Molecular Genetic Analysis of Morphogenesis in *Drosophila*: Functions of the *hindsight* Locus

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Abstract

In order to form complex three-dimensional body structures, multicellular organisms have to be able to coordinate the activities of all cells involved; in metazoa the control of various morphogenetic movements during gastrulation are particularly important. *Drosophila* embryogenesis provides an excellent model. The *Drosophila hindsight* gene function is required for germband retraction. Embryos lacking *hindsight* activity have a normal body plan and undergo normal morphogenetic movement prior to the onset of germband retraction. However, they fail to retract their germbands. *hindsight* encodes a large nuclear protein of 1920 amino acids. Sequence analysis reveals that it contains fourteen C_2H_2 type zinc-fingers, arranged in widely spaced clusters. Additional features of the HINDSIGHT protein, such as glutamine-rich and proline-rich domains, suggest it functions as a transcription factor. Embryonic expression of *hindsight* is complex: it is found in the endoderm (anterior and posterior midgut), amnioserosa, subsets of the central nervous system, the peripheral nervous system and the tracheal system. However, it is the expression of *hindsight* in the midgut that is important for germband retraction since mutations which abolish *hindsight* endodermal expression also affect germband retraction. Although *hnt* is not expressed in ectoderm, it is these cells that undergo the cell shape changes that accomplish germband retraction. We propose that *hindsight* activity regulates a signal produced by the endoderm that is responsible for the coordination of morphogenetic cell shape changes and movements in ectoderm. In addition, *hindsight* is also required for normal eye development. HINDSIGHT protein is initially detected in the morphogenetic furrow of the developing *Drosophila* eye. HINDSIGHT is expressed in all photoreceptor cells as they are recruited into the ommatidial cluster. Mosaic analysis of *hindsight* in the larval eye disc reveals that homozygous *hindsight* mutant patches contain regularly spaced ommatidial clusters with variable numbers of photoreceptor cells. Analysis of these photoreceptor cells using cell-specific and general developmental markers indicates that their differentiation is abnormal. The presumptive R8 cells fail to express

BOSS protein and the presumptive R2-5 cells do not express ROUGH protein. Genetically, *hindsight* shows synergistic interaction with *Star*, a gene also involved in photoreceptor specification. Taken together, these results demonstrate an early role for *hindsight* in photoreceptor development. Furthermore, *hindsight* is expressed throughout eye development and its activity is required late in pupal development when photoreceptor cells undergo morphological changes, such as apical-basal extension and rhabdomere separation.

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Chapter 1

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The Terminal Gene Hierarchy of *Drosophila* and the Genetic Control of Tissue Specification and Morphogenesis

Running head: *Drosophila* terminal gene hierarchy

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Abstract

The cells at the anterior and posterior termini of the *Drosophila* embryo form the gut, the brain, parts of the peripheral nervous system, the head skeleton and the unsegmented epidermal structures that reside in the termini. A maternally encoded signal transduction pathway instructs these cells to adopt asegmental terminal - as distinct from central segmental - fates. The consequent regulation of zygotic transcription of numerous genes leads to the detailed specification of cells in different parts of the termini to follow particular developmental programs. Genetic and molecular analyses of mutations that affect distinct aspects of this program have provided insights into how a general signal is transduced into specific cell fates.

I. Introduction

In *Drosophila* the establishment of cell fates along the dorso-ventral and antero-posterior axes of the embryo is accomplished by genetic hierarchies that act both maternally and zygotically (for reviews see Lipshitz, 1991; St Johnston and Nüsslein-Volhard, 1992). While only one pathway is required for the specification of dorso-ventral fates, three interacting pathways confer fates along the antero-posterior axis: these have been referred to as the 'anterior' (head and thorax), 'posterior' (abdomen) and 'terminal' (asegmental termini) pathways (Nüsslein-Volhard et al., 1987).

Axis specification is initiated during oogenesis and involves interactions between the developing oocyte and the surrounding, somatically-derived follicle cells. Two main consequences are (1) the asymmetric deposition of localized determinants within the oocyte: *bicoid* RNA for the anterior pathway and *nanos* RNA for the posterior pathway (St Johnston and Nüsslein-Volhard, 1992; Ding and Lipshitz, 1993), and (2) local deposition (or activation) in the extracellular matrix that surrounds the oocyte, of ligands for uniformly distributed receptors that are synthesized in the early embryo: TOLL for the dorso-ventral system and the TORSO receptor tyrosine kinase for the terminal pathway (Lipshitz, 1991; St Johnston and Nüsslein-Volhard, 1992).

