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Role of the Amnioserosa in Germ Band Retraction of the *Drosophila melanogaster* Embryo

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As the germ band shortens in *Drosophila melanogaster* embryos, cell shape changes cause segments to narrow anteroposteriorly and to lengthen dorsoventrally. One of the genes required for this retraction process is the *hindsight* (*hnt*) gene. *hnt* encodes a nuclear Zinc-finger protein that is expressed in the extraembryonic amnioserosa and the endodermal midgut prior to and during germ band retraction (M. L. R. Yip, M. L. Lamka, and H. D. Lipshitz, 1997, *Development* 124, 2129–2141). Here we show, through analysis of *hnt* genetic mosaic embryos, that *hnt* activity in the amnioserosa—particularly in those cells that are adjacent to the epidermis—is necessary for germ band retraction. In *hnt* mutant embryos the amnioserosa undergoes premature cell death (L. C. Frank and C. Rushlow, 1996, *Development* 122, 1343–1352). We demonstrate that prevention of premature apoptosis in *hnt* mutants does not rescue retraction. Thus, failure of this process is not an indirect consequence of premature amnioserosal apoptosis; instead, *hnt* must function in a pathway that controls germ band retraction. We show that the *Krüppel* gene is activated by *hnt* in the amnioserosa while the *Drosophila* insulin receptor (INR) functions downstream of *hnt* in the germ band. We present evidence against a physical model in which the amnioserosa "pushes" the germ band during retraction. Rather, it is likely that the amnioserosa functions in production, activation, or presentation of a diffusible signal required for retraction.

Key Words: hindsight;* germ band retraction; *Drosophila;* amnioserosa; apoptosis; insulin receptor (*inr*); *Krüppel.

INTRODUCTION

During animal development, morphogenetic events are driven by cell shape changes, cell movements, cell death, and polarized cell division (Fristrom, 1988). In *Drosophila melanogaster,* both genetic and molecular analyses can be brought to bear on a mechanistic dissection of these processes. Morphogenetic events in *Drosophila* begin soon after cellularization of the blastoderm embryo and play a major role in the processes of gastrulation, germ band extension and retraction, dorsal closure, and head involution (Campos-Ortega and Hartenstein, 1997). The cellular mechanisms underlying these morphogenetic movements are diverse. For example, formation of the ventral furrow during gastrulation and dorsal closure of the epidermis later in embryogenesis require coordinate cell shape changes (Leptin and Grunewald, 1990; Kam *et al.*, 1991; Sweeton *et al.*, 1992; Costa *et al.*, 1993; Young *et al.*, 1993). Germ band extension, on the other hand, is driven largely by local cell rearrangements (Costa *et al.*, 1993; Irvine and Wieschaus, 1994). During germ band retraction, the extended, "u-shaped" germ band shortens, bringing the caudal end of the germ band to the posterior of the embryo (Campos-Ortega and Hartenstein, 1997). Cell shape changes in the epidermis are sufficient to account for most of the shortening of the germ band during retraction (Martinez-Arias, 1993).

A number of genes have been defined in *Drosophila* that play specific roles in coordinating morphogenetic events. For example, the secreted protein encoded by *folded gastrulation* is postulated to bind to a cell surface receptor that is associated with the *concertina* Gα subunit protein, ultimately resulting in the coordinate cell shape changes that drive ventral furrow formation and posterior midgut invagination (Parks and Wieschaus, 1991; Costa *et al.*, 1993, 1994). Genes in the JUN-amino terminal kinase and *decapentaplegic* pathways have been shown to control dorsal closure through regulation of the actin-myosin cytoskeleton (Affolter *et al.*, 1994; Glise *et al.*, 1995; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996; Glise and Noselli, 1997; Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar and Hafen, 1997).

Mutations in a number of genes disrupt the process of

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germ band retraction. One of these, *hindsight* (*hnt*) (Wieschaus *et al.*, 1984; Frank and Rushlow, 1996; Goldman-Levi *et al.*, 1996; Yip *et al.*, 1997), encodes a putative Zinc-finger transcription factor that is expressed in the midgut and amnioserosa, but not in the epidermis, prior to and during germ band retraction (Yip *et al.*, 1997). Since it is the epidermis that undergoes morphological changes during germ band retraction (Martinez-Arias, 1993), we speculated that HNT expression in the midgut or amnioserosa might regulate a signaling process required for epidermal cell shape changes during retraction (Yip *et al.*, 1997). Embryonic lethal mutations in *hnt* result not only in germ band retraction defects, but premature *reaper*-mediated apoptosis of the amnioserosa (Frank and Rushlow, 1996). Mutations in the *Drosophila* homolog of the insulin receptor (*inr*) also result in failure of germ band retraction (Fernandez *et al.*, 1995). The INR is expressed throughout the germ band but is absent from the amnioserosa (Fernandez *et al.*, 1995).

Here we investigate the role of the amnioserosa in germ band retraction. We demonstrate that *hnt* activity in the amnioserosa—particularly in those cells that abut the epidermis—is necessary for retraction. We find that premature apoptosis of the amnioserosa is not the cause of failed germ band retraction in *hnt* mutants. We demonstrate that the *Krüppel* gene is activated by HNT in the amnioserosa. Furthermore, we find that overexpression of the INR can rescue the germ band retraction defects in *hnt* mutants despite the fact that the amnioserosa still undergoes premature apoptosis. These results suggest that the INR transduces a retraction signal that originates in the amnioserosa and is received by the germ band.

MATERIALS AND METHODS

Drosophila Strains

The following embryonic lethal *hnt* alleles were used: hnt^{XE81}, hnt^{EH704a}, and hnt^{XO01} (Wieschaus *et al.*, 1984; Eberl and Hilliker, 1988; Yip *et al.*, 1997). *Df(3L)H99*, a small deficiency that removes *reaper, head involution defective*, and *grim*, was used to suppress apoptotic cell death (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1997). The doubly mutant stocks *y* hnt^{XO01} or *y* hnt^{XE81}*/FM7 (ftz-lacZ); Df(3L)H99,* kni^{ri-1} p^{p}*/TM6B, Tb ca P[Ubx-lacZ,* w^{+}*]* were produced to assay germ band retraction in *hnt/Y; Df(3L)H99* embryos. The *HS-inr17,* $Df(3R)e^{D7}$ line carries a heat-inducible *inr* minigene, *HS-inr17,* and a deficiency, $Df(3R)e^{D7}$, that removes the *inr* gene (Fernandez *et al.*, 1995). The *HS-inr17* insertion produces high levels of ectopic INR protein throughout embryos even in the absence of heat shock (Fernandez *et al.*, 1995). The srp^{PZ} enhancer trap (01549, Rehorn *et al.*, 1996) was used to assay *srp* expression in *hnt* mutants. The $puc^{A251.1F3}$ enhancer trap (Martin-Blanco *et al.*, 1998) was used to assay *puc* expression in *hnt* mutants.

