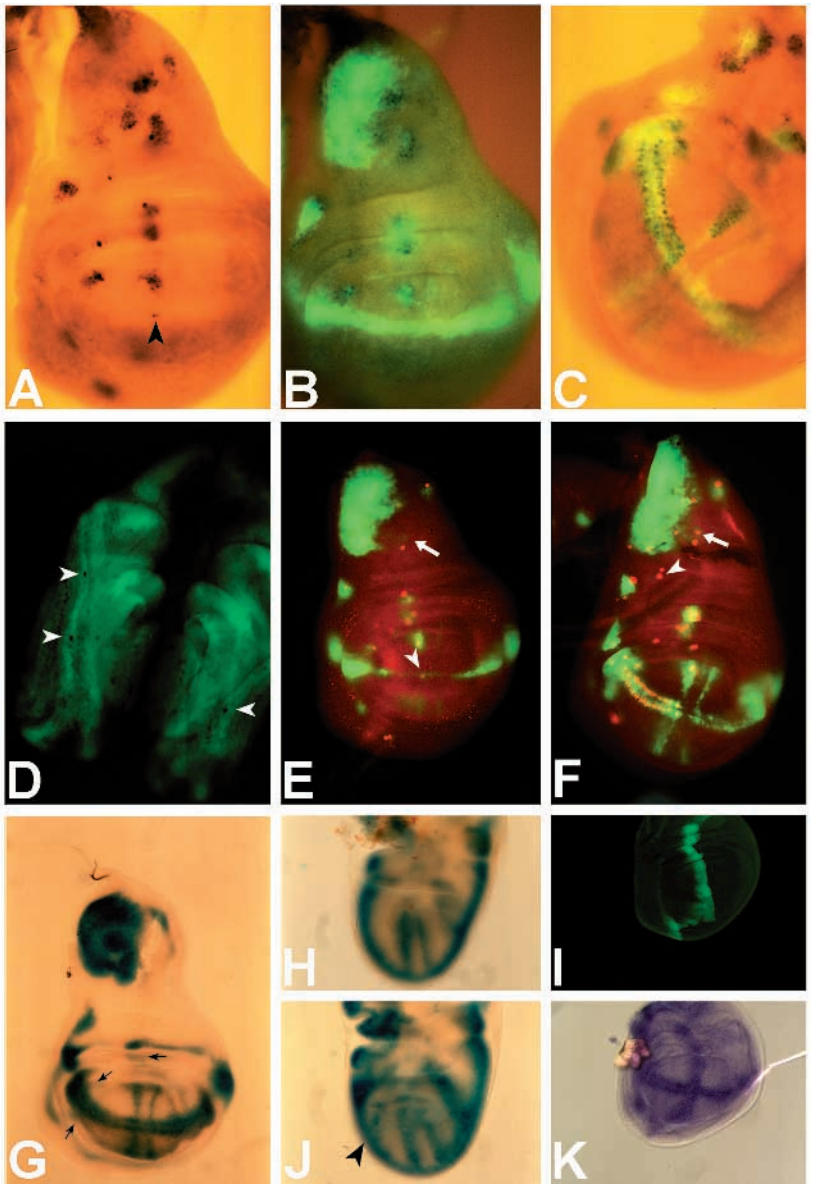


Fig. 4. (A-D) The relationship between the expression patterns of *klumpfuss* and *achaete*. The expression of *klumpfuss* is detected by the fluorescence of the GFP in wing discs carrying the P-Gal4 insertion klu^{G410} and UAS-GFP. (A) Mid third-instar disc stained with anti-ACHAETE antibody to reveal the proneural clusters. The arrowhead points to the first ACHAETE-positive cell at the wing margin. (B) Superimposition of the GFP pattern on the ACHAETE pattern shows that the proneural clusters are included in the *klumpfuss* expression domains. (C) The wing region of a late third instar disc shows complete overlap of the expression of ACHAETE and *klumpfuss* in the wing region. (D) A third instar leg disc shown from the side. Arrowheads pointing towards the ACHAETE-positive SOPs arising within the concentric expression domains of *klumpfuss*. (E-F) The relation of *klumpfuss* expression pattern and SOP positions in the wing disc. The SOPs are marked by A101 expression; the SOPs red, *klumpfuss* expression green. E Mid third instar; F late third instar. The SOPs arise within the expression domains of *klumpfuss*, with one exception indicated by the arrowhead in F. Arrowhead in E point towards the already developed SOPs at the wing margin and the notum. The arrows in E and F highlight the region where the apa, tr1 and psa SOPs are arising, as in F visible. The comparison of the pictures shows that *klumpfuss* expression precedes generation of the SOPs. (G) The expression of *klumpfuss* in a $sc^{10\text{-}1}$ wing disc, revealed by the β-galactosidase expression from klu^{P212}. The arrows indicate positions where *klumpfuss* expression is lost or weak. All affected positions are in the wing region; the main expression domains are unaffected (compare also with Fig. 3G and H). (H) Expression of *klumpfuss* is detectable in veins 3-5 in a klu^{P212} wing disc, 3 hours apf (X-Gal staining). (J) Expression of klu^{P212} in a *Hw49c* disc of the same age as in H, where *achaete* and *scute* are ectopically expressed along wing vein 2. In these discs *klumpfuss* is also ectopically expressed along vein 2 (arrowhead in J). (I) The expression pattern of *ptc*Gal4 revealed by UAS-GFP fluorescence. (K) Ectopic expression of *UAS-l'sc* driven by the *ptc*Gal4 line leads to ectopic activation of *klumpfuss* (detected by in situ hybridization) in a broad stripe along the anterior/posterior compartment border and closes the gap between the third and fourth wing vein, where it is normally expressed

spreading later over the whole leg (6 hours after puparium formation). Expression in the antennal and dorsal prothoracic discs also occurs in concentric domains (Fig. 3B). In the eye disc, expression starts behind the morphogenetic furrow and extends through the whole anlage (Fig. 3B). *klumpfuss* is further expressed in the parts that form the head capsule (Fig. 3B). Expression in the larval brain is restricted to the neuroblasts and the proliferation zone of the optic lobes (Fig. 3C).