In this review we focus on the terminal pathway. Over 40 genes have been defined genetically and/or molecularly as residing in the terminal pathway and more than 20 additional identified genes are likely to function in this hierarchy (Table 1). Since neither the genetic nor the molecular screens have been saturating, this list represents only a subset of the loci that function in the terminal pathway. The initial components of the pathway, from ligand production and activation through receptor activation and cytoplasmic signal transduction, are encoded by maternally synthesized molecules. This signal results in the control of differential zygotic gene expression. We begin our description of the pathway at the level of the transmembrane receptor, TORSO, that receives the terminal signal (section II). We describe what is known about ligand production and activation, and how the signal

is transduced by a cytoplasmic phosphorylation cascade. We then focus (section III) on the zygotic genes that implement detailed aspects of terminal cell fate specification. Finally (section IV), we consider how the outcome of reading the same signal is modulated within the termini along their antero-posterior and dorso-ventral axes, as well as how cells in the anterior terminal region are programmed to undergo distinct developmental fates from those in the posterior terminal region.

II. Maternally Encoded Components of the Terminal Pathway

A. The Transmembrane Receptor

The *torso* gene encodes a transmembrane receptor tyrosine kinase related to those of the PDGF family (Table 1, Figure 1) (Casanova and Struhl, 1989; Sprenger et al., 1989) that receives the extra-embryonic terminal signal and transduces it into the embryo. Since expression of the *torso* gene is restricted to the germline of the mother, *torso* mutations are strictly maternal effect in nature. Loss of function *torso* ($torso^{lof}$) alleles result in an inability of cells at both the anterior and posterior termini of the embryo to adopt terminal cell fates. Instead the cells in the termini adopt central fates: the segmented region expands into the termini (Figure 1) (Schüpbach and Wieschaus, 1986b). The $torso^{lof}$ alleles encode proteins with no functional kinase activity. Gain of function *torso* alleles ($torso^{gof}$) result in the reciprocal phenotype: the terminal fate map expands into the central region with consequent loss of segments (Figure 1) (Klingler et al., 1988; Schüpbach and Wieschaus, 1989; Strecker et al., 1989). The $torso^{gof}$ alleles behave genetically as hypermorphs (Strecker et al., 1989) and have alterations in the extracellular domain that result in constitutive, ligand-independent activation of their kinase activity (Figure 1) (Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993). The reciprocal phenotypes of *torso* gain of function versus *torso* loss of function alleles indicate that the *torso* gene has dual functions during embryogenesis: to promote terminal cell fates in the polar regions and to

repress central, segmented cell fates in the termini (Strecker et al., 1989; Strecker and Lipshitz, 1990).

The *torso* gene is expressed maternally and its transcripts are uniformly distributed in the egg and early embryo (Casanova and Struhl, 1989; Sprenger et al., 1989). These are translated after fertilization and TORSO protein is found on the surface of the entire early embryo (Casanova and Struhl, 1989). How then is the terminal signal transduced only in the termini? All experimental data so far support the model that TORSO's ligand (or its activity) is spatially restricted to the perivitelline space at the termini (see next section). Since the TORSO receptor is present in excess, it will sequester the limited amount of ligand and prevent it from diffusing away from the termini (Casanova and Struhl, 1993). As a result the terminal signal is only transduced at the poles. It is assumed, by analogy to what is known about vertebrate receptor tyrosine kinases, that ligand binding results in receptor oligomerization with consequent transient phosphorylation of the TORSO receptor's cytoplasmic domain on tyrosine residues, probably through autophosphorylation (Yarden and Ullrich, 1988; Schlessinger and Ullrich, 1992). This activation of the kinase activity of the receptor initiates a cytoplasmic signal transduction cascade. Before detailing this cascade, we discuss what is known about the TORSO receptor's ligand and its activation.