Genetic Mosaic Analysis

The *Fs(3)Horka* mutation was used to generate *hnt* embryonic gynandromorphs (Erdélyi and Szabad, 1989; Szabad *et al.*, 1995). Embryos from a mating of *y* hnt^{XE81}*/FM7 (ftz-lacZ)* females and *Fs(3)Horka/TM3* males were fixed and stained with anti-HNT monoclonal antibodies to identify *hnt* mosaics. *Fs(3)Horka* causes loss of the paternal (wild-type) X chromosome in the embryo (Erdélyi and Szabad, 1989; Szabad *et al.*, 1995). Since germ band retraction is complete by stage 13 (Campos-Ortega and Hartenstein, 1997), embryos that were stage 13 or older were selected for analysis. The anterior and posterior midgut and amnioserosa were scored for HNT staining in germ band retracted and unretracted *hnt* mosaic embryos. Three categories were used: (i) all cells in the tissue were genotypically wild type (hnt^{+}), (ii) all cells in the tissue were genotypically mutant (hnt^{-}), or (iii) the tissue was mosaic, including both hnt^{+} and hnt^{-} cells. In those unretracted embryos in which amnioserosal tissue could not be morphologically identified due to premature apoptosis (Frank and Rushlow, 1996), the amnioserosa was scored as hnt^{-}. As a control for germ band retraction defects unrelated to loss of *hnt* in mosaics, embryos from a mating of Canton S females and *Fs(3)Horka/TM3* males were fixed and stained with anti-HNT monoclonal antibodies. These embryos were scored for success or failure of germ band retraction.

Phenotypic Rescue Experiments

y hnt/FM7 (ftz-lacZ) females were crossed to *w; HS-inr17,* $Df(3R)e^{D7}$*/TM3* males. Two different methods were used to analyze *hnt* mutant progeny in tests for rescue of germ band retraction defects. In the first method, embryos were collected for 5–8 h at 25°C and then allowed to age at least 12 h at 25°C. They were then transferred to a water-filled dish in which they were left overnight to complete embryonic development and to drown hatching first instar larvae. The following day all unhatched embryos and larvae were collected and their cuticles were mounted for analysis (see below). *hnt* mutant cuticles were identified by the presence of the *y* marker. In the second method, embryos were collected for 3 h at 25°C and then allowed to age for an additional 12 h at 25°C before fixation. They were then double immunostained with monoclonal anti-HNT and anti-EN antibodies. In a separate experiment, embryos were collected for 3 h at 25°C, aged 3.5 h, heat shocked for 2 × 30 min at 36°C (with a 1-h recovery period between heat shocks), and allowed to recover for an additional 1.5 h. They were then fixed and processed for hybridization with antisense-*reaper* RNA probe followed by anti-HNT immunostaining.

Cuticle Preparations

Stage 17 embryos were dechorionated in 50% bleach for 2 min, devitellinized in 1:1 heptane:methanol while vortexing for 1 min, fixed in 4:1 acetic acid:glycerin at 60°C for at least 15 min, and then stored in the acetic acid:glycerin mixture until mounted. Cuticles were mounted according to Lamka *et al.* (1992).

In Situ Hybridization, Immunostaining, and Tdt-Mediated Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Procedures

Embryos were prepared and stained according to the procedures described in Patel (1994) except in the case of anti-dp-ERK staining for which embryos were fixed in 8% formaldehyde. Mouse monoclonal anti-HNT (Mab 27B8 1G9) (1:10 dilution, see Yip *et al.*, 1997), anti-EN (MAb 4D9) (1:10 dilution, see Patel *et al.*, 1989), anti-dp-ERK (from Sigma Chemical, used at 1:150 dilution, see Gabay *et al.*, 1997a,b), and anti-β-galactosidase antibodies (1:300 dilution; Promega) were detected using a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:300 dilution; Jackson ImmunoResearch). Rabbit polyclonal anti-KR (1:300, see Gaul *et*

al., 1987) and affinity-purified rabbit anti-DM-JUN (1:1000 dilution, see Bohmann *et al.*, 1994) were detected using a HRP-conjugated anti-rabbit antibody (1:300 dilution; Jackson Immuno-Research). For double labeling with anti-HNT and a second antibody, sequential HRP reactions were performed, and detection of HNT was enhanced by the addition of 0.064% nickel chloride to the staining solution. Embryos were mounted in glycerol.

For *in situ*/antibody double-labeling experiments, *in situ* hybridization was followed by antibody staining. *In situ* hybridization was performed as described (O'Neill and Bier, 1994). Digoxigenin-labeled *reaper*, *ush*, and *scab* antisense RNA probes were synthesized by in vitro transcription off of the T3 promoter, respectively, from the p13B2 plasmid (White *et al.*, 1994), Bluescript *ush* *Kpn*I–*Hin*dIII plasmid (Cubadda *et al.*, 1997), and Bluescript comp αPS3 plasmid (Stark *et al.*, 1997) using the Ambion Megascript kit. Digoxigenin-labeled *pnr* antisense RNA probe was synthesized by *in vitro* transcription off of the T7 promoter from the Bluescript *pnr* *Hin*dIII–*Eco*RI plasmid (Ramain *et al.*, 1993) using the Ambion Megascript kit. Digoxigenin-labeled antisense *vn* probe was provided by Dr. A. Simcox. After transcript visualization, embryos were washed, dehydrated in an ethanol series, and stored overnight at 4°C in 70% ethanol as described (Cohen and Cohen, 1992; Lehmann and Tautz, 1994). Embryos were rehydrated the following day, immunostained for HNT as described above, and mounted in glycerol.

For TUNEL/antibody double-labeling experiments, embryos were fixed according to the procedure described in Patel (1994). TUNEL was performed as previously described (White *et al.*, 1994) except that digoxigenin-16–dUTP was used for incorporation. Label was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody at a dilution of 1:2000. Following detection, embryos were washed and dehydrated as above. After rehydration the following day, embryos were immunostained for HNT as described above and then mounted in glycerol.

