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Original Article

Cellular pathways acting along the germband and in the amnioserosa may participate in germband retraction of the *Drosophila melanogaster* embryo

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ABSTRACT **Germband retraction in *Drosophila melanogaster*, like most embryonic morphogenetic events in this organism and in higher eukaryotes, is not well understood. We have taken several approaches to study the relationships between previously identified mutations (*u-shaped, serpent, hindsight* and *tailup*) that selectively cause germband retraction defects in homozygous embryos, and a more pleiotropically acting locus, DER/*faint little ball*. Our observations from genetic, immunohistochemical, and embryo culture experiments suggest that the former four loci are elements of at least two parallel and partially redundant cellular pathways that affect germband retraction by acting in amnioserosal development or maintenance. An additional discrete and unique pathway, represented by DER/*faint little ball*, is likely to function in the germband itself. While the role of the amnioserosa during germband retraction appears to be permissive, the action of DER in the germband may be mediated by the cytoskeleton.**

KEY WORDS: *germband retraction defective loci, amnioserosa, genetic interactions, embryo culture, Drosophila melanogaster*

Introduction

Two sets of processes that comprise embryonic development of *Drosophila melanogaster*, as of higher organisms, are morphogenetic movements, that generate the overall forms of and relationships among cell populations in the embryo, and differentiation, in which individual cells and tissues obtain their final molecular/biochemical identity. Much knowledge has accumulated about the chain of molecular events that bring about cell differentiation. More recently, the cellular mechanisms and molecular circuitry of the processes which bring about cell rearrangements that are responsible for two early morphogenetic events in the *Drosophila* embryo, gastrulation and germband extension, have also begun to be investigated (Leptin and Grunewald, 1990; Parks and Wieschaus, 1991; Sweeton *et al.*, 1991; Reuter *et al.*, 1993; Irvine and Wieschaus, 1994). Interestingly, in both instances, multiple parallel pathways have been shown to contribute to the final morphogenetic results (Leptin and Grunewald, 1990; Irvine and Wieschaus, 1994).

After germband extension, the next morphogenetic event, which occurs relatively late in embryonic development (at stage 12, 7:20 to 9:20 h when development takes place at 25°C), is germband retraction, or shortening. During this process the extended, U-shaped metameric region of the embryo returns to its previous anatomical topology and brings the caudal end of the embryo back to the posterior tip (see Campos-Ortega and Hartenstein, 1985 for description). The mechanism(s) by which germband retraction occurs are not understood. We have taken several approaches in examining this process in wild type embryos and embryos homozygous for mutations that cause abortion of germband retraction. We have asked the following questions: what structures and tissues of the germband extended embryo might be involved in germband shortening? How many cellular pathways carry out this process? Is germband retraction dependent on the integrity of the cytoskeleton?

We have immunohistochemical evidence suggesting that two anatomical regions of the germband extended embryo are important in carrying out retraction, the germband itself and the amnioserosa, but that the latter may be acting passively. Our genetic data demonstrates that a unique pathway is implemented in the germband, while at least two overlapping pathways are necessary, probably in the amnioserosa, to permit shortening to occur. Finally, we have shown that germband shortening is a cytoskeletal dependent process.

Abbreviations used in this paper: ush, U-shaped; tup, tail up; srp, serpent; hnt, hind sight; DER/flb, Drosophila EGF receptor/faint little ball; fas III, fasciclin III.

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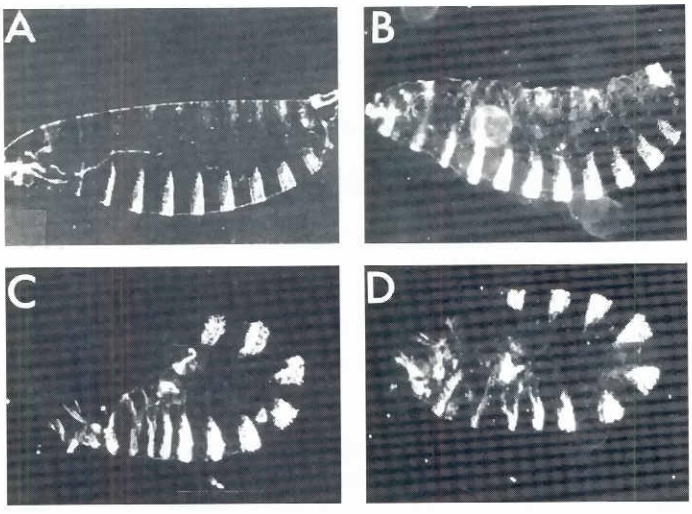

Fig. 1. Embryonic cuticular morphology of wild type embryos and embryos which display weak, intermediate and severe germband retraction defects viewed parasagittally. *The wild type embryo* **(A)** *exhibits a fully retracted germband and all the denticle bands are on the ventral side. In embryos displaying weak germband retraction defects, such as embryos homozygous for the srp P insertion allele shown in* **(B)**, *one or no ventral setae remain on the dorsal aspect of the embryo. In embryos with an intermediate germband retraction defective phenotype, such as* tup^{IIIB29} *homozygous embryos* **(C)**, *the ventral setae of two to three abdominal segments can be seen on the dorsal aspect, and in embryos such as* ush^{IIA108} tup^{IIIB29} *double mutants with a severe phenotype four segments remain dorsally* **(D)**. *In this and all other figures, anterior is to the left and dorsal is up.*

Results

Characterization of the cuticular phenotypes of the germband retraction defective mutants

In order to be able to correlate between the extent of failure of the germband to retract and specific tissue defects, and to determine whether modifications of the phenotype of one locus occur in the presence of one or two copies of a mutation in a second locus, we quantified the germband retraction phenotype of the alleles of the loci that we employed. This was done by determining the distribution of cuticular germband retraction defective phenotypes (weak, intermediate and severe, see Materials and Methods and Fig. 1) typical for the nonviable embryos mutant in each allele. The germband retraction defective loci which we examined have previously been identified based on the U-shaped cuticle of the dead embryos homozygous in these loci (Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984). On the other hand, other than DER/*flb*, these loci cause no segmental pattern defects that can be observed in cuticle preparations. We found that embryos homozygous for alleles of the various loci have cuticular phenotypes of different severities.