B. The Extra-embryonic Ligand

Mutations in four maternal genes [*fs*(1)*Nasrat*, *fs*(1)*polehole*, *torso-like* and *trunk*] produce phenotypes closely resembling those of *torso* mutations (Table 1, Figure 2) and behave genetically as if they act upstream of the TORSO receptor based on their inability to suppress $torso^{gof}$ phenotypes. Three of these [*fs*(1)*Nasrat*, *fs*(1)*polehole* and *trunk*] are expressed in the germline (Perrimon and Gans, 1983; Schüpbach and Wieschaus, 1986a) while the fourth, *torso-like*, is expressed in the follicle cells that surround the oocyte (Stevens et al., 1990). A clone of between 6 and 30 mutant *torso-like* follicle cells at the

posterior end of the developing oocyte is sufficient to cause deletion of the telson in the posterior terminal region (Stevens et al., 1990). Consistent with this spatially restricted requirement, expression of the *torso-like* gene is restricted to the anterior border cells and posterior follicle cells, and ectopic expression of TORSO-LIKE during oogenesis generates phenotypes similar to those produced by $torso^{gof}$ mutations (Savant-Bhonsale and Montell, 1993; Martin et al., 1994). While the TORSO-LIKE protein has an amino-terminal signal sequence and is secreted, it bears no obvious similarity to other proteins. Thus, the nature of the localized information provided by TORSO-LIKE is unclear: it may be a ligand for TORSO or it may function to activate an otherwise uniformly-distributed inactive ligand for the TORSO receptor (Figure 2). The latter possibility appears most likely since preliminary data suggest that *trunk* may encode a secreted, inactive ligand for TORSO (Figure 2) (cited in Casanova and Struhl, 1993).

While the *torso-like* and *trunk* loss of function mutant phenotypes are identical to those of *torso* loss of function phenotypes, those produced by *fs(1)Nasrat* and *fs(1)pole hole* are more pleiotropic. Females homozygous for most alleles of *fs(1)Nasrat* or *fs(1)pole hole* lay normal-looking eggs that collapse soon after deposition, are permeable to neutral red and burst upon removal of the chorion (Degelmann et al., 1990). These alleles are assumed to be genetically amorphic. One mutant allele at each locus [$fs(1)Nasrat^{211}$ and $fs(1)pole\ hole^{1901}$], results in the typical terminal class mutant phenotype, while two alleles of *fs(1)Nasrat* [$fs(1)Nasrat^{1}$ and $fs(1)Nasrat^{DH1}$] result in both the collapsed egg and the embryonic terminal phenotypes. Interestingly, $fs(1)Nasrat^{DH1}$, which is a temperature-sensitive allele, results in phenotypes that range from ones similar to those produced by *torso* loss of function alleles to ones similar to weak *torso* gain of function phenotypes. It has been speculated that *fs(1)Nasrat* and *fs(1)pole hole* encode vitelline membrane proteins or extracellular matrix-associated proteins that interact with the TORSO protein or its ligand (Figure 2) and/or provide structural support for the oocyte (Degelmann et al., 1990; Lipshitz, 1991).

C. Cytoplasmic Signal Transduction

The cytoplasmic signal transduction pathway initiated by the TORSO receptor shares many components with other receptor tyrosine kinase (RTK)-mediated pathways, such as those initiated by SEVENLESS in the developing eye (Hafen et al., 1994) and by the *Drosophila* homolog of the epidermal growth factor receptor (DER) in various tissues (Shilo and Raz, 1991; Shilo, 1992). The known components are listed in Table 1 and Figure 2 and are summarized here.

The *downstream of receptor kinases* (*drk*) gene encodes a protein containing SRC homology 2 (SH2) and SRC homology 3 (SH3) domains (Olivier et al., 1993; Simon et al., 1993). *drk* mutations were initially identified as extragenic modifiers of SEVENLESS receptor tyrosine kinase activity in the eye. SH2 domains are capable of binding receptor tyrosine kinases *in vivo* and *in vitro* in a phosphorylation-dependent manner (Pawson and Schlessinger, 1993). Consistent with such a role, DRK protein has been shown to bind SEVENLESS protein *in vitro* and *in vivo* (Olivier et al., 1993; Simon et al., 1993). A role for DRK in the embryonic terminal gene hierarchy is suggested by the fact that $torso^{gof}$ phenotypes are suppressed by the $drk^{E(sev)2B}$ allele; such a genetic interaction would place DRK downstream of the TORSO receptor. Consistent with this interpretation, the $drk^{E(sev)2B}$ allele has a single amino acid substitution in the SH2 domain (Doyle and Bishop, 1993; Olivier et al., 1993); thus it is likely to interfere with binding of DRK to the activated TORSO receptor, hence affecting the efficiency of coupling to downstream signaling molecules.