RESULTS

hindsight Function Is Required in the Amnioserosa for Germ Band Retraction

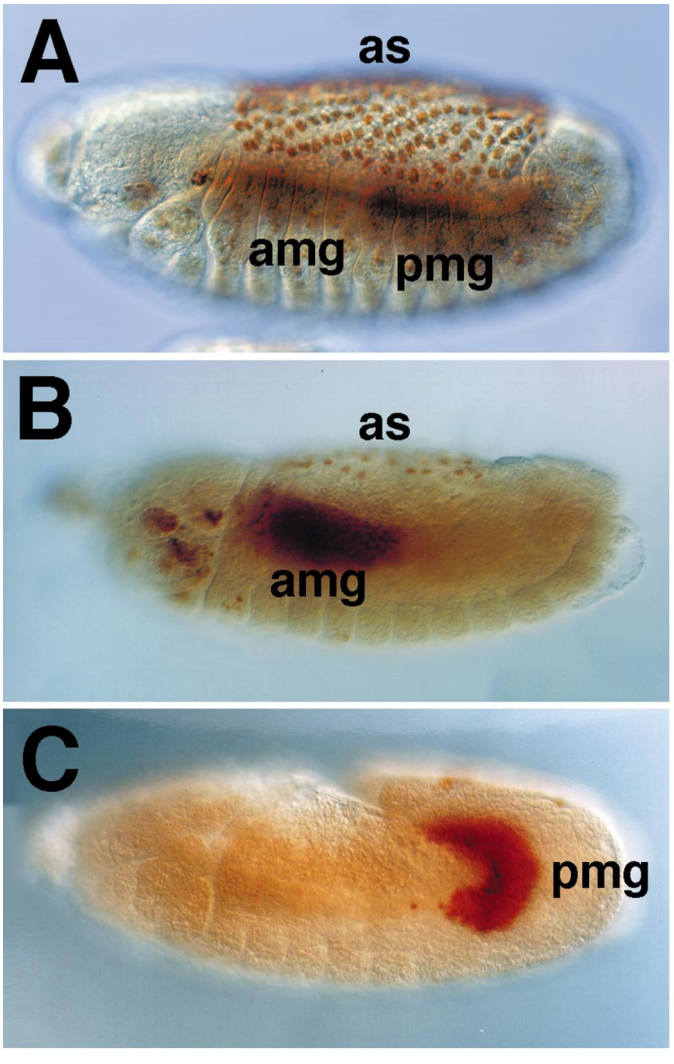
HNT is expressed in the amnioserosa and in the anterior and posterior midgut of wild-type embryos prior to and during germ band retraction (Yip *et al.*, 1997). To define the spatial requirements for HNT activity in germ band retraction, embryos genetically mosaic for *hnt* were produced by inducing wild-type X chromosome loss in heterozygous (hnt^{XE81}/+) embryos (Szabad *et al.*, 1995). Hemizygous (hnt^{XE81}/0) mutant tissues were identified by the absence of staining with anti-HNT antibodies (Fig. 1). The results of analysis of 77 *hnt* mosaic embryos are shown in Fig. 2. There is a strong correlation between the genotype of the amnioserosa and success or failure of germ band retraction: 100% of the mosaics in which germ band retraction was successful had hnt^+ tissue in the amnioserosa (n = 22) while 100% of the embryos with a fully mutant amnioserosa failed to undergo germ band retraction (n = 34). Overall, 96% of the *hnt* mosaics that failed to complete germ band retraction possessed *hnt* mutant patches that included all or part of the amnioserosa (n = 55). A total of 4.5% of wild-type control embryos were defective in germ band retraction (n = 332), a similar frequency to that observed for unretracted mosaics with a fully hnt^+ amnioserosa (3.6%, n = 55). The genotype of the anterior or posterior midgut is uncorrelated with success or failure of germ band retraction. For example, 47% of the embryos with a completely mutant anterior midgut failed, while 53% succeeded, in germ band retraction (n = 19). Similarly, 55% of the embryos with a completely mutant posterior midgut failed, while 45% succeeded, in retraction (n = 11). Since the anterior and posterior midgut primordia are at opposite ends of the fate map, none of the 77 mosaic embryos was completely mutant in both of these tissues; thus we cannot exclude the possibility that in such a case germ band retraction would fail. Despite this caveat, our results prove that *hnt* activity in the amnioserosa is necessary for germ band retraction.

hindsight Function Is Important in the Amnioserosal Cells That Abut the Epidermis

We next assayed spatial requirements for *hnt* function within the amnioserosa. To do this we mapped the hnt^+ and hnt^- cells in the amnioserosas of 10 embryos that had genetically mosaic amnioserosas and had successfully completed germ band retraction (Figs. 2 and 3). There was a clear bias toward genotypically wild-type cells residing at the edge of the amnioserosa. The average percentage of wild-type cells in the 10 amnioserosas was 60% (range 16–96%; see Fig. 3). In contrast the average perimeter occupied by

FIG. 1. *hnt* genetic mosaic embryos. (A) Stage 13 wild-type and (B, C) hnt^{XE81} mosaic embryos immunostained with anti-HNT antibody to reveal wild-type (stained) and mutant (unstained) tissue. (A) Wild-type embryo (after germ band retraction) showing the presence of HNT in nuclei of several tissues. Labeled are the anterior midgut (amg), posterior midgut (pmg), and amnioserosa (as). (B) Stage 13 hnt^{XE81} mosaic embryo with a mutant patch that includes the posterior midgut (unstained) and part of the amnioserosa (partially stained) but not the anterior midgut (stained). This embryo successfully underwent germ band retraction. (C) Late stage 15 hnt^{XE81} mosaic embryo with a mutant patch that includes the anterior midgut and amnioserosa (both unstained) but not the posterior midgut (stained). This embryo failed to complete germ band retraction. In this and subsequent figures embryos are shown with anterior to the left and dorsal toward the top of the page.

FIG. 2. Analysis of germ band retraction in hnt^{XE81} mosaic embryos. Each row represents a single genetically mosaic embryo while each column represents a different tissue type. Embryos are grouped according to whether the germ band was retracted (left) or unretracted (right). Note that in those unretracted embryos in which amnioserosal tissue could not be morphologically identified due to premature apoptosis (Frank and Rushlow, 1996), the amnioserosa was scored as hnt^-.