klumpfuss is involved in a variety of developmental processes

Flies homozygous for the insertions klu^{P212} and klu^{P819} are viable, but show loss of bristles at some positions and fusion of tarsal segments. Excision of the P-element insertions in both lines was attempted using either the Δ2-3 transposase (Robertson et al., 1988) or X-rays. In the first case, a total of 200 revertants were analysed, 170 of which were wild-type, indicating precise excision of the P-elements. In the remaining 30, phenotypic defects were observed in homozygous flies or in combination with the original insertion; all 30 mutants fell into one complementation group. Twelve of these latter excisions were analysed in detail (see M&M). For the X-ray reversion screen, an isogenic strain of klu^{P212} was used. Several alleles were recovered, among them the two deficiencies $Df(3L)klu^{XR17}$ (67E; 68B) and $Df(3L)klu^{XR19}$ (68A). The cytogenetic analysis of all these rearrangements, as well as $Df(3L)vin^{2}$ (67F; 68D6), $Df(3L)vin^{5}$ (68A2-3; 68F3-6), $Df(3L)lxd^{2}$ (68A2-3; 68C5-7) and $Df(3L)lxd^{8}$, together with the results of complementation crosses between the deficiencies and the amorphic allele $klu^{212lR63}$, localized the gene at 68A1-2.

Even the strongest alleles are semilethal when homozygous, some animals developing to adulthood but dying shortly after hatching. However, most of the homozygotes die at the end of the third instar. No defects were detected in either cuticle preparations or antibody staining with 22C10, 44C11 or BP-104 of embryos homozygous for the smallest deficiency klu^{XR19} or for

other strong alleles. Among the mutant larvae, defects were detected in the mouth-hooks, where some teeth were missing, and in the larval brain, the morphology of which is obviously abnormal (not shown).

klumpfuss is required to specify epidermal cells as SOPs and for bristle differentiation

We found that a number of macrochaetae are missing in head and thorax, particularly from the anterior margin of the wing, the wing veins, antennae and legs of homozygotes for all of the alleles (Figs 5, 6, 8). Non-innervated bristles at the margin of the alula are also affected. In addition, differentiation defects with incomplete penetrance were observed at some bristle positions (Fig. 6). To assess the phenotypic variability, 19 machrochaete positions on head and notum were studied in about 50 flies from each of the crosses between 13 different *klumpfuss* alleles and *Df(3L)vin*^{2}. We found that weak alleles are more variable in their phenotype than strong alleles; moreover, penetrance is position dependent: only one of the 19 bristles considered in this analysis, the anterior sternopleural bristle, is consistently missing in these individuals. However, in each mutant individual, a significant number of bristles is missing. The proximal costa is most frequently affected, the number of bristles there always being severely reduced (Fig. 6A-D). Therefore, due to the characteristic location and penetrance of the phenotypic defects, we concentrated our analysis on this position.

Using various markers (see Fig. 6A-D and Materials and methods), two different defects can be distinguished. In most cases, the lack of bristle apparatus is correlated with a lack of all the bristle cells (Fig. 6A-D). As mentioned, in strong *klumpfuss* mutants, the number of bristles in the proximal costa is strongly reduced; we found a strong reduction in the number of A101-positive cells in this region at 26 hours apf, i.e. around the time when, in wild type, most SOPs are detectable (Fig. 6E,F). In the wild type, three recurved bristles are present in the proximal region of the costa; in all *klu*^{212lR51C} / *klu*^{XR19} animals, there are only two of these bristles and only two A101-positive cells are visible at this position. Some 70% of the *klu*^{212lR51C} / *klu*^{XR19} animals lack the presutural bristle; the corresponding A101 cells are absent in the majority of the imaginal discs stained (not shown). The same phenotype is detected in *klu*^{G410} / *klu*^{819lR5} and *klu*^{G410} / *klu*^{212lR51C} heterozygotes.

In some cases, e. g., at the dorsocentrals and in the proximal region of the costa, remnants of the bristle apparatus are still visible at higher magnification; in these positions, bristle cells are detectable with various markers (see Fig. 6G-I). This indicates defects in cyto-differentiation of the cells of the sensillum. The results therefore suggest that, in some positions, *klumpfuss* is required in order for epidermal cells to develop as SOPs and, in other positions, for proper differentiation of the progeny cells.

klumpfuss is required for cell differentiation in the leg imaginal discs

In *klumpfuss* mutants, the distal regions of the leg segments are preferentially affected. In homozygotes for hypomorphic alleles, tarsal segments 3-5, on the one hand, and trochanter and femur, on the other, are fused in all three leg