RAS, a protein with GTPase activity, is involved in many intracellular signal transduction pathways (Lowy and Willumsen, 1993). Its activity is regulated by bound guanine nucleotide (Bourne et al., 1990; Bourne et al., 1991): RAS is active when bound to GTP and inactive when GDP is bound. Cycling between the two states largely depends on two opposite activities: (1) a RAS guanine nucleotide exchange factor that catalyzes the

exhange of GDP for GTP and (2) a RAS GTPase activating protein (GAP) that stimulates the intrinsic GTPase activity of RAS protein to hydrolyze bound GTP. A *Drosophila* homolog of RAS, RAS1, was first implicated in the SEVENLESS signaling pathway (Simon et al., 1991). Loss of function mutations in the *Ras1* gene cause embryonic lethality and function as dominant suppressors of the $torso^{gof}$ phenotype (Doyle and Bishop, 1993), while maternal expression of a dominant activated RAS1 protein ($RAS1^{Glu13}$) phenocopies the $torso^{gof}$ phenotype (Lu et al., 1993). Further, direct injection of variants of mammalian $p21^{ras}$ protein into *Drosophila* embryos have indicated a role in the TORSO signaling pathway: activated $p21^{ras}$ ($p21^{v\text{-}ras}$) partially rescues the $torso^{lof}$ phenotype while dominant-negative forms of $p21^{ras}$ ($p21^{rasN17}$) block the terminal signal in wildtype embryos and generate a $torso^{lof}$ phenotype (Lu et al., 1993).

Mutations in the *Drosophila Gap1* gene, which encodes a protein with homology to mammalian GTPase activating protein (GAP), were identified as producing extra R7 cells in a sensitized *sevenless* genetic background (Gaul et al., 1992; Rogge et al., 1992) and as causing hyperinnnervation of the R7/R8 retinotopic map (Buckles et al., 1992). The homology to mammalian GAP suggested a function in the SEVENLESS pathway through regulation of RAS1 activity. GAP1 may also function as a negative regulator in the TORSO-mediated pathway since *Gap1* mutations enhance $torso^{gof}$ phenotypes (Doyle and Bishop, 1993).

The *Drosophila* homolog of RAS guanine nucleotide exchange factor is encoded by the *Son of sevenless* (*Sos*) gene. Gain of function alleles of *Sos* were isolated originally as dominant suppressors of *sevenless* mutations (Rogge et al., 1991; Simon et al., 1991; Bonfini et al., 1992). Most loss of function alleles of *Sos* are lethal and embryos derived from germline clones of Sos^{lof} show either a terminal class phenotype (zygotic genotype: *Sos*/+) or very little differentiation (zygotic genotype: *Sos*/*Sos*) (Lu et al., 1993). Thus the $torso^{lof}$ phenotype produced by direct injection of a dominant negative form of $p21^{ras}$ (see above) is probably due to competition with endogenous RAS for binding to the SOS

protein (Lu et al., 1993). *Sos*^{lof} alleles act as dominant suppressors of *torso*^{gof} phenotypes (Doyle and Bishop, 1993).

l(1)pole hole [distinct from *fs(1)pole hole*, discussed above] encodes the *Drosophila* homolog of the RAF serine/threonine kinase (D-RAF) (Nishida et al., 1988; Ambrosio et al., 1989b). D-RAF is expressed and required both maternally and zygotically, but it is maternally expressed D-RAF that functions specifically in the terminal signal transduction pathway. Embryos derived from homozygous *l(1)pole hole* germline clones show a typical terminal class phenotype (Ambrosio et al., 1989b) and embryos that are deprived of both maternal and zygotic *l(1)pole hole* function show incomplete development and massive cell death (Ambrosio et al., 1989a; Melnick et al., 1993). While suppression of the *torso*^{gof} phenotype by *l(1)pole hole* mutations places D-RAF downstream of the TORSO receptor (Ambrosio et al., 1989b), the failure of injection of activated p21^{ras} to rescue embryos that lack maternal D-RAF activity suggests that D-RAF functions downstream of RAS in the terminal pathway (Lu et al., 1993). Similar conclusions have been reached from molecular genetic analyses of RAS/RAF-mediated vulval induction in *Caenorhabditis elegans* (Sternberg, 1993). Recent data have demonstrated a GTP-dependent direct interaction between the amino-terminal domain of RAF and the effector domain of RAS (Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993).