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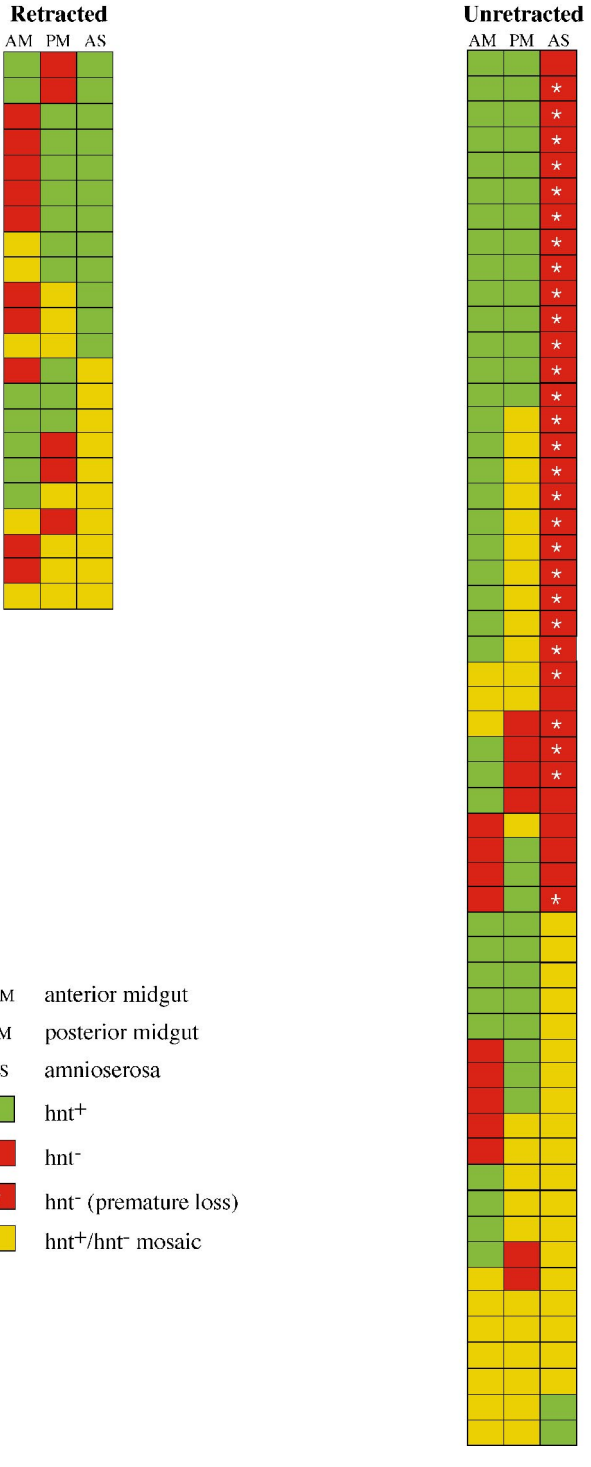

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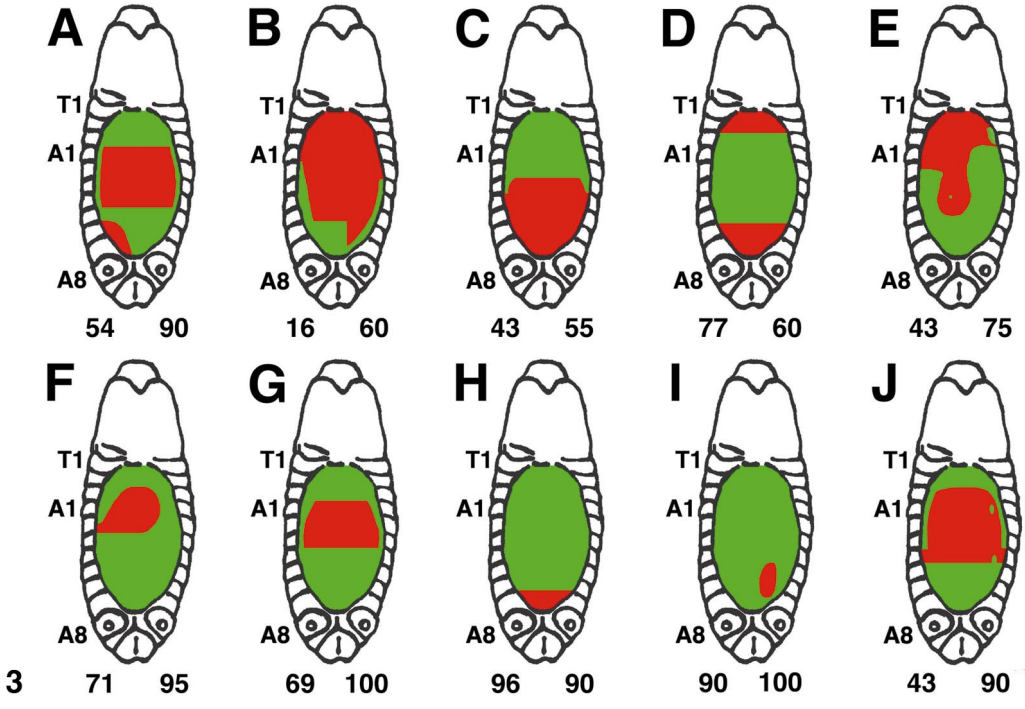

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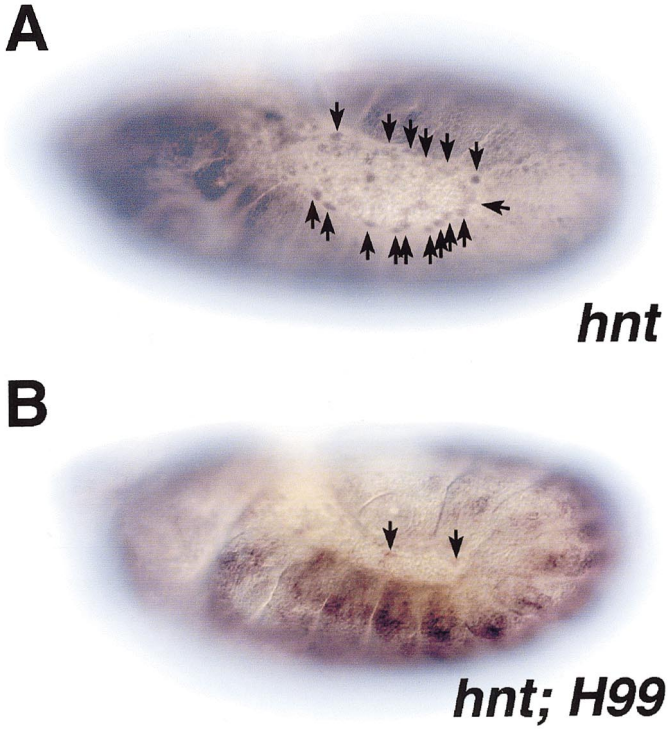