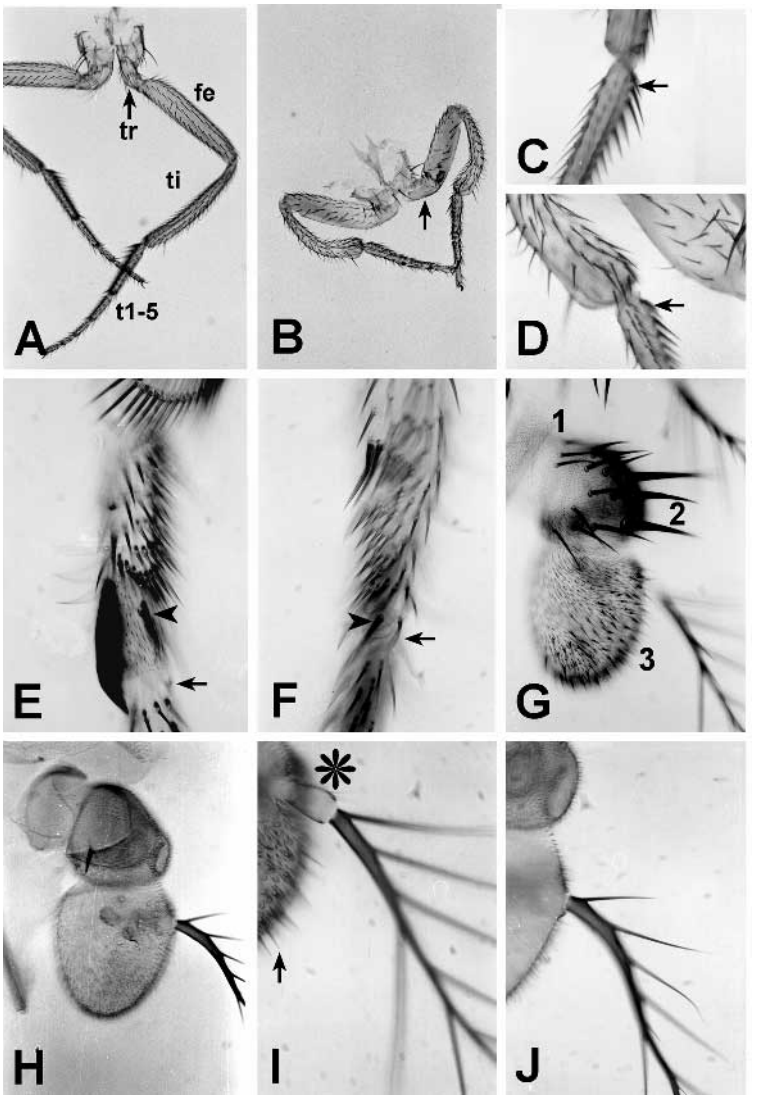


Fig. 5. Phenotypes of *klu* mutants. (A): Wild type metathoracic legs. (B) Metathoracic legs of *klu*^{212lR51C}. The trochanter is fused to the femur (arrow, see also Fig. 7H and 9A); fusions are also evident in the tarsal region and the size of the leg segments is reduced. The bristle number in the region of the femur is also reduced (see also Fig. 9A). (C-F): Analysis of the first tarsal segment in mutants suggests that the size reduction is due to a loss of the distal region. (C) The arrow indicates a characteristic bristle in the proximal region of a wild-type first tarsal segment. This bristle is also present in *klu*^{212lR51C} mutants (D). (E) The distal region of the first tarsal segment of a wild-type male: arrowhead points to a macrochaete in this region, the arrow highlights the distal segment border. On the left the outlines of the sex comb bristles are visible. (F) The same region as in E in a *klu*^{212lR51C} mutant. The sex combs and the corresponding distal region of the segment have been lost as indicated by the much shorter distance between the arrowhead and the arrow in comparison to that in E. The distal part of the tibia is also affected (compare C and D). (G) A wild-type antenna. The numbers indicate the three segments. (H) The number of bristles on the antenna is strongly reduced in *klu* mutants (*klu*^{212lR51C}/*Df(3L) vin2*). (I) The basal cylinder of the third antennal segment indicated by the asterisk (wild-type). This structure is severely reduced in *klu* mutants (J, *klu*^{212lR51C}/*Df(3L) vin2*).

pairs. In homozygotes for severe alleles, the defects are stronger and partial fusions of the first tarsal segment to the tibia and/or the second tarsal segment occur occasionally (Fig. 5A-F). There are fewer sex comb bristles and first tarsal segment bristles in homozygotes for weak alleles; in those bearing severe alleles, the first tarsal segments, including the sex combs, are lost (Fig. 5E-F). Bristles characteristic of the proximal part of other segments are still present, although reduced in number and sometimes showing cytodifferentiation defects (Fig. 5C-F).

We detected the first visible leg defects shortly after evagination of the discs (0-1 hour apf), i.e., when tarsal segments become normally recognisable (Fristrom and Fristrom, 1975). The use of various P-lacZ insertions as markers, helps to analyse the occuring defects: *klu*^{P212}, *odd-skipped*, expressed in the 1st and 5th tarsal segments, and *disconnected (disco)*, expressed in concentric stripes in the distal region of all tarsal segments and the tibia (Cohen, 1993), allow one to distinguish the divisions of the leg disc (see Fig. 7). In leg discs of homozygous larvae, one can still discriminate remnants of the tarsal segments with those markers (Fig. 7B,C,E), indicating that the initial subdivision of the leg segments takes place. Especially the *disco* expression pattern is of particular use to monitor the defects, since it is expressed in the distal region of all tarsal segments and the tibia (Cohen, 1993; see Fig. 7A). In late third instar, the tarsal segments seem to be present and one can detect all concentric rings of *disco*-expression (Fig. 7C). However, slight defects in the expression pattern are detectable (arrow in Fig. 7C). Later, approximately 1 hour apf, the expression pattern is severely disturbed and the morphology of the tarsal region becomes very abnormal. At this time, we observe massive cell death in this region (Fig. 7F,G).

All these data indicate that *klumpfuss* is not involved in the proximal-distal pattern formation of the leg disc, rather it is required for the differentiation of the distal tarsal region. In addition to the leg defects, the size of the basal cylinder of the antenna, which is homologous to the tarsal segments (Postlethwait and Schneidermann, 1971), is also reduced (Fig. 5I-J). However, we did not analyse the development of the defects in the antenna.