The *Drosophila* CORKSCREW protein bears homology to cytoplasmic tyrosine phosphatases that also contain two SH2 domains (Perkins et al., 1992). As is the case for D-RAF and SOS, CORKSCREW activity is required both maternally and zygotically. Embryos derived from eggs produced by homozygous *corkscrew* germline clones show characteristic terminal class pattern defects, as well as twisted gastrulation and germband retraction defects reminiscent of those exhibited by embryos derived from *torso*^{lof} mothers (see also III below). Mutations in the *corkscrew* gene suppress *torso*^{gof} phenotypes, suggesting that CORKSCREW lies downstream of TORSO in the terminal pathway;

however the actual position of CORKSCREW in the cytoplasmic signal transduction pathway is uncertain. As for null *l(1)pole hole* alleles, null *corkscrew* alleles do not completely abolish expression of *tailless* (see III below). Embryos from which maternal contributions of functional D-RAF and CORKSCREW have been eliminated, lack posterior *tailless* expression; this was initially taken to suggest a possible interaction of CORKSCREW with D-RAF (Perkins et al., 1992). However, injection of $p21^{v\text{-}ras}$ protein into embryos lacking maternal CORKSCREW can rescue the phenotype, positioning CORKSCREW upstream of RAS1 (Lu et al., 1993), whereas similar experiments (see above) place D-RAF downstream of RAS1. Recent biochemical data from mammalian cells have shown that GRB2 (the DRK homolog), can either directly bind activated PDGF receptor (the mammalian TORSO homolog) (Arvidsson et al., 1994) or form a complex with SHPTP2 (the mammalian homolog of CORKSCREW; also known as SYP/PTP1D/PTP2C) and then bind activated PDGF receptor (Bennett et al., 1994; Li et al., 1994). That the same is likely to hold true for the *Drosophila* terminal pathway is supported by the data that positions CORKSCREW upstream of RAS1.

The *Dsor1* gene encodes the *Drosophila* homolog of the mammalian MAP kinase activator (MEK) and yeast PBS2, STE7 and BYR1 (Tsuda et al., 1993). Mutations in *Dsor1* were identified as dominant suppressor of *l(1)pole hole* and were subsequently found to suppress other terminal class mutants [*fs*(1)*pole hole*, *torso-like*, *trunk* and *corkscrew*]. Elimination of functional maternal DSOR1 from embryos produces phenotypes similar to those in embryos produced by *l(1)pole hole* germline clones. These results suggest that DSOR1 acts downstream of D-RAF.

A *Drosophila* homolog of MAP kinase (Biggs III and Zipursky, 1992) is encoded by the *rolled* locus. *rolled* mutants exhibit defects in multiple receptor tyrosine kinase signaling pathways (Biggs III et al., 1994; Brunner et al., 1994b). Gain of function mutations in *rolled* cause similar phenotypes to those produced by $torso^{gof}$ mutations. Loss

of function mutations in *rolled* can suppress terminal class phenotypes caused by *torso*^{gof} alleles (Brunner et al., 1994b).

The position of D-SRC, the *Drosophila* homolog of mammalian C-SRC, in the terminal signal transduction pathway remains unclear. Ectopic expression of wildtype and mutant forms of D-SRC interfere with eye development and with germband retraction in the embryo (Kussick et al., 1993). This phenotype is dependent on functional RAS1 activity. In mice, the oncogenic form of C-SRC, V-SRC, can constitutively phosphorylate and enhance SYP/PTP1D (CORKSCREW) activities (Feng et al., 1993; Vogel et al., 1993). Moreover, RAF and SRC coimmunoprecipitate when expressed *in vitro*, indicating possible direct interactions between RAF and SRC (Cleghon and Morrison, 1994). By analogy, D-SRC may function downstream of the TORSO receptor and interact with CORKSCREW and D-RAF.

Four of the segment polarity genes (see III(C) below) are expressed maternally and mutations result in terminal defects in embryos derived from germline clones. Two of these (*shaggy/zeste-white3* and *fused*) encode serine/threonine kinases, one (*dishevelled*) a novel protein, and one (*porcupine*) has not yet been studied molecularly. For logistical reasons these are considered in our discussion of pair-rule genes; however, it should be noted here that their gene products may reside in the maternally encoded signal transduction pathway.

D. Conservation of Signal Transduction Pathways

As we have emphasized, the terminal signal transduction pathway in the *Drosophila* embryo shares many components with related receptor tyrosine kinase-mediated pathways both in *Drosophila* and in other metazoa. The best studied examples include: the SEVENLESS-mediated pathway in *Drosophila* eye development (Hafen et al., 1994); various growth factor responses in mammalian cells (Yarden and Ullrich, 1988; Schlessinger and Ullrich, 1992); vulval induction in *Caenorhabditis elegans* (Sternberg,

1993); and the pheromone response in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kurjan, 1993). Typically, ligands activate the pathway by binding to transmembrane receptors, inducing oligomerization and conformational changes that are transmitted across the cell membrane and catalyze the phosphorylation and activation of the receptor's kinase activity. Phosphorylation of cytoplasmic signal transduction molecules follows. The participation of small GTP-binding proteins such as RAS, or of G proteins, in these pathways is important. Downstream of the GTP-binding proteins are RAF and the MAP kinase cascade which eventually modulate the activities of transcriptional regulators through phosphorylation.