As can be seen in Table 1, the majority of nonviable ush^{IIA108}, tup^{IIIB29} or hnt^{XE} embryos born from a cross of balanced males and females have an intermediate to weak germband retraction defective phenotype. This indicates that there is a greater requirement for the activity of their gene products in later stages of germband retraction than in its initiation. While the proportion of embryos displaying a severe phenotype is low for all of these three loci, it is highest for ush^{IIA108}. This proportion increases among embryos transheterozygous for ush^{IIA108} and a deficiency which uncovers the *ush* locus, whereas it does not increase among embryos transheterozygous for tup^{IIIB29} and a deficiency chromosome, which we have shown, does not complement *tup*. Thus, we conclude that, with respect to the germband retraction defective phenotype, ush^{IIA108} is a hypomorph and tup^{IIIB29} is an apparent functional null allele. Embryos bearing the *srp* allele which we generated and employed, srp^{AS282^-} (see Materials and Methods), display intermediate to severe retraction defects that are significantly more pronounced than the weak defects of the previously available allele, srp^{9L06}, as well as ush^{IIA108}, tup^{IIIB29} or hnt^{XE}.

The cuticular profile of embryos homozygous for the amorphic DER/*flb* allele, flb^{JE1}, is strikingly different from that of all the other loci examined with respect to germband retraction. flb^{JE1} embryos have an extremely severe phenotype in which virtually all the embryos are curled into round, denticle-less balls (Raz *et al.*, 1991). This latter phenotype, with a penetrance that approaches 100%, is the strongest germband retraction defective phenotype that we have observed. The severity and high penetrance of the flb^{JE1} phenotype point to the possibility that DER/*flb* acts uniquely along a cellular pathway that implements germband retraction. This is in contrast with the loci described above whose partially penetrant, largely intermediate, phenotypes suggest that there is redundancy in their function. An additional *flb* allele that we used, flb^{JE3}, does not display a germband retraction defective phenotype.

Abnormalities are exhibited by the amnioserosa of embryos homozygous for each of the germband retraction defective loci

The amnioserosa, the dorsalmost tissue of the embryo which is abutted by the extended germband, has previously been suggested to be aberrant in embryos carrying the germband retraction defective loci (Clifford and Schupbach, 1992; Ray and Gelbart, unpublished). More recently, using expression of the *Race* gene as a marker for amnioserosa differentiation, Frank and Rushlow (1996) showed that the tissue generally begins to differentiate normally in *tup*, *ush*, *hnt*, and *srp* mutant embryos, but is lost prematurely in these embryos following germband extension. We therefore examined the integrity of the amnioserosa immediately prior to the initiation of germband retraction in the germband retraction defective alleles of interest to us, particularly the severe *srp* allele that we have generated, by employing anti-Krüppel antibodies which stain the nuclei of the large amnioserosal cells (Gaul *et al.*, 1987). In Figure 2A one of the two lateral wings of the amnioserosa in a wild type embryo is shown. The nuclei of the amnioserosal cells appear elongated and many are concentrated at the periphery of the tissue.

We found that each of the different germband retraction defective loci is associated with abnormalities of the amnioserosa, but that these vary in severity between the different loci. In germband extended embryos homozygous for the *srp* P excision allele (Fig. 2B) or for tup^{IIIB29} (Fig. 2C), there were fewer Krüppel cross-reactive nuclei than in wild type embryos. The nuclei that were present, either dispersed in the central part of the amnioserosa, in the case of *srp* embryos, or at the periphery of the tissue, in the case of *tup* bearing embryos, were, however, of normal shape. In ush^{IIA108} (Fig. 2D) embryos there was also a reduced number of nuclei. The remaining nuclei were concentrated in the internal part of the amnioserosa and were irregular in shape, implying that the amnioserosal cells had an aberrant morphology. In embryos

TABLE 1

CHARACTERIZATION OF EMBRYONIC LETHAL PHENOTYPES OF GERMBAND RETRACTION MUTANTS

Genotype	Number of unhatched embryos of each phenotypic class	
	weak or intermediate	severe (%)
1. ush^{IIA108} /ush^{IIA108}	139	18 (11.5%)
2. ush^{IIA108} /*Df* (2L)*al*	59	51 (46.4%)
3. tup^{IIIB29} /tup^{IIIB29}	84	3 (3.4%)
4. tup^{IIIB29} /*Df*(2L)OD15	99	4 (3.9%)
5. srp^{AS282^-}/srp^{AS282^-}	105	36 (25.5%)
6. hnt^{XE}/ hnt^{XE}	90	5 (5.3%)

Embryonic lethal phenotypes were determined by examining the cuticles of unhatched embryos after wild type embryos had hatched. At least two embryo collections from heterozygous balanced strains or from two independent crosses between two balanced strains (in the case of 2 and 4 above) were examined and the results were pooled since they displayed the same tendencies.

carrying the flb^{JE1} allele (Fig. 2E), the Krüppel expressing nuclei, mostly in the perimeter of the amnioserosa, appeared to be degenerating. This agrees with the observations of Clifford and Schupbach (1992), who reported that cell death occurs in the amnioserosa of *flb* germband extended embryos beginning from stage 10. Hemizygous hnt^{XE} embryos (Fig. 2E) had almost no amnioserosal nuclei that were recognized by the anti-Krüppel antibody. The reduction in number of amnioserosal nuclei that express the Krüppel marker in embryos mutant in all of the germband retraction loci is consistent with the idea that these loci are necessary either for differentiation or maintenance of the amnioserosa.