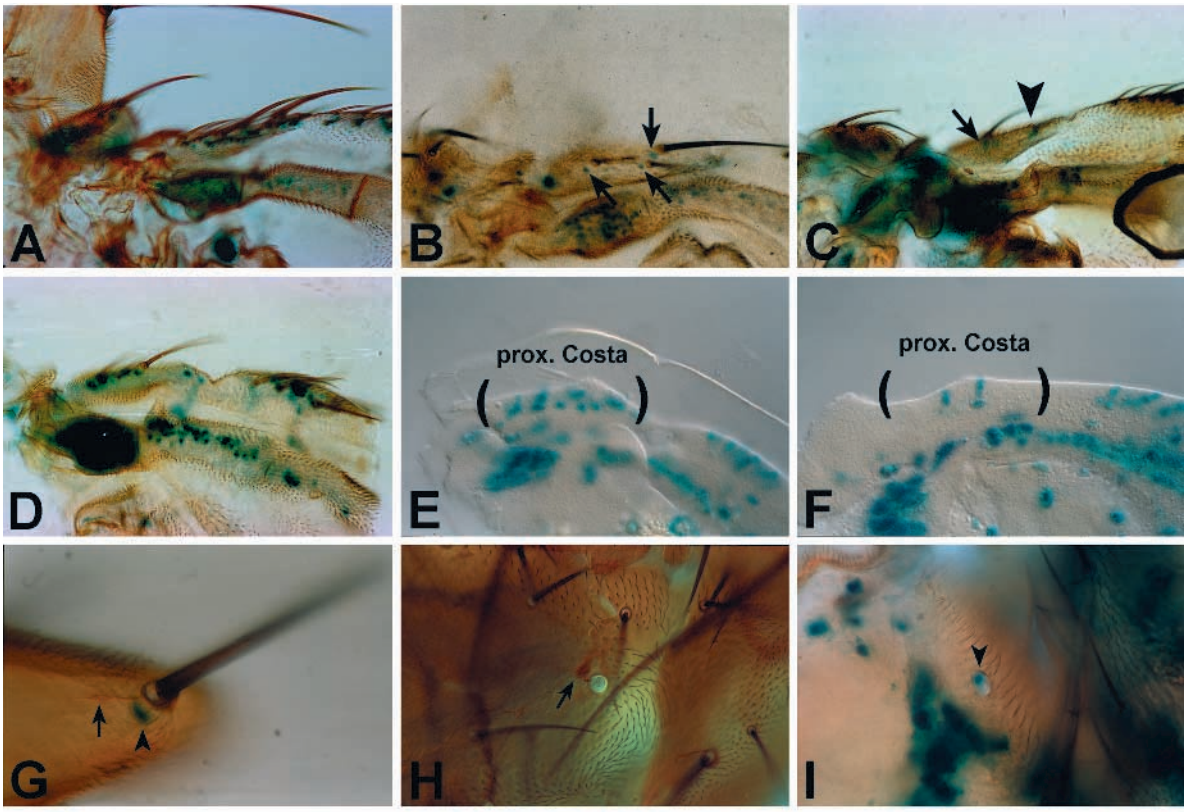


Fig. 6. Analysis of the bristle defects of *klu* mutants. (A-F) The region of the proximal costa is shown as an example. (A) A101 staining of a wild-type. A labelled socket cell nucleus is associated with each bristle. (B) The same region of a *klu*^{XR19}/*klu*^{212lR51C} animal. The number of bristles is severely reduced and the stained socket cells are associated with the remaining bristles (arrows). The same phenotype is observed with other markers, such as A37 (C) which labels the socket cell, and A293 (D) which labels all four cells of a sensillum. The analysis suggests that, concomitantly with the cuticular structure, the corresponding cells are lost. The arrowhead in C points to a bristle sensillum which has not differentiated properly. In this case the corresponding cells are still detectable. (E) The region of the proximal costa (brackets) 24 hours apf, stained with the A101 marker, labelling the SOPs of the region. (F) The same region in a *klu*^{G410}/*klu*^{212lR51C} mutant 26 hours apf. The number of SOPs is severely reduced. (G-I): Analysis of the differentiation phenotype of *klu* mutants (*klu*^{212lR51C}/*Df(3L)vin2*). (G) A101/22C10 double staining of a wild-type scutellar macrochaete. Arrowhead points to the A101-positive nucleus of the socket cell; the arrow points to the 22C10-positive neuron innervating the sensillum. (H-I): A dorso-central bristle sensillum, which has failed to differentiate the cuticular structures. The neuron and socket cell of the sensillum are still detectable by 22C10 (H) and A101 (I).

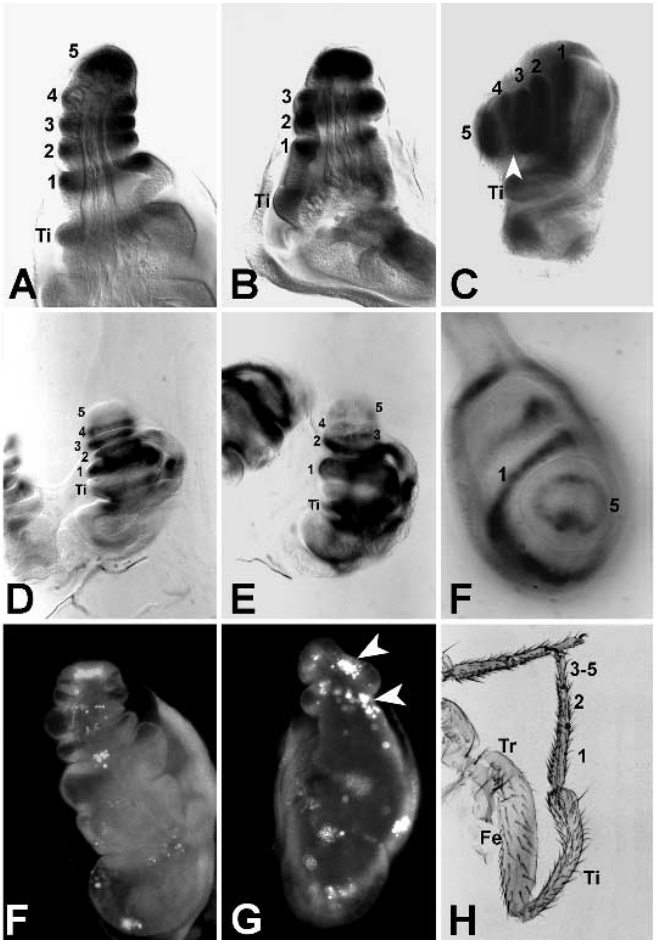


Fig. 7. Analysis of the leg phenotype of *klu* mutants. (A) Expression of *disco* P-lacZ in a wild-type evaginating leg 1 hours apf. Expression is seen in the proximal region of each tarsal segment (1-5) and the tibia (Ti). (B) In a $klu^{212lR51C}$ evaginating leg the *disco* pattern in the tarsal region is severely disturbed and only the first and second tarsal segments are distinguishable. Note that *disco* expression in the third tarsal segment is still recognizable on the left side, whereas it is missing on the other side. The lack of expression on this side is correlated with a change in morphology, indicating that the integrity of the segment on this side is also disturbed. (C) *disco* expression in a late third instar leg disc of a $klu^{212lR51C}$ mutant. At this time, the tarsal segments are still distinguishable with *disco*. The arrowhead points to a region in the fourth tarsal segment where the expression has begun to fade. The comparison with the older disc shown in B suggests that the defects in the mutant become more severe with time and the integrity of the tarsal segments is finally lost. This is also manifest in the expression of the klu^{P212} insertion. (D) Expression of klu^{P212} in a heterozygous leg disc. All tarsal segments are distingishable. (E) A disc homozygous for klu^{P212}. Some of the tarsal expression domains are collapsing, but remnants of expression are still visible (D-E, 1 hours apf). (F) *odd-skipped* P-lacZ expression in a $klu^{212lR51C}/klu^{XR19}$ late third instar leg disc. The expression is identical to that in wild-type discs (not shown). (F) Acridine orange staining of a wild-type leg disc 1 hours apf. Dead cells are revealed by the fluorescence of the incorporated acridine orange. (G) In a $klu^{212lR51C}$ leg disc of the same age a much larger number of dead cells is detectable, especially in the tarsal region (arrowheads). (H) The leg of a $klu^{212lR51C}$ animal showing the terminal phenotype: The tarsi 3-5 are fused, the distal part of the tibia is abnormal and the trochanter and femur are also fused. In addition, the number of bristles, especially in the femur, is reduced.