Studies in other systems have identified transcriptional regulators that are modulated by the MAP kinase cascade. In mammalian systems, the activity of transcription factors - such as ELK-1 (which belongs to the ETS-family), C-JUN and C-MYC - is affected by their phosphorylation state, and this state is regulated by the MAP kinase cascade (Hunter and Karin, 1992). In the *Drosophila* SEVENLESS-mediated pathway, it has been shown that a *Drosophila* homolog of C-JUN as well as two ETS-family proteins (YAN and POINTED) are required for R7 development and that the latter can be phosphorylated *in vitro* by the MAP kinase cascade (Brunner et al., 1994a; O'Neill et al., 1994). In the *Drosophila* tracheal system - development of which is regulated by the *Drosophila* homolog of the FGF receptor, BREATHLESS - POINTED functions to program cell growth and migration (Klämbt, 1993).

At this point, little is known about transcription factors in the *Drosophila* embryo that might be the mediators of the terminal signal into the nucleus; however, it is highly likely that one or more of the above proteins (or related proteins yet to be identified) will be involved. One unanswered question involves the nature of the control exerted by such activated (or repressed) transcriptional control proteins. Clearly not all of the genes that they turn on and off can be the same in all tissues and at all times. In other words, an outstanding question - and one of our major areas of ignorance - is how conserved

cytoplasmic signal transduction pathways and their target transcription factors result in differential gene regulation. The next section (III) will focus on the specific readout of the terminal signal in terms of zygotic genes that respond to it. We then go on (section IV) to consider how cells are instructed as to their positions along the dorso-ventral and anterior-posterior axes within the termini, as well as how the anterior terminal region is elevated from the 'ground state' represented by the posterior terminus through interaction of the terminal and anterior genetic pathways.

III. The Zygotic Effectors of Terminal Cell Fates

A. Subdivision of the Termini into Distinct Tissues

Before describing the assorted zygotic terminal pathway genes and their roles, it is first useful to define some of the details of terminal development. Much of the emphasis in discussions of antero-posterior axis specification in *Drosophila* has been on the central, segmented part of the embryo. The maternal hierarchies involved in these regions are the so-called 'anterior' and 'posterior' systems, and the developmental problem in the central region is largely one of subdividing a domain into repeating units - parasegments/segments - and then elaborating differences among those repeated units (St Johnston and Nüsslein-Volhard, 1992; Martinez Arias, 1993; Pankratz and Jäckle, 1993). [Parasegments are segment-sized embryonic developmental units that are out of phase with the actual morphologically defined segments by half of a segment (Martinez Arias and Lawrence, 1985).] This is a problem specific to insects that use the long germband mode of development in which the entire segmented region is specified from a single field of cells at the blastoderm stage. In contrast, short germband insects and other metazoa do not face this problem; segmental repeats either are not present or they develop sequentially with consequent differences in the nature of positional specification and the mechanisms used to implement it.

In contrast to the antero-posterior axis in the segmented region of *Drosophila*, cell fate specification mechanisms along the dorso-ventral axis and in the embryonic termini more closely resemble those implemented generally in other metazoa. That is, cells must be instructed as to their position and then must develop into different tissues (rather than metameric units) dependent on their location. Along the dorso-ventral axis, this largely involves subdivision into (from ventral to dorsal): mesoderm, neuro-ectoderm, epidermal ectoderm and extra-embryonic tissues. In the termini, this process involves subdivision into (from terminus toward center) endoderm, intestinal ectoderm, epidermal ectoderm and (in the anterior) brain. Since these processes more closely resemble those in other metazoa, it is probably no accident that, in terms of molecular pathways, the maternally encoded components of both the dorso-ventral and the terminal systems use cell-cell interaction and signal transduction mechanisms to instruct cells as to their fates, rather than the more specialized system of localized cytoplasmic determinants used by the maternal anterior and posterior systems (Lipshitz, 1991; Ding and Lipshitz, 1993).

Given this view, we shall focus on the effectors of terminal fates, not in terms of drawing arbitrary boundaries on a field of cells, but rather