Only embryos mutant in DER/flb exhibit ventral germband defects that may contribute to abortion of germband retraction

We were prompted to examine the integrity of the internal tissues of the germband in embryos carrying each of the retraction defective loci because of abnormalities observed previously along the ventral aspect of DER/*flb* mutant embryos (Clifford and Schupbach, 1989, 1992; Schejter and Shilo, 1989; Zak *et al.*, 1990; Raz and Shilo, 1992, 1993). In *flb* embryos the fate of the ventralmost cells which usually express fasciclin III (Patel *et al.*, 1987) is replaced by more lateral fates. These are visualized by the *disco* marker of the C50.ISI enhancer trap that is expressed in the precursor's of the Keilin's organs (Raz and Shilo, 1993). When we used the anti-fasciclin III (fas III) antibody to simultaneously follow the fate of the segmental neural columns and glial cells and the visceral mesoderm which surrounds the gut, no defects were observed in germband extended embryos homozygous in srp^{AS282^-} (Fig. 3C and D), tup^{IIIB29} (Fig. 3E and F) or ush^{IIA108} (Fig. 3G and H), or hemizygous in hnt^{XE} (Fig. 3I and J; compare with flb^{JE1} embryos that have lost fas III expression from the neuroectoderm, Fig. 3K and L). Neither did we observe ectopic expression of the *disco* marker on a srp^{AS282^-} background (data not shown), nor any alterations in the distribution of DER protein, present in the segmented mesoderm and, to a lesser extent, in the ectoderm, of extended embryos, on *ush*, *srp* and *tup* mutant backgrounds (data not shown). In addition to demonstrating the absence of defects along the germband, these results imply that the gene product of DER/*flb* is not acting downstream to any of these latter loci.

Zygotic genetic interactions between ush and tup suggest that they represent two molecular pathways functioning in parallel

In order to begin to dissect out the number of pathways that may be involved in germband retraction, we took a genetic approach. We examined the phenotypes of ush^{IIA108} tup^{IIIB29} double mutant embryos to determine whether a shift in the distribution of phenotypes relative to that of single mutants has occurred. Among the *ush tup* double mutant embryos, over 25% had a severe phenotype (Table 2) indicating a greater than additive effect of the two mutations and the requirement for both loci in the initiation of germband retraction. When we examined the effect of the presence of a single mutant tup^{IIIB29} allele on the homozygous ush^{IIA108} phenotype and vice versa, we found a similar proportion of severe mutants in the progeny of these crosses as in the double mutants (Table 2). These results indicate that *ush* and *tup* are dominant enhancers of one another. Since the *tup* allele that we are employing appears to be amorphic, enhancement of the *tup* phenotype by mutations in *ush* suggest that *ush* is acting in a parallel pathway. *tup* and *ush* thus define two separate but partially redundant pathways which contribute jointly to germband retraction (with *ush* playing a slightly predominant role, as indicated by the larger number of severely defective progeny among embryos mutant in *ush* alone).

Zygotic genetic interactions among the loci ush, hnt and srp imply that they lie on the same or closely related pathways

Having established that *ush* and *tup* are likely to be components of two distinct parallel genetic pathways, we determined whether there are differences in the interactions of each of these loci with the other retraction defective loci. Indeed, while we observed multiple interactions for *ush* with *srp* and *hnt*, no interactions were observed for *tup* with these loci. The ush^{IIA108} and srp^{AS282^-} loci acted as reciprocal dominant enhancers of the germband retraction defective phenotype and each of these loci was also able to dominantly enhance the phenotype of hnt^{XE} hemizygous male embryos. When parallel crosses were carried out to examine for dominant interactions between tup^{IIIB29} and srp^{AS282^-} and tup^{IIIB29} and hnt^{XE} (Table 2), no such interactions were observed. Thus, we have observed that *ush*, but not *tup*, interacts with *srp* and *hnt* and that the latter two, both of which display dominant interactions with *ush*, also interact with each other.

The absence of genetic interactions between DER/flb and tup or elements of the ush pathway strengthens the notion that DER/flb may define a uniquely acting molecular pathway

Both the extreme cuticular phenotype of embryos homozygous for flb^{JE1} and the results of our immunohistochemical analysis showing that only flb^{JE1} embryos have defects along the germband point to the possibility that this locus may be functioning uniquely at a different anatomical site from the other loci to implement germband retraction. We sought additional genetic support for this suggestion. When either the weak DER/*flb* allele, JE3, or severe allele, JE1, were introduced onto a tup^{IIIB29}/tup^{IIIB29} background (the former in one copy or two, not shown, and the latter in a single copy, Table 2), there was no enhancement of the tup^{IIIB29}/tup^{IIIB29} phenotype. This is evidenced by the very small number of embryos with a severe phenotype, in virtually the identical proportion as the number of embryos displaying a severe phenotype among tup^{IIIB29}/tup^{IIIB29} progeny. In reverse crosses, designed to test the ability of *tup* to dominantly modify the distribution of phenotypes among *flb*