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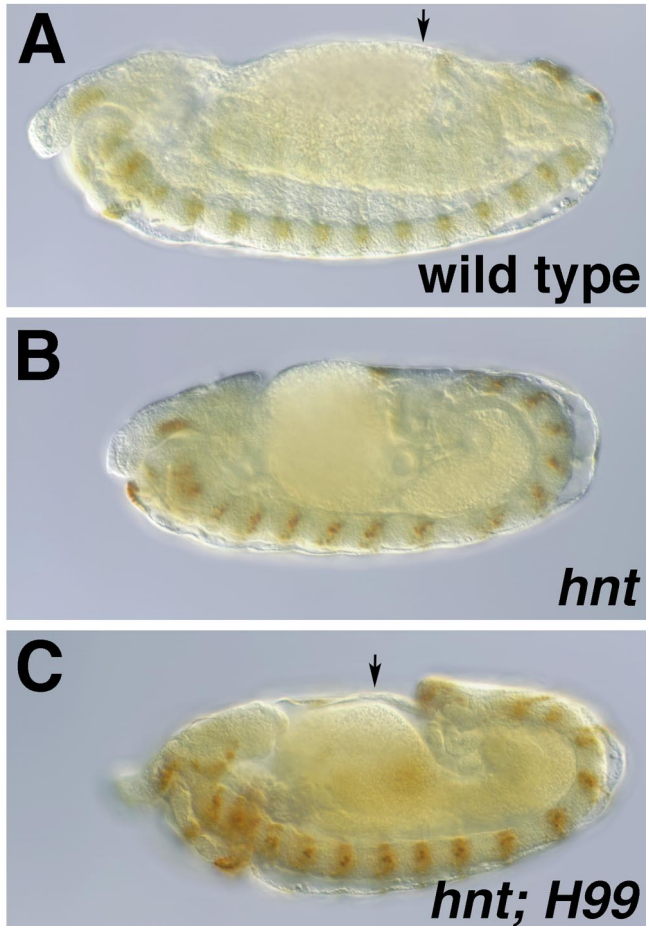

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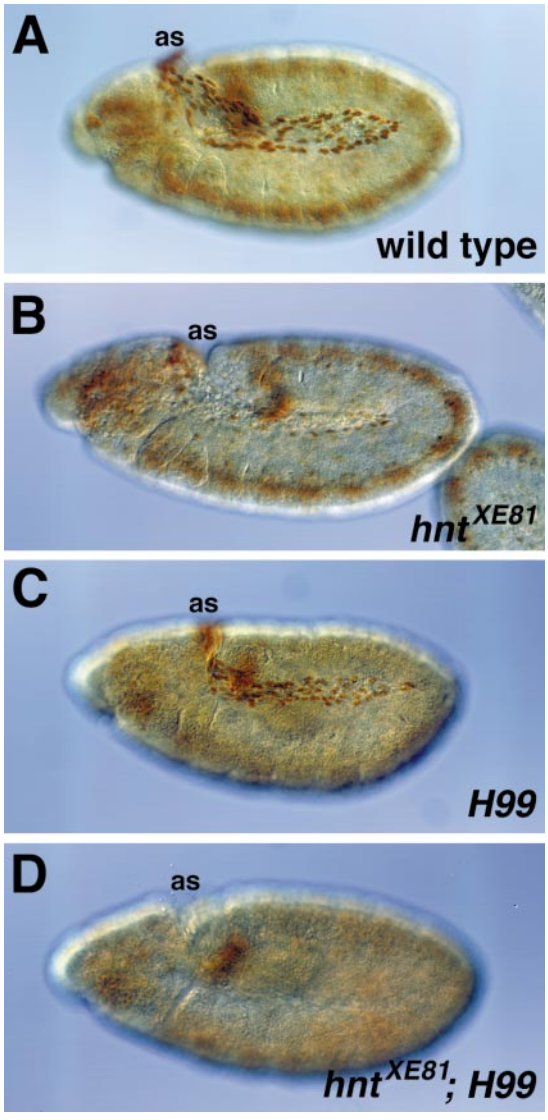

FIG. 6. The *Krüppel* gene is regulated by *hnt* in the amnioserosa (as) of *hnt; H99* double mutants. Stage 11 wild-type (A), hnt^{XE81} (B), *H99* (C), and hnt^{XE81}*; H99* (D) embryos immunostained for KR protein. KR is present in the amnioserosa in wild type (A) and *H99* (C) mutants. However, KR is absent from most, but not all, amnioserosal nuclei in *hnt* mutants (B) and is absent from the amnioserosa of *hnt; H99* double mutants (D) despite rescue of amnioserosal survival.

genotypically wild-type cells was 82% (range 55–100%; see Fig. 3). Particularly striking were two cases (Figs. 3B and 3J) with small patches of wild-type cells but a high fraction of amnioserosa perimeter occupied by these cells (Fig. 3B, patch 16%, perimeter 60%; Fig. 3J, patch 42%, perimeter 90%). The complete hnt^+ versus hnt^- cell distribution in the amnioserosas of unretracted embryos with mosaic amnioserosas could not be mapped because of the folded nature of their amnioserosas; however, the genotypes of cells at the perimeter of 17/19 of these amnioserosas could be scored unambiguously. The average perimeter occupied by genotypically wild-type cells in these unretracted embryos was 42% (range 4–90%), one-half the average in retracted embryos. Together these results suggest that the presence of hnt^+ amnioserosal cells adjacent to the epidermis is required for germ band retraction.

hindsight Function Is Required in the Amnioserosa for Germ Band Retraction Independent of Its Role in Preventing Premature Apoptosis