Most traits of the *klumpfuss* loss-of-function phenotype is rescued by the CNB4 cDNA

We conducted a rescue experiment using the Gal4 system (Brand and Perrimon, 1993) to verify that the cloned transcription unit is *klumpfuss*. As activator, we used the Gal4 insertion klu^{G410}, which drives *lacZ* expression in a pattern essentially identical to that of β-galactosidase in klu^{P212}, although somewhat patchy. It induces a strong *klumpfuss* phenotype at 17˚C, which is not complemented by any of several *klu* alleles tested. Activation of *UAS-*$klu^{myctag11B}$ in either a klu^{G410} / klu^{819lR5} or a klu^{G410} / $klu^{212lR51C}$ background leads to complete rescue of the bristle phenotype and partial rescue of the leg phenotype (Fig. 8A-F). The incomplete rescue of the leg phenotype is most probably due to the patchy activation of UAS-*klumpfuss* by klu^{G410} (Fig. 3F). The effects of ectopic expression of *klumpfuss* will be described elsewhere.

Phenotypic interactions reveal functional relationships of *klumpfuss* with the proneural genes

To detect possible functional interrelationships with the proneural genes, we combined *klumpfuss* mutations with mutations of the *achaete-scute* complex (AS-C, Campuzano and Modolell, 1992). $In(1)ac^{3}$ animals lack most microchaetae and several macrochaetae in notum and legs (Fig. 9A; Lindsley und Zimm, 1992). This phenotype is strongly enhanced in flies homozygous for the severe allele $klu^{212lR63}$, and less so in homozygotes for the hypomorph klu^{P212}. Synergistic effects are obvious in the leg of these animals (Fig. 9C). The mutation Hw^{49C} causes strong ectopic expression of ACHAETE and SCUTE (Skeath und Carroll, 1991), which leads to development of ectopic bristles. Thus, in Hw^{49C} mutants, multiple bristles develop instead of the single anterior sternopleural bristle. In Hw^{49C};klu^{P212} double mutants no bristles develop at the anterior sternopleural position. Hence, klu^{P212} is epistatic to Hw^{49C}.

ase^{1} is a 19 kb deletion that removes the *asense* gene (Brand et al., 1993; Dominguez und Campuzano, 1993). ase^{1} flies have a reduced number of bristles on anterior wing margin and abdomen; in addition, some of the wing margin bristles exhibit differentiation defects (Fig. 9E). In *klu* mutants, we observe a slight reduction in the number of bristles at the wing margin (Fig. 9D), which is strongly enhanced in double mutant combinations (Fig. 9F and G). Thus, ase^{1};$klu^{212lR51C}$ flies lack almost all bristles at the anterior wing margin and abdominal microchaetae are missing (Fig. 9). Since these defects have never been observed in the single mutants, a close functional relationship between *klumpfuss* and *asense* is probable. Interestingly, *asense* has a proneural function in the anterior wing margin but not in other regions of the body.

The phenotypic interactions described suggest functional interrelationships between *klumpfuss* and the AS-C. The epistatic relations between Hw^{49c} and klu^{P212} suggest that *klumpfuss* requires the activity of *achaete* and *scute* for its role in bristle development. Therefore, we examined the β-galactosidase expression of klu^{P212} in *achaete* and *scute* mutants, and *vice versa*.

Ectopic expression of ACHAETE and SCUTE in Hw^{49c} is particularly strong along the second wing vein (Skeath und Carroll, 1991). Ectopic β-galactosidase expression was found in the territory of the second wing vein of Hw^{49c};klu^{P212}

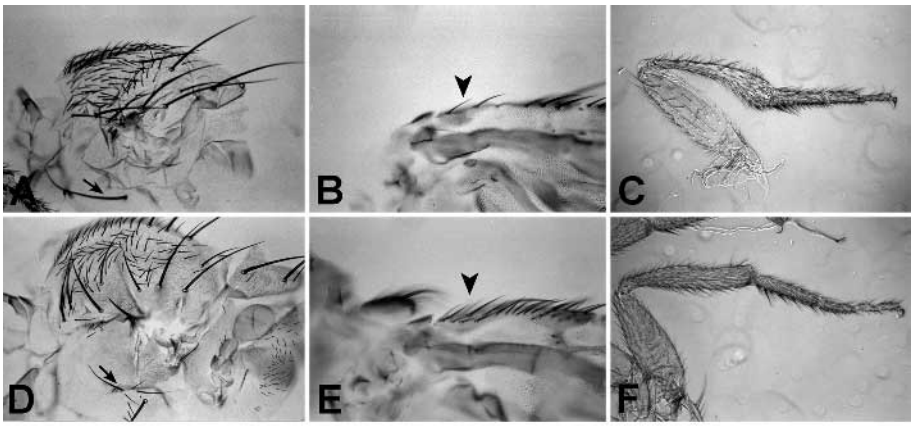


Fig. 8. Rescue of *klu* mutants by UAS-*klu*. (A-C) Mutant phenotype of klu^{G410} / klu^{212lR5} heterozygotes; (D-F) in presence of UAS-klu. The phenotype at the positions shown is completely penetrant and is therefore used to demonstrate rescue. (A) The arrow points to the position where the anterior sternopleural macrochaete is located. It is lost in the mutant, together with other surrounding bristles. These bristles form when UAS *klumyc11B* is present, as seen in D. (B) Proximal region of the costa of the wing (arrowhead). The number of bristles is strongly reduced in the mutant, but are rescued in the presence of the UAS-*klu* construct (E). (C) The typical *klu* mutant leg phenotype. The tarsal segments are reduced in size and segments 3-5 are fused together. The tibia is malformed. Also in this case a substantial rescue of the defect is observed in the presence of UAS-*klumyc 11B*, as shown in F. The incomplete rescue of the leg defects is probably due to the patchy expression of the UAS construct (see Fig. 3F).

imaginal discs. We next expressed UAS-*lethal of scute* driven by *patched-Gal4* (Hinz et al., 1994) and tested whether *klumpfuss* is ectopically expressed. Under these conditions, expression of *lethal of scute* in wild-type background induces bristle development within the *patched* domain (Hinz et al., 1994). We found ectopic activation of *klumpfuss* to be restricted to the *patched* expression domain in the wing disc and absent from the other regions. This indicates that *lethal of scute* is able to activate *klumpfuss* expression, but apparently only in the wing region.