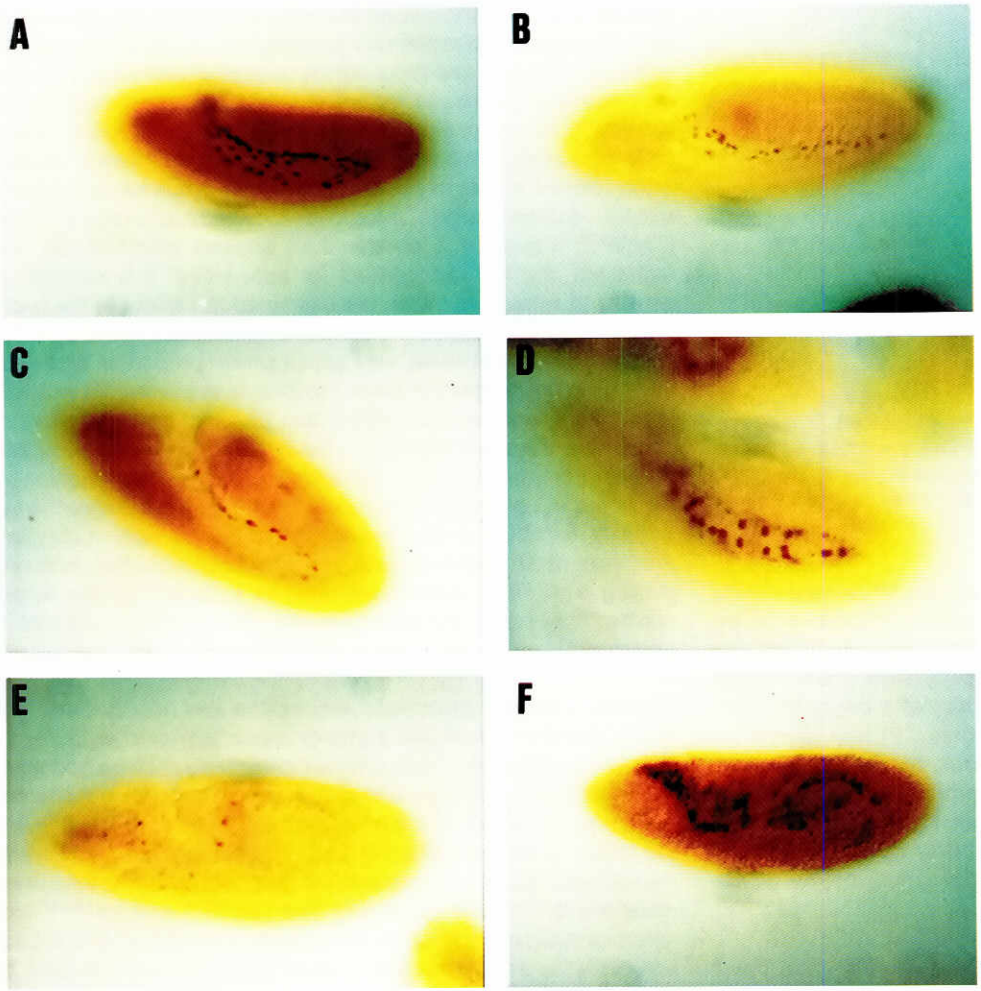

Fig. 2. Visualization of the amnioserosa in wild type and mutant fully extended embryos by immunohistochemical staining with anti-Krüppel antibodies. *Since the Krüppel antigen appears in cells of the neurogenic region at the onset of germband retraction, it was possible to recognize homozygous fully extended embryos that had not yet begun to shorten their germbands. The amnioserosa is present as two lateral wings of tissue that have been pushed aside by the lengthened germband. Parasagittal optical sections which provide visualization of the maximum number of amnioserosal cells in* **(A)** *wild type,* **(B)** *$srp^{AS282^{-}}$,* **(C)** *tup^{IIIB29},* **(D)** *ush^{IIA108},* **(E)** *hnt^{XE} and* **(F)** *flb^{JE1} embryos are shown.*

embryos, no evidence of a phenotypic shift was observed on either a homozygous flb^{JE3} background or a homozygous flb^{JE1} background (not shown). DER/*flb* also did not act as a dominant modifier of two elements of the *ush* pathway, *srp* or *hnt* (Table 2). Nor did *srp* act as a dominant modifier of the weak or strong DER/*flb* phenotype (not shown).

Sensitivity of germband retraction to cytoskeletal inhibitors

Clearly, changes must occur in the germband during retraction. Many of the protein products that carry out these changes, like elements of the cytoskeleton, are likely to have earlier embryonic functions. Consequently, mutations in the genes that encode these proteins will cause lethality before the stage of germband shortening. In order to show involvement of the cytoskeleton in carrying out germband retraction, we applied cytoskeletal inhibitors to live embryos in culture using a recently published embryo culture technique (Strecker *et al.*, 1994). Embryos at the germband extended stage were treated with colchicine or cytochalasin D and examined three hours later for the effects of the drugs on retraction. Most of the control embryos mock-treated with ethanol had undergone retraction by 3 h after treatment (Table 3). This was not the case for the embryos treated with colchicine or cytochalasin D. As can be seen in Table 3, a statistically significant proportion of the drug-treated embryos displayed a completely unretracted phenotype three hours after exposure to the drugs.

The observed results are not due to large-scale cell death. When drug-treated embryos were examined for cell death by inclusion of 0.05% acridine orange in the incubation medium, there was no increase in the number of acridine orange staining cells over control embryos (data not shown). Furthermore, the effects of the cytoskeletal inhibitors are reversible since among drug treated-embryos allowed to develop overnight and examined for germband retraction, about half of the embryos had undergone some degree of retraction (data not shown). These results demonstrate the feasibility of applying the embryo culture technique to short term studies of *Drosophila* embryos exposed to cytoskeletal inhibitors or other drugs. Since both colchicine and cytochalasin D are able to specifically phenocopy the germband retraction mutant phenotype, both the microtubule and microfilamentous cytoskeleton are implicated as participating in the process of retraction either actively or in response to external cues.

Discussion

In an immunohistochemical examination of embryos homozygous for each of four different loci involved in germband retraction, we, like others (Frank and Rushlow, 1996) found that all of the loci, *tup*, *ush*, *srp* and *hnt*, are associated with abnormalities of the amnioserosa. Because of the pivotal position of the

amnioserosa at the interface of the extended germband with other tissues, and since anomalies in the amnioserosa, but not other organs, are shared by *tup*, *ush*, *srp* and *hnt* embryos, we consider it likely that these anomalies are responsible for the common failure to undergo complete germband retraction. Interestingly, *srp* has been shown to promote morphogenesis and differentiation of the endoderm into anterior and posterior midgut (Reuter, 1994). It may also be acting in the dorsal part of the embryo to specify differentiation of cells into amnioserosa since Frank and Rushlow (1996), using a different *srp* allele, have observed earlier abnormalities in the expression of an amnioserosal marker gene, *Race*, in *srp* mutants than in any other germband retraction defective class.