It has been suggested that failure of germ band retraction in *hnt* mutants is an indirect consequence of premature amnioserosal loss (Frank and Rushlow, 1996). To test this hypothesis, we blocked amnioserosal cell death in *hnt* mutant embryos with the small deletion *Df(3L)H99* (White *et al.*, 1994). *Df(3L)H99* removes the three cell death genes *reaper, head involution defective,* and *grim* (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996). *Df(3L)H99* single mutants complete germ band retraction (data not shown, and see White *et al.*, 1994). TUNEL, a marker of cells that are undergoing programmed cell death, confirmed that premature apoptosis of the amnioserosa is suppressed in *hnt; Df(3L)H99* double mutants (Fig. 4). While many amnioserosal nuclei are TUNEL positive in hnt^{XE81} single-mutant embryos (Fig. 4A), few are labeled in wild-type (data not shown and see Frank and Rushlow, 1996) or in hnt^{XO01}*; Df(3L)H99* and hnt^{XE81}*; Df(3L)H99* double mutant embryos (Fig. 4B). Despite amnioserosal survival, *hnt; Df(3L)H99* double mutant embryos failed to retract their germ bands (Fig. 5). A total of 78% of the hnt^{XO01}*; Df(3L)H99* double mutants (n = 72) and 78% of sibling control embryos (n = 150) failed to undergo retraction. Similarly 85% of hnt^{XE81}*; Df(3L)H99* double mutant embryos (n = 13) and 80% of sibling control embryos (n = 20) failed to undergo retrac-

FIG. 3. Spatial analysis of hnt^+ and hnt^- patches in the amnioserosas of germ band retracted hnt^{XE81} mosaic embryos. (A–J) Schematically represent the distribution of hnt^+ cells (green) and hnt^- cells (red) of the 10 retracted embryos with genetically mosaic amnioserosas in Fig. 2. The percentage of hnt^+ cells for each amnioserosa is shown (left number) along with the percentage of hnt^+ cells at the perimeter (right number). T1, first thoracic segment; A1, first abdominal segment; A8, eighth abdominal segment. Embryos are viewed from the dorsal side with anterior toward the top of the page.

FIG. 4. Premature apoptosis of the amnioserosa is blocked in *hnt; H99* double mutants. Stage 13 (A) hnt^{XE81} and (B) hnt^{XE81}*; Df(3L)H99* mutant embryos. Many amnioserosal nuclei are TUNEL positive in hnt^{XE81} mutant embryos (A) but not in hnt^{XE81}*; Df(3L)H99* mutant embryos (B). Arrows point to TUNEL-positive cells.

FIG. 5. Germ band retraction fails in *hnt; Df(3L)H99* double mutant embryos. Stage 14 embryos were immunostained for EN protein to mark the germ band. (A) Wild-type embryos complete germ band retraction. (B) hnt^{XE81} mutant embryos fail to undergo germ band retraction. (C) hnt^{XE81}*; Df(3L)H99* double mutant embryos show a similar "u-shaped" phenotype to hnt^{XE81} mutants. Note, however, that the amnioserosa is morphologically present in wild-type and hnt^{XE81}*; Df(3L)H99* double mutant embryos (arrows) but not in hnt^{XE81} mutant embryos.

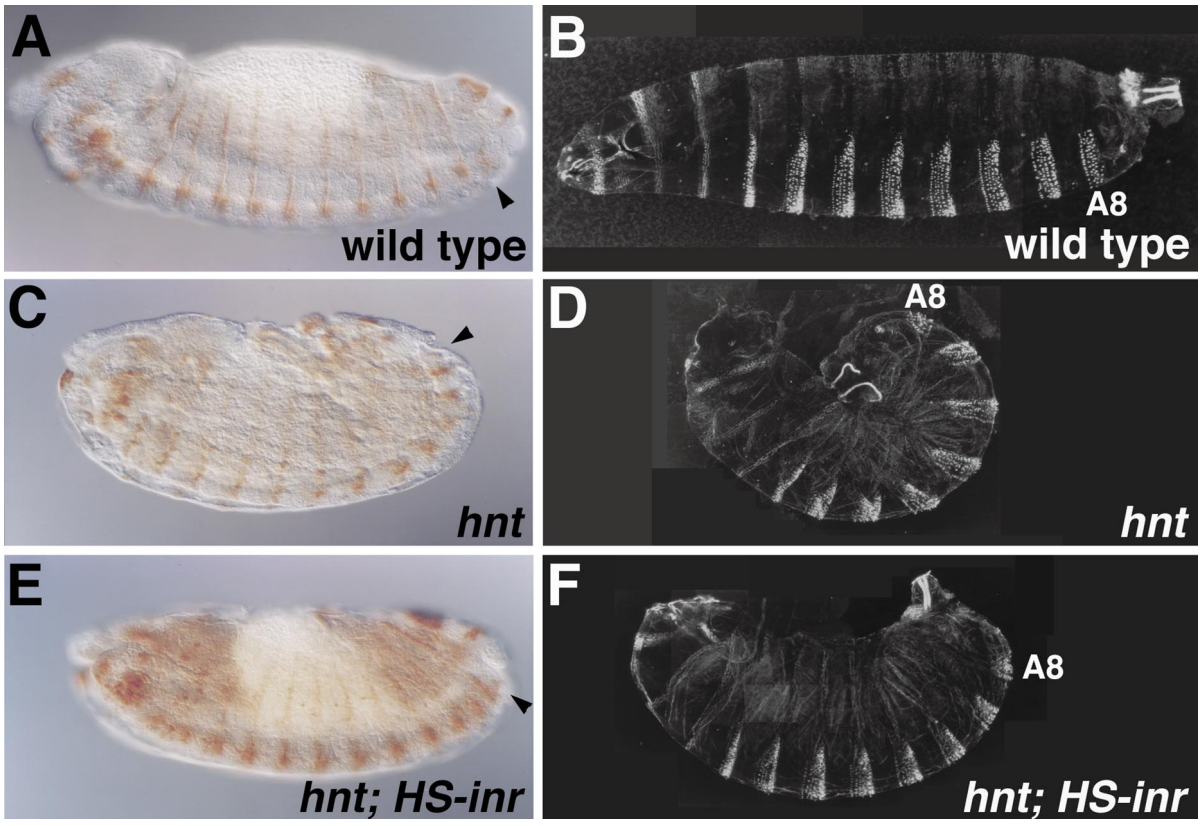

FIG. 7. Germ band retraction defects in *hnt* mutants are rescued by overexpression of the *Drosophila* insulin receptor, INR. (A, B) Wild-type, (C, D) hnt^{XO01}, and (E, F) hnt^{XO01}; *HS-inr*, $Df(3R)e^{D7}$/+. Embryos (stage 14 or older) immunostained for EN protein to mark the germ band (A, C, E) and cuticles from stage 17 embryos (B, D, F). Arrowheads mark A8, the eighth abdominal segment.

tion. We conclude that the failure of germ band retraction in *hnt* mutants is not an indirect consequence of amnioserosal loss; rather, *hnt* plays a more direct role in germ band retraction.