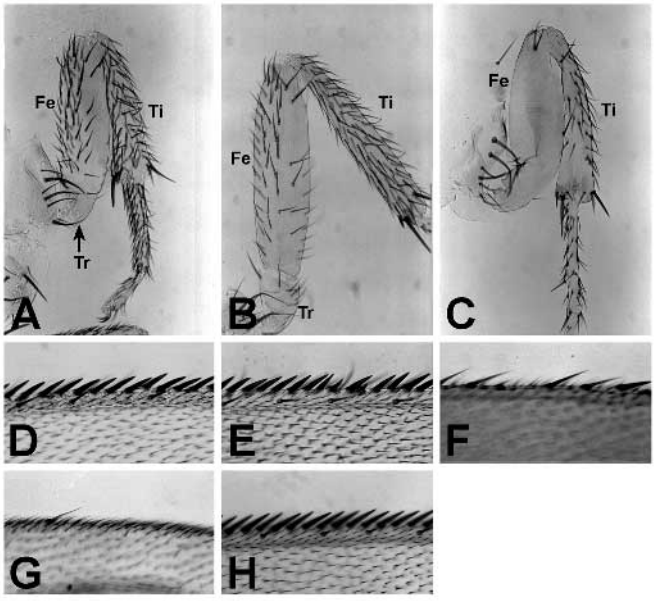


Fig. 9. Genetic interactions between alleles of *klu* and the AS-C. (A) A metathoracic leg of a $klu^{212lR63}/Df(3L)vin2$ animal. The arrow indicates fusion of the trochanter and femur. The number of bristles in the region of the femur is slightly reduced (compare with Fig. 5A). The same is true for the legs of ac^3 animals (B). The combination of both mutations (C) results in a loss of nearly all bristles in the femur and a strong reduction in the number of bristles in the tibia. (D) There is a slight reduction in the number of bristles on the anterior wing margin of a $klu^{212lR51c}/Df(3L)vin2$ animal as compared with the wild-type (H). The same is true for an ase^1 hemizygous animal (E). In addition bristle differentiation defects are visible. (F) The anterior wing margin of a ase^1; klu^{819lR5}/klu^{XR19} mutant. The number of hairs is strongly reduced. (G) The same region in a ase^1; $klu^{212lR63}/klu^{XR19}$ mutant. All bristles except one are lost.

In $In(1)sc^{10-1}$ flies, where no ACHAETE or SCUTE protein is detectable (Skeath and Carroll, 1991) and all innervated bristles of head and thorax are missing (Garcia-Bellido, 1979), the overall *klumpfuss* expression pattern in imaginal discs is normal. However, we found that a few expression domains are weaker or absent in the wing region (arrows in Fig. 5G). Conversely, no abnormality in the ACHAETE expression pattern could be detected in *klu* mutants (data not shown).

These results indicate a weak influence of AS-C genes on *klumpfuss* expression, which is restricted to the wing area of the wing disc. However, the overall expression pattern of *klumpfuss* is largely independent of proneural genes. Consequently, the functional relationship revealed by the genetic interactions, is likely to occur at a post-transcriptional level.

DISCUSSION

klumpfuss is a member of the EGR family of transcription factors

Sequence analysis predicts a protein of 750 amino acids with four zinc-finger domains. Sequence comparison revealed a high homology of zinc fingers 2-4 with those of the members of the EGR family. All members of this family bind the DNA consensus sequence GCGC(G/T)GGGCG. Although DNA-binding data are still lacking, it is likely that KLUMPFUSS also binds to this sequence, since all amino acids that make contacts with the EGR target sequence, as well as the aspartic acid following the first arginine contacting the target sequence of zinc finger 3 of WT-1, are conserved in KLUMPFUSS. This residue is essential since exchanges at the aspartic acid position abolish the DNA-binding capacity of WT-1 (Pelletier et al., 1991). Interestingly, an aspartic acid at the corresponding position in the other two zinc fingers is completely conserved among the EGR proteins, WT-1 and KLUMPFUSS (see Fig. 2A). Like WT-1, KLUMPFUSS has an additional, fourth zinc finger which is unrelated to those of other members of the family (Call et al., 1990; Gessler et al., 1990). However, it seems improbable that KLUMPFUSS represents a true orthologue of WT-1, since WT-1 homologues in vertebrates (Larson et al.,

1995) and KLUMPFUSS share no other significant homology outside the zinc finger region.

So far only two other proteins are described as EGR-like in invertebrates the *Drosophila* genes *stripe* (*sr*) and *huckebein* (*hkb*, Broenner et al., 1994). However, whereas the zinc finger of STRIPE have the characteristic amino acids for the binding of the DNA consensus sequence, in the zinc fingers of HUCKEBEIN four out of the six amino acids are not conserved. Therefore, from these data, it appears improbable that HUCKEBEIN binds to the same target sequence as the EGR proteins and is probably a more distantly related protein. Outside the zinc-finger region all proteins of the EGR family are different; thus, on the basis of the sequence, it is not possible to decide about whether KLUMPFUSS acts as a transcriptional activator, like the EGR proteins, or as a repressor.

klumpfuss is involved in initial steps of differentiation processes

Certain bristles and the corresponding A101-positive (SOP) cells were frequently missing in *klumpfuss* mutants. One may of course ask whether lack of A101 expression indeed signals the lack of the SOP, and whether this is due to functional deficits of the genes of the AS-C in *klumpfuss* mutants. If loss of *klumpfuss* function were to lead to functional defects of AS-C gene function, commitment of epidermal cells as SOPs would not occur. However, single cells expressing ACHAETE, i.e., with the characteristics of SOPs, were indeed present in the proneural clusters of *klumpfuss* mutants. Therefore, SOP development appears to be initiated correctly. The data on *klumpfuss* expression in flies mutant for the AS-C point to a role for *klumpfuss* in early steps of bristle development, but downstream of the proneural proteins. We assume that the cells that would normally have become SOPs switch fate before they activate A101 expression and probably die. The assumption that SOPs enter apoptosis is supported by the observation of abundant cell death in other developing organs of *klu* mutants, like the legs. All these data thus suggest that at certain bristle positions, such as that of the anterior sternopleural, *klumpfuss* is required during early bristle development immediately after proneural gene function, in order to allow a particular epidermal cell to develop as a SOP.