Previously described genetic evidence also points to a role for some of the germband retraction defective loci in amnioserosal development. *hnt*, *ush* and *tup* act as suppressors of the *torso* dominant phenotype in which central regions take on terminal fates in the embryo (Strecker *et al.*, 1991). The mode of suppression by *hnt*, *ush* and *tup* is likely to be by inactivating the ectopically activated *zerknüllt (zen)* pathway in these regions since simultaneous activity of *Toll* and *torso* in the progeny of *torso* dominant mothers allows *zen* to be active where it would normally be repressed by the *Toll* pathway alone (Jiang *et al.*, 1993; Kirov *et al.*, 1993; Lehming *et al.*, 1994), and it has already been suggested that *ush*, *tup* and *hnt*, as well as *srp*, act downstream of *zen* in specification of the amnioserosa (Ray and Gelbart, unpublished).

The mechanism by which the amnioserosal defects cause abortive germband retraction is unclear since Pannier deficient embryos that lack the amnioserosa (Ramain *et al.*, 1993), and display no amnioserosal staining with anti-Krüppel antibodies (data not shown), do not exhibit germband retraction defects. This implies that the role of the amnioserosa in germband retraction is passive and that defects in particular qualities of the amnioserosa can prevent germband retraction from occurring even while its total absence does not affect the process. Accordingly, there does not appear to be a direct correlation between the overall severity of the abnormalities seen with the Krüppel amnioserosal marker and the shortening defects