Krüppel Functions Downstream of hindsight in the Amnioserosa

In *hnt* mutant embryos, *reaper* expression in the amnioserosa begins at stage 9 and the amnioserosa begins to show morphological defects at stage 11 (Frank and Rushlow, 1996). To define genes that are regulated directly or indirectly by HNT, we surveyed a set of amnioserosal markers in hnt^{XE81} or hnt^{XO01} mutant embryos at or before stage 11. These included loci required for germ band retraction—*serpent* (*srp*, Rehorn *et al.*, 1996) and *u-shaped* (*ush*, Nüsslein-Volhard *et al.*, 1984); loci that function in dorsal closure–*jun amino-terminal kinase* (*jun*, Bohmann *et al.*, 1994), *pannier* (*pnr*, Ramain *et al.*, 1993), *puckered* (*puc*, Martin-Blanco *et al.*, 1998), and *scab* (Stark *et al.*, 1997); and a locus encoding a TGFα-like protein that functions in cell–cell communication—*vein* (*vn*, Schnepp *et al.*, 1996). Possible effects on *vn* expression were investigated in light of the fact that EGF receptor mutants fail in germ band

TABLE 1

Rescue of Germ Band Retraction in *hindsight* Mutants by Overexpression of the *Drosophila* Insulin Receptor Homolog, INR

Embryo genotype	% Mutant embryos with retracted germ bands (n)[a]	Fold rescue
y hnt^{XO01}/*Y*	1.4 (144)	—
y hnt^{XO01}/*Y*; *HS-inr17*, $Df(3R)e^{D7}$/+	66 (109)	47×
y hnt^{EH704a}/*Y*	0 (126)	—
y hnt^{EH704a}/*Y*; *HS-inr17*, $Df(3R)e^{D7}$/+	16 (126)	16×
y hnt^{XE81}/*Y*	0 (140)	—
y hnt^{XE81}/*Y*; *HS-inr17*, $Df(3R)e^{D7}$/+	13 (180)	13×

[a] n, total number of hnt^{-} embryos scored.

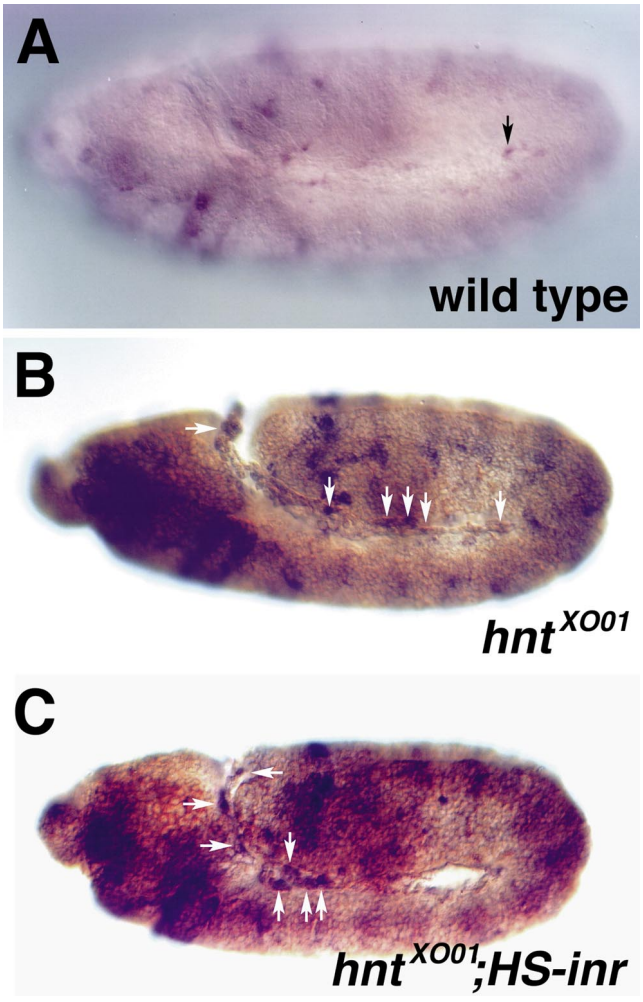

FIG. 8. INR overexpression does not rescue *reaper*-mediated apoptosis of the amnioserosa. (A) Wild-type embryo with a small number of *reaper*-positive amnioserosal cells. In hnt^{XO01} (B) and hnt^{XO01}*; HS-inr* (C) embryos there are many *reaper*-positive amnioserosal cells. Arrows point to *reaper*-positive cells.

retraction (Clifford and Schüpbach, 1989; Raz *et al.*, 1991). For all six markers there was no detectable change in amnioserosal expression.

We showed previously that amnioserosal expression of *Krüppel* (*Kr*) is altered in *hnt* mutants (Yip *et al.*, 1997): KR protein is first detected in the amnioserosa of wild-type embryos during germ band extension (Fig. 6A) (Gaul *et al.*, 1987); however, in *hnt* mutants, KR is clearly absent from most amnioserosal cells by stage 11 (Fig. 6B) (Goldman-Levi *et al.*, 1996; Yip *et al.*, 1997). Here we address whether loss of KR in *hnt* mutants is an indirect consequence of premature apoptosis of the amnioserosa. To do this we assayed KR expression in the amnioserosa of *Df(3L)H99* single (Fig. 6C) and hnt^{XE81}*; Df(3L)H99* double (Fig. 6D) mutants. KR expression is normal in *Df(3L)H99* single mutants but is absent in the *hnt; Df(3L)H99* double mutants (Fig. 6D). Thus loss of KR in *hnt* mutants is not a result of premature apoptosis of the amnioserosa. Rather the *Kr* gene resides downstream of HNT in the *hnt* genetic hierarchy in the amnioserosa. A specific role for *Kr* in germ band retraction remains to be defined (see Discussion).