Our data imply that *klumpfuss* activity is restricted to the time of SOP specification, expression being switched off when the cell initiates SOP development. Since *klumpfuss* is apparently not expressed in bristle cells later in development, the gene has an influence on cells in which it is apparently not expressed. Such a delayed effect is also found for proneural genes. Dominguez and Campuzano (1993) showed that bristle differentiation defects at the anterior wing margin in *asense* mutants can be rescued by duplications of the *achaete* and *scute* genes. These latter genes are, however, switched off in the nascent SOP (Cubas et al., 1991); consequently, the effect on *asense* has to be indirect. This implies that a differentiation program of which *klumpfuss* takes part is initiated during specification of the SOP, and is needed for the proper differentiation of the SOP and the cells that it generates. In certain bristle positions, e.g. the dorsocentrals, all bristle cells are still detectable with specific markers, but only remnants of the bristle are present. Granted that *klumpfuss* expression probably occurs in all proneural clusters that give rise to bristles, its role obviously varies in importance depending on the site. It might nevertheless act as a general factor in bristle development, both at intitial steps of SOP specification and during differentiation of its progeny cells.

Following overexpression of proneural genes, supernumerary bristles arise within the regions in which bristles normally develop in the wild type. Rodriguez et al. (1991) interpreted this observation as a manifestation of spatially restricted competence of the imaginal discs to develop sensory organs. That is to say, imaginal disc cells require a certain competence in order to respond to proneural gene activity. *klumpfuss* expression domains include most, or all, proneural clusters that give rise to bristles in the imaginal discs; however, with the exception of the wing area, its expression seems to be largely independent of proneural gene activity in the imaginal discs. It is tempting to speculate that *klumpfuss* may act to confer on the imaginal neurogenic regions the competence to respond to proneural gene activity.

With respect to the leg defects, similar conclusions can be drawn. Prior to, and at the time when the defects arise in the mutants (0-1 hpf), expression of *klumpfuss* is confined to the distal region of each leg segment. *klu* mutants lack distal structures in tarsal segments, which are frequently fused. These defects are visible as early as the time at which the tarsal segments become recognizable and, at this time, clusters of apoptotic cells are seen. Therefore, although the initial subdivision of leg segments takes place, the cells in the distal part of the tarsal segments eventually enter apoptosis. On this basis, we propose that *klumpfuss* is required for the onset of differentiation of the distal part of the tarsal segments. In addition, *klumpfuss* is also required for bristle development within proximal regions of the leg discs.

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REFERENCES

Bellen, H. J., Vaessin, H., Bier, E., Kolodkin, A., D'Evelyn, D., Kooyer, S. and Jan, Y. N. (1992). The *Drosophila* couch potato gene, an essential gene required for normal adult behavior. *Genetics* **131**, 365-375

Bier, E., Ackermann, L., Barbel, S., Jan, L. and Jan, Y. N. (1988). Identification and characterization of a neuron-specific nuclear antigen in *Drosophila*. *Science* **240**, 913-916

Bier, E., Vaessin, H., Sherperd, S., Lee, K., MC Call, V., Barbel, S., Ackermann, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y. and Jan, Y. N. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. *Genes Dev.* **3**, 1273-1287

Bier, E., Vaessin, H., Younger-Sherperd, S., Jan, L. Y. and Jan, Y. N. (1992). *deadpan*, an essential pan-neural gene in *Drosophila*, encodes a Helix-Loop-Helix protein similar to the *hairy* gene product. *Genes Dev.* **6**, 2137-2151

Blair, S., Giangrande, A., Skeath, J. B. and Palka, J. (1992). The development of normal and ectopic sensilla in the wings of *hairy* and *Hairy wing* mutants of *Drosophila*. *Mech. Dev.* **38**, 3-16

Boulianne, G. L., de la Concha, A., Campos-Ortega, J. A., Jan, L. Y. and Jan, Y. N. (1991). The *Drosohila* neurogenic gene *neuralized* encodes a novel protein and is expressed in precursors of larval and adult neurons. *EMBO J.* **10**, 2975-2983

Brand, M. and Perrimon, N. (1993). Targeted expression as a means of

altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brand, M., Jarman, A. P., Jan, L. Y. and Jan, Y. N. (1993). *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* **119**, 1-17

Broenner, G., Chu-LaGraff, Q., Doe, C. Q., Cohen, B., Weigel, D., Taubert, H. and Jackle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in *Drosophila*. *Nature* **369,** 664-668.

Brown, N. M. and Kafatos, F. C. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Molec. Biol.* **203**, 425-437

Call, K. M., Glaser, T., Ito, C,Y. Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., Jones, C. and Housemann, D. E. (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilm´s tumor locus. *Cell* **60**, 509-520

Campuzano, S. and Modolell, J. (1992). Patterning of the Drosophila nervous system: the acaete-scute gene complex. *Trends Genet. Sci.* **8**, 202-208

Cohen, S. M. (1993). Imaginal disc development. In *Development of Drosophila* (ed. A. Martinez-Arias and M. Bate). New York: Cold Spring Harbor Press.

Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008

Dominguez, M. and Campuzano, S. (1993). The *asense* gene, a member of the *Drosophila achaete-scute* complex, is a proneural and neural differentiation gene. *EMBO J.* **12**, 2049-2060

Fristrom, D. and Fristrom, J. W. (1975) The mechanism of evagination of imaginal discs of *Drosophila meanogaster*. I. General considerations. *Dev. Biol.* **43,** 1-23

Frommer, G., Vorbrueggen, G., Pasca, G., Jaeckle, H. and Volk, T. (1996). Epidermal egr-like finger protein of *Drosophila* participates in myotube guidance. *EMBO J.***15**, 1642-1649

Fujita, S. C., Zipursky, S. L., Bencer, S., Ferrus, A.and Shotwell, S. L. (1982). Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natn. Acad. Sci., USA* **79**, 7929-7933

Garcia-Bellido, A. (1979). Genetic analysis of the achaete-scute system of *Drosopila melanogaster*. *Genetics* **91**, 491-520

Gessler, M., Poustka, A., Cavenee, W., Neye, R. L., Orkin, S. and Bruns, G.A.P. (1990). Homozygous deletion in Wilm´s tumors of a zinc-finger gene identified by chromosome jumping. *Nature* **343**, 774-778

Ghysen, A. and Dambly-Chaudiere, C. (1989). Genesis of the *Drosophila* nervous system. *Trends in Genet.* **5**, 251-255

Ghysen, A., Dambly-Chaudiere, C., Jan, L.Y. and Jan, Y.N. (1993). Cell interactions and gene interactions in peripheral neurogenesis. *Genes Dev.* **7**, 723-733.