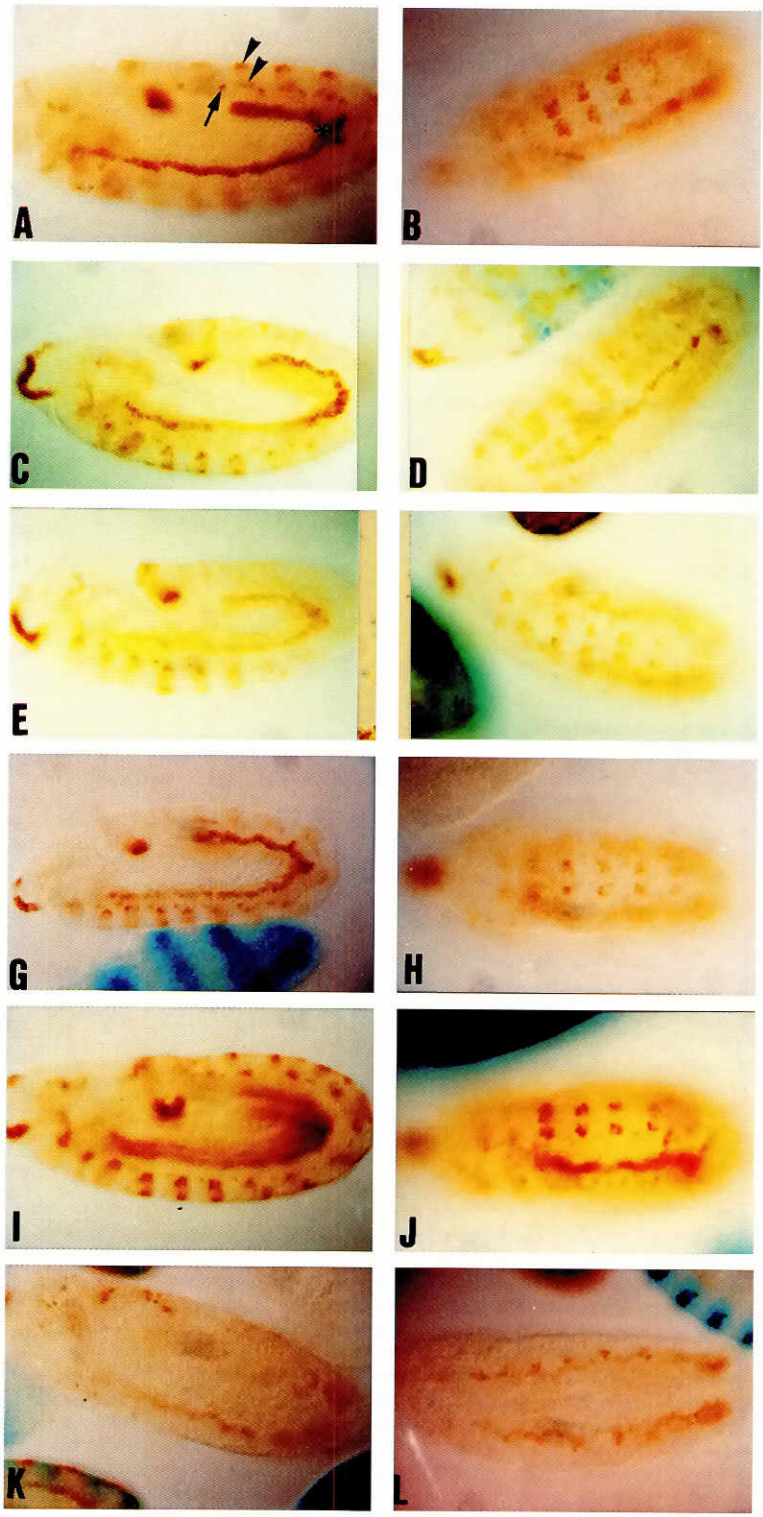

Fig. 3. Visualization of germband structures in wild type and mutant fully extended embryos by immunohistochemical staining with anti-fas III antibodies which recognize several tissues along the germband of extended embryos. *In germband extended embryos, fas III is expressed in segmentally repeated patches of neuroepithelial cells. It is not found in the neuroblasts which delaminate from the neuroepithelial cells and divide to give rise to ganglion mother cells, but is present on the surface of the ganglion mother cells themselves and in the neurons which they generate. Thus beneath each fas III positive outer epithelial patch, and derived from it, is a column of neuroblasts, which do not express the antigen, and deeper ganglion mother cells and neurons which do. Between the patches of fas III expressing neurons, there are also small clusters of additional, probably glial, cells, that are recognized by the anti-fas III antibody. Since fas III appears on the surface of epidermal cells in the segmental grooves immediately before the initiation of retraction, mutant embryos that did not yet exhibit segmental groove staining were examined for their pattern of expression of fas III (Patel et al., 1987). Maximally germband extended embryos were viewed parasagittally (the first of each pair) and ventrally (the second of each pair). The embryos shown are* **(A,B)** *wild type,* **(C,D)** *srp^{AS282}*, **(E,F)** *tup^{IIIB29}*, **(G,H)** *ush^{IIA108}*, **(I,J)** *hnt^{XE}* and **(K,L)** *flb^{JE1}. In* **(A)** *the outer neuroepithelial cells and the inner layer of ganglion mother cells and neurons are shown with arrowheads, a glial cell cluster is shown with an arrow and the visceral mesoderm is indicated by a star.*

TABLE 2

CHARACTERIZATION OF LETHAL PHENOTYPES OF DOUBLE MUTANT EMBRYOS AND HOMOZYGOUS OR HEMIZYGOUS MUTANT EMBRYOS IN THE PRESENCE OF A SINGLE ALLELE OF A SECOND LOCUS

Genotype	Number of unhatched embryos of each phenotypic class: weak or intermediate	severe (%)	Chi square (significance level)
A. Interactions between *ush* and *tup*			
1. ush^{IIA108} tup^{IIIB29} /ush^{IIA108} tup^{IIIB29}	34	12 (26.1%)	
2. ush^{IIA108} tup^{IIIB29} /ush^{IIA108} +	67	31 (31.6%)	14.54* (0.001)[a]
3. ush^{IIA108} tup^{IIIB29} /+ tup^{IIIB29}	84	26 (23.6%)	14.20* (0.001)[b]
B. Interactions between *ush*, *srp* and *hnt*			
1. $\frac{ush^{IIA108}\ /+}{+}$; $\frac{srp^{AS282^-}}{srp^{AS282^-}}$	59	35 (37.2%)	3.13* (0.100)[c]
2. $\frac{ush^{IIA108}}{ush^{IIA108}}$; $\frac{srp^{AS282^-}/+}{+}$	241	65 (21.2%)	6.09* (0.02)[a]
3. $\frac{hnt^{XE}}{\rightarrow}$; $\frac{ush^{IIA108}\ /+}{+}$	79	15 (16.0%)	4.64* (0.05)[d]
4. $\frac{hnt^{XE}}{\rightarrow}$; $\frac{srp^{AS282^-}/+}{+}$	139	27 (16.1%)	5.81* (0.02)[d]
C. Interactions between *tup*, *srp* and *hnt*			
1. $\frac{tup^{IIIB29}\ /+}{+}$; $\frac{srp^{AS282^-}}{srp^{AS282^-}}$	129	55 (29.9%)	0.55 (0.50)[c]
2. $\frac{tup^{IIIB29}}{tup^{IIIB29}}$; $\frac{srp^{AS282^-}/+}{+}$	124	1 (0.8%)	N.D.[b,e]
3. $\frac{hnt^{XE}}{\rightarrow}$; $\frac{tup^{IIIB29}\ /+}{+}$	129	5 (3.7%)	0.06 (0.80)[d]
D. Interactions between DER/*flb* and other loci			
1. tup^{IIIB29} flb^{JE1}/tup^{IIIB29} +	101	1 (1.0%)	N.D.[b,e]
2. $\frac{flb^{JE1}/+}{+}$; $\frac{srp^{AS282^-}}{srp^{AS282^-}}$	53	17 (24.3%)	0.008 (0.98)[c]
3. $\frac{hnt^{XE}}{\rightarrow}$; $\frac{flb^{JE1}/+}{+}$	115	11 (8.7%)	0.52 (0.50)[d]

The desired progeny class for characterization of lethal phenotypes was obtained by collecting the unhatched progeny of a self cross of heterozygous double mutants (A.1) or the unhatched progeny resulting from a cross between a double mutant strain and a second strain heterozygous for a mutation in one of the two loci (A.2, A.3, D.1). In these cases, the unhatched progeny were all of the class of interest. In crosses involving hnt^{XE} (B.3, B.4, C.3, D.3) and in the remaining cases, the desired progeny were obtained as described in Materials and Methods. In these instances, the genotypic class of interest comprised (theoretically) only half of the lethal progeny and was indistinguishable from the other half. The two possible resulting genotypic classes are separated by a large, bold slash (**/**) and the existence of these two classes was taken into account in the statistical analyses, as described in Materials and Methods. All egg collections were carried out on at least two independent crosses, and, since they displayed the same tendencies, the results were pooled. [a]Chi square value when compared to phenotype of ush^{IIA108}/ush^{IIA108} embryos; [b]Chi square value when compared to phenotype of tup^{IIIB29} /tup^{IIIB29} embryos; [c] Chi square value when compared to phenotype of srp^{AS282^-}/srp^{AS282^-} embryos; [d] Chi square value when compared to phenotype of hnt^{XE}/→ embryos; [e] Chi square value cannot be determined because there are too few embryos in the severe class, but clearly no significant differences as compared to the distribution of tup^{IIIB29} / tup^{IIIB29} embryos; *significant values

of mutant embryos of different genotypes. For example, the srp^{AS282^-} allele, with a weak amnioserosal phenotype, causes pronounced intermediate to severe retraction defects, and the total absence of cells expressing Krüppel in hnt^{XE} embryos is associated with only an intermediate to weak cuticle phenotype. The specific characteristics of the amnioserosa that are necessary to permit shortening of the germband to take place are not known.