The Insulin Receptor, INR, Functions Downstream of hindsight to Mediate Germ Band Retraction

The *Drosophila* homolog of the mammalian insulin receptor tyrosine kinase, INR, is required for germ band retraction (Fernandez *et al.*, 1995). The INR is expressed in all embryonic tissues except the amnioserosa (Fernandez *et al.*, 1995), raising the possibility that the INR functions to transduce a retraction signal in the germ band. An antibody that recognizes the activated form of MAP kinase (MAPK) has been reported (Gabay *et al.*, 1997b); this antibody highlights regions of high-level activation of this signaling pathway in *Drosophila* embryos (Gabay *et al.*, 1997a) but does not include a component that reflects the reported expression pattern of the INR (Fernandez *et al.*, 1995). In hnt^{XE81} mutant embryos we saw no alterations in the high-level MAPK activation pattern (M.L.L. and H.D.L., data not shown).

In cell culture experiments, high-density conditions have been shown to be sufficient to activate receptor tyrosine kinases in a ligand-independent manner (Samanta *et al.*, 1994). Therefore, we tested whether ubiquitous overexpression of the wild-type INR could rescue germ band retraction defects in *hnt* mutant embryos. Using a *HS-inr* transgene (Fernandez *et al.*, 1995) to overexpress the wild-type insulin receptor, we observed nearly complete rescue of germ band retraction in *hnt* mutants (Fig. 7). The extent of rescue depended on the strength of the *hnt* allele used (Table 1): For example, 47-fold rescue was achieved in embryos carrying the weak hnt^{XO01} allele (Yip *et al.*, 1997) versus 16-fold rescue in embryos carrying the intermediate hnt^{EH704a} allele (Yip *et al.*, 1997) and 13-fold rescue in embryos carrying the strong hnt^{XE81} allele (Yip *et al.*, 1997). The fact that high levels of the INR can rescue germ band retraction in *hnt* mutants, together with the fact that the extent of rescue by the INR inversely correlates with the strength of the *hnt* allele tested, is consistent with the possibility that the INR functions downstream of *hnt* in a germ band retraction pathway.

The Amnioserosa Is Not Required to "Push" the Germ Band during Retraction

Our results using *hnt; Df(3L)H99* double mutants demonstrated that survival of the amnioserosa is not sufficient for germ band retraction (see above; Figs. 4 and 5). Given that INR overexpression rescues germ band retraction in *hnt* mutants, we asked whether rescue of germ band retraction in this situation correlated with rescue of premature apoptosis in the amnioserosa. Analysis of *reaper* expression in *hnt; HS-inr* embryos showed that the amnioserosa underwent premature apoptosis as in *hnt* mutant embryos

(Fig. 8). We conclude that activation of the INR in the germ band bypasses the requirement for an amnioserosa. In other words, the amnioserosa is not required to physically push the germ band during retraction; rather, a diffusible signal produced by the amnioserosa is likely to be transduced by the INR.

DISCUSSION

Role of Amnioserosal HNT Expression in Germ Band Retraction

HNT is expressed in several tissues prior to and during germ band retraction (Yip *et al.*, 1997). Most notable among these are the amnioserosa and the midgut. Here we have used genetic mosaic analysis to demonstrate unambiguously that HNT expression in the amnioserosa is necessary for germ band retraction.

Previous analyses of *hnt* mutants led to the suggestion that failure of germ band retraction might be an indirect consequence of premature apoptosis of the amnioserosa (Frank and Rushlow, 1996). We have blocked apoptosis in the amnioserosa of *hnt* mutant embryos using the *H99* deficiency. Strikingly, germ band retraction still fails, demonstrating that this failure cannot simply be a consequence of amnioserosal apoptosis. In light of recent studies implicating cell tension in cell survival (Chen *et al.*, 1997), we speculate that apoptosis is an indirect consequence of defects in *hnt* mutant amnioserosal tissue.

Analysis of amnioserosal genetic mosaics in which germ band retraction was successful, along with comparison to those in which germ band retraction failed, suggests that HNT expression in the cells at the perimeter of the amnioserosa is particularly important. Since these are the cells that abut the germ band, this result supports the hypothesis that successful germ band retraction requires contact or signaling between the amnioserosa and the germ band (Yip *et al.*, 1997).

The hindsight Genetic Pathway in Germ Band Retraction

HNT is a nuclear Zn-finger protein that has the characteristics of a transcription factor (Yip *et al.*, 1997). Thus its role in the amnioserosa is likely to be regulation of downstream genes that ultimately control or coordinate germ band retraction (Yip *et al.*, 1997). To date *Kr* is the only gene we have identified whose expression is altered in *hnt* mutants. Further, since KR expression in the amnioserosa is not restored in *hnt; H99* double mutant embryos, loss of KR expression in the amnioserosa of *hnt* mutants cannot simply be a consequence of these cells entering apoptosis. *Kr* mutant embryos show no premature apoptosis in their amnioserosas, neither is there any effect of *Kr* mutations on HNT expression (M.L.L., unpublished data). Taken together, then, all the evidence points toward the *Kr* gene residing downstream of HNT in the germ band retraction regulatory hierarchy in the amnioserosa. Since the germ band undergoes very little extension in *Kr* mutants (Irvine and Wieschaus, 1994) it is not possible to assay whether germ band retraction fails in these mutants. Neither have we uncovered any genetic interactions between *hnt* and *Kr* mutations in double heterozygotes. Thus the exact functions of *Kr* in the HNT regulatory hierarchy remain unknown.

In light of the possibility that communication between the amnioserosa and the germ band is crucial for retraction, we investigated whether a transmembrane receptor tyrosine kinase—INR—functions downstream of HNT in germ band retraction. We focused on the INR because *inr* mutants fail to undergo germ band retraction and the INR is normally expressed only in the germ band and not the amnioserosa (Fernandez *et al.*, 1995). Unlike in *hnt* mutants, in *inr* mutants the amnioserosa do not undergo premature apoptosis (M.L.L., unpublished data). We have shown here that overexpression of INR rescues the retraction defect in *hnt* mutants, consistent with a possible role for INR in transducing a retraction "signal" within the germ band.

Role of the Amnioserosa in Germ Band Retraction

The amnioserosa could serve either of two functions in germ band retraction (Yip *et al.*, 1997): Physical interaction between the amnioserosa and the germ band might be necessary for retraction (e.g., shape changes in the amnioserosa might push the germ band during retraction). Alternatively, physical contact might not be required but, rather, a retraction signal might diffuse from the amnioserosa to the germ band. The first possibility is excluded by our demonstration that germ band retraction can occur in the absence of the amnioserosa when the INR is overexpressed in *hnt* mutants. We speculate that HNT may control a gene hierarchy in the amnioserosa that results in production, activation, or presentation of a retraction signal that is subsequently transduced in the germ band by the INR, thus leading to the coordinate cell shape changes that drive retraction.

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