Gonzalez, F., Romani, S., Cubas, P., Modolell, J. and Campuzano, S. (1989). Molecular analysis of the *asense* gene, a member of the *achaete-scute complex* of *Drosophila melanogaster*, and its novel role in optic lobe development. *EMBO J.* **8**, 3553-3562

Haber, D.A., Sohn, R. L., Buckler,A. J., Pelletier, J., Call, K. and Housman, D. E. (1991). Alternative splicing and genomic structure of the Wilm's tumor gene, WT1. *Proc. Natn. Acad. Sci. USA* **88**, 9618-9622

Han, K. and Manley, J. L. (1993). Transcriptional repression by the *Drosophila* even-skipped protein: definition of a minimal repression domain. *Genes Dev.* 7, 491-503

Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic Helix-Loop-Helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87

Hortsch, M., Bieber, A. J., Patel, N. H. and Goodman C. (1990). Differential splicing generates a nervous system-specific form of *Drosophila neuroglian*. *Neuron* **4**, 697-709

Huang, F., Dambly-Chaudiere, C. and Ghysen, A. (1991). Emergence of sense organs in the wing disc of *Drosophila*. *Development* **111**, 1087-1096

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* nervous system. *Cell* **73**, 1307-1321

Klambt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117,** 163-176.

Larson et al (1995). Subnuclear localization of WT-1 in splicing or transcription factor domains is regulated by altrnative splicing. *Cell* **81**, 391-401

Lee, J. C., VijayRaghavan, K., Celniker. S. E. and Tanouye, M. A. (1995). Identification of a Drosophila muscle development gene with structural homology to mammalian early growth response transcription factors. *Proc. Natn. Acad.Sci., USA* **92**, 10344-10348

Licht, J. D., Grossel, M. J., Figge, J and Hansen, U. M. (1990) *Drosophila* Krüppel protein is a transcriptional repressor. *Nature* **353**, 569-571

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego, New York, Boston, London, Sydney, Tokto, Toronto: Academic Press Inc.

Madden, S. L. and Rauscher, F. J. (1993). Positive and negative regulation of transcription and cell growth mediated by the EGR family of zinc-finger gene products. *Ann. N. Y. Acad. Sci.* **684**, 75-84

Martinez, C., Modolell, J. and Garrel, J. (1993) Regulation of the proneural gene *achaete* by HLH protein. *Mol. Cell. Biol.* **13,** 3514-3521

Masucci, J. D., Miltenberger, R. J. and Hoffman, F. M. (1990) Patternspecific expression of the *Drosophila decapentaplegic* gene in imaginal discs is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4**, 2011-2023

Mitchell, P. J. and Tijan, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378

Miyagawa, K., Kent, J., Schedl, A., van Heyingen, V. and Hastie, N. (1994). Wilm´s tumor – a case of disrupted development. *JCB Supplement* **18**, 1-5

Mueller, H.-J., Skerka, C., Bialonski, A. and Zipfel, P. (1991). Clone pAT 133 identifies a gene that encodes another human member of a class of growth factor-induced genes with almost identical zinc-finger domains. *Proc. Natn. Acad.Sci., USA* **88**, 10079-100983

Nardelli, J., Gibson, T. and Charnay, P. (1992). Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis. *Nucleic Acid Res.* **20**, 4137-4144

Norbeck, B. A. and Denburg, J. L. (1991). Pattern formation during insect leg segmentation: Studies with a prepattern of a cell surface antigen. *Roux's Arch. Dev. Biol.* **199**, 476-491

Pavlevitch, N. P. and Pabo, C. O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* **252**, 809-817

Pelletier, J., Bruening, W., Kashtan, C. E., Mauer, S. M., Manivel, J. C., Striegel, J. E., Houghton, D. C., Junien, C., Habib, R., Fouser, L., Fine. R. N., Silverman, J. L., Haber, D. A. and Housman, D. (1991). Germline mutations in the Wilm's tumor suppressor gene are associated with abnormal urogenital development in Denys-Dash Syndrome. *Cell* **67**, 437-447

Pirrotta, V., Hadfield, C. and Pretorius, H. J. (1983). Microdissection and cloning of the white locus and the 3B1-3C2 region of the *Drosophila* X-chromosome. *EMBO J.* **2**, 927-934

Postlethwait, J. H. and Schneidermann, H. A. (1971). Pattern formation and determination in the antenna of the homeotic mutant Antennapedia of *Drosophila melanogaster*. *Dev. Biol.* **25**, 606-640

Robertson, H. M., Preston, C. R., Phillis, R. W., Jhonson-Schlitz, D., Benz, W. K. and Engels, W. R. (1988). A stable source of P-element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470

Rodriguez, J., Hernandez, R., Modolell, J. and Ruiz-Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **11**, 3583-3592

Romani, S., Campuzano, S., Macagno, E. R. and Modolell, J. (1989). Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev.* **3**, 997-1007

Sambrook, J., Fritch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*, Second edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schubiger, G. (1974). Acquisition of differentiation competence in the imaginal leg of *Drosophila*. *Roux's Arch. Dev. Biol.* **174**, 303-311

Skeath, J. B. and Carrol, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev.* **5**, 984-995

Tautz, D. and Pfeifle, C. (1989). A non radioactive *in situ* hybridization method for localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85

Villares, R. and Cabrera, C. V. (1987). The *achaete-scute* gene complex of *Drosophila melanogaster*, conserved domains in a subset of genes required for neurogenesis and their homology to myc. *Cell* **50**, 415-424

Yeh, E., Gustafson, K. and Boulianne, G. L. (1995) Green fluorescent protein as a vital marker and reporter of gene expression in *Drosophila*. *Proc. Natn. Acad.Sci., USA* **92**, 7036-7040

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