Although it is possible that the amnioserosal defects of DER/*flb* embryos is also the cause of their severe phenotype, we would like to suggest that it is the unique activity of DER/*flb* at a discrete, alternative site, the germband itself, that contributes the overriding factor to the complete inability of homozygous flb^{JE1} embryos to retract. Many lines of evidence support this hypothesis. One is the extreme phenotype of embryos carrying the flb^{JE1} allele. Unlike embryos bearing any of the other alleles that we have examined, all flb^{JE1} embryos have a severe retraction defective phenotype. Another line of evidence is our immunohistochemical observations. Using both fas III and the DER protein itself as markers, we showed that only embryos mutant in DER/*flb* display defects along the germband. The ventral effects of mutations in DER/*flb* along the germband have already been recognized (Raz and Shilo, 1993). Indirectly, they have also been demonstrated to be relevant to germband retraction. First, the temperature sensitive period of an *flb* temperature sensitive allele for ventral fate changes coincides with that for defects in germband retraction. Secondly, and more convincingly, weak mutations in DER/*flb* are synergistic in their effect on the germband retraction phenotype with members of the *spitz* group of genes, which includes *spitz (spi)* and *Star (S)*. Mutations in the *spitz* group act in the ventral ectoderm. They cause longitudinal strips of the ventral or ventrolateral ectoderm to be lacking. On their own they do not give rise to germband retraction defects (Mayer and Nüsslein-Volhard, 1988). In contrast to the interactions observed between DER and the *spitz* group, we have not observed any synergistic effects on germband retraction between tup^{IIIB29} and *Df*(2L)*S-3* (data not shown). A final line of evidence suggesting that the function of DER/*flb* in a discrete anatomical site constitutes its major contribution to germband retraction is the series of genetic interactions that we have documented among the other retraction defective loci but fail to observe between them and DER/*flb* (see below).

TABLE 3

EFFECTS OF CYTOSKELETAL INHIBITORY DRUGS ON GERMBAND RETRACTION

	Number of embryos with each phenotype: unretracted	partially retracted	fully retracted	Chi square (significance level)
embryos at time of application of drugs	61	7	12	
control (ethanol treated)	27	18	51	19.7 (<0.001) **
10 µg/ml cytochalasin D	41	19	3	
control (ethanol treated)	12	7	68	17.2 (<0.001) **
20 µg/ml colchicine	37	15	33	

[a]Chi square values obtained by comparing number of (partially retracted + fully retracted) *vs.* unretracted embryos in drug treated and control groups; *significant values.

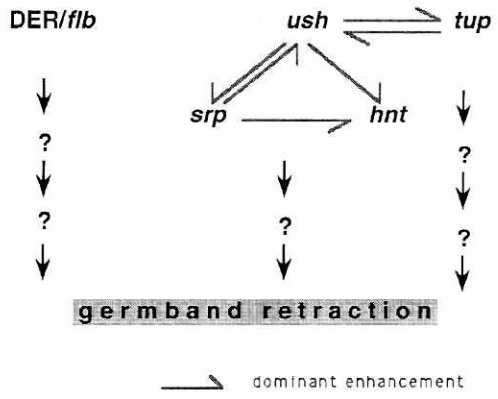

Fig. 4. Model of the molecular pathways that are proposed to be active in germband retraction. *Only those instances in which genetic interactions between two loci were observed are indicated. In all cases where dominant enhancement was observed, it occurred at less than the expected 50% penetrance. This is due, we believe, to the simultaneous operation of parallel pathways. Possible enhancement effects of hnt on other loci could not be tested because of the location of hnt on the first chromosome which would cause a large number of different classes of lethal progeny to be found among the offspring of a cross introducing hnt onto the background of the other germband retraction defective loci.*

The genetic interactions that we have documented are summarized in Figure 4. These suggest that *ush*, which acts as a mutual dominant enhancer of an amorphic *tup* allele, is functioning in a parallel, but partially redundant, pathway to the *tup* pathway. In addition, they provide evidence that *srp* and *hnt* may be acting in the same or closely related pathways with *ush*. In this regard, it is interesting that Frank and Rushlow (1996) have reported that amnioserosal cell loss is a consequence of programmed cell death in *ush*, *hnt* and *srp* mutants, but not in embryos that are mutant in *tup*. Elucidation of the exact epistatic relationships between the *ush*, *srp* and *hnt* loci will have to await additional genetic experiments (e.g. to see whether lack of one gene product can be rescued by additional doses of a second) or cloning of the different genes, which will make it possible to examine the relative times of gene expression and changes of expression of one gene product on the background of mutations in the other loci. The multiplicity of dominant interactions among the loci *ush*, *srp* and *hnt* and, to a lesser extent, *tup*, suggests that the process of germband shortening is, in general, sensitive to the dosage of the gene products of many of the germband retraction defective loci and that the observed results are not allele specific. At the same time, the absence of genetic interactions between DER/*flb* and any of the other germband retraction defective loci strengthens the notion that the gene product of DER/*flb* functions in a distinct pathway.

We have demonstrated that germband retraction probably requires the simultaneous activity of cellular pathways that function in the dorsal amnioserosal tissue and one pathway acting along the germband itself. The gene products of *ush*, *srp*, *hnt* and *tup* appear to be components of at least two interdependent pathways that determine the proper differentiation or maintenance of the amnioserosa, including characteristics that are necessary to allow germband retraction to be carried out. DER/*flb* may uniquely represent a pathway functioning in the active part of the germband extended embryo — along the entire length of the extended germband on its ventral aspect. Ultimately cell shape changes mediated by the cytoskeleton are likely to be responsible for movement of the germband. The nature of these cytoskeletal changes and whether they are directly triggered by the DER/*flb* pathway or require the prior activation of this pathway in establishing ventral identity, remains to be studied. Nonetheless, we believe that the relationships between the germband retraction defective loci revealed by our genetic and immunohistochemical studies can serve as a basis for the further unraveling of the molecular pathways that carry out this morphogenetic process, including the involvement of additional loci that we have not investigated.

Materials and Methods

Drosophila melanogaster stocks

Fly stocks, most of which are described in Lindsley and Zimm (1992), were in general obtained from the Bloomington Stock Center with the following exceptions: the JE alleles of DER/*flb* which originate from J. O'Donnel, the *Star* deficiency, *Df*(2L)*S-3*, from N. Perrimon. The C50.1S1 enhancer trap line originates from S. Cohen and W. Gehring (Cohen *et al.*, 1991). The transposon has integrated in the region of the *disco* gene and displays an expression pattern similar to that gene. It was used in this work for labeling the precursors of the Keilin's organs. The *serpent (srp)* presumptive imprecise excision allele that we employed, $srp^{AS282^{*}}$, was generated in our laboratory (details to be reported elsewhere) by remobilizing the transposable element in P element insertion strain AS282 (Cooley *et al.*, 1988), which we found does not complement srp^{9L06} and is thus allelic to it. We chose to work with this allele because the srp^{9L06} allele (Nüsslein-Volhard *et al.*, 1984) displays only a weak germband retraction defective phenotype. In addition, the presence of second site mutations cause it to be very prone to non-specific enhancing interactions (data not shown).

Immunohistochemical procedures

To discriminate between wild-type and mutant embryos, balancer chromosomes carrying P elements expressing *lacZ* were used. Expression of *lacZ* was detected by standard X-gal staining. Antibody staining was carried out by previously described protocols (Zak *et al.*, 1990). Anti-Krüppel antibodies (6840) were provided by Chris Rushlow and used at a 1:1500 dilution. Mouse antibodies against fasciclin III were received from T. Volk (unpublished) and used at a 1:3 dilution. The anti-DER antibodies have been described previously (Zak *et al.*, 1990). Stained embryos were observed under Nomarski optics and photographed.

Genetic procedures

The loci that we examined are *U-shaped (ush)*, *tailup (tup)* and DER/*faint little ball(flb)* on the second chromosome, maintained over *CyO, hindsight (hnt)* on the X chromosome, maintained over *FM7* and *serpent (srp)* on the third chromosome maintained over *TM3, Sb*. Double mutants between loci on the same chromosome were generated by meiotic recombination. When checking for dominant enhancement between mutations in two loci that are not on the same chromosome, the desired progeny were obtained in two crosses — the first cross generated the double heterozygotes, which were viable in all the cases we tested, and the second cross introduced an additional copy of one of the two mutant alleles into the population. In crosses involving hnt^{XE}, heterozygous hnt^{XE} females were crossed to males heterozygous for the second locus of interest. The lethal progeny were all males hemizygous for hnt^{XE}, half of which were also heterozygous for the additional locus. Thus, in all crosses between loci that are not on the same chromosome, the progeny of interest constituted (theoretically) 50% of the entire population of lethal embryos and could not be distinguished from the remaining 50%. This fact was taken into account in the statistical analyses described below.

For cuticle preparations, embryo collections on sugar/agar/apple juice plates supplemented with yeast paste were allowed to develop until all wild type embryos hatched. Unhatched embryos were dechorionated in bleach, mounted in 1:1 Hoyer's-lactic acid and allowed to clear overnight at 60°C. Cuticles were examined under darkfield illumination and photographed with T-Max 100.

Statistical analyses

The results of our genetic crosses did not yield a clear new progeny class. We checked for significant differences between populations by looking for changes in the distribution of progeny among the germband retraction defective categories. The cuticular phenotype was scored as being severe, intermediate or weak, by drawing an imaginary line between the two arms of the folded metameric germband and counting the number of ventral denticle bands that remained dorsal to this line. We define a "severe" phenotype as one in which the posteriormost four ventral denticle bands (A5-A8) have remained on the dorsal side of the embryo (Fig. 1D), i.e. virtually no germband retraction has taken place. In an "intermediate" phenotype, two to three denticle bands are dorsally displaced (Fig. 1C), indicating that some retraction has occurred, and in a "weak" phenotype either one band is dorsal or all segments have retracted but the cuticle still does not appear wild type (for example the filzkorper has not everted, Fig. 1B; compare with wild type in Fig. 1A). We present the phenotype of each allele as the distribution of severe versus (intermediate plus weak) mutant progeny since we found that this was the best way to determine whether enhancement of the mutant phenotype had occurred in the test group versus the control group. The comparison was done by conducting a 2x2 Chi square analysis with a continuity factor. In crosses that were carried out in which theoretically only 50% of the progeny are of the genotype of interest, with the remainder being of the control group, we took a significance level of 0.1 to be indicative of a significant difference between the experimental population and the controls.

Embryo culture

Embryo culture during and after treatment with cytoskeletal perturbing drugs was carried out essentially as described in Strecker *et al.* (1994), using hexane to permeabilize embryos. To obtain embryos at full germband extension, embryos were collected over a period of 1 h and allowed to continue to develop for 7 h at 25°C. The germband extended embryos were placed into live embryo culture and treated for 15 min with cytoskeletal inhibitors. The drugs were removed and the embryos were maintained in culture for an additional 3 h prior to fixation and microscopic examination. At the end of the incubation period embryos were fixed as for immunohistochemical procedures and mounted in Permount. They were scored for the degree of germband retraction by observation under Nomarski optics with a Zeiss Axioskop microscope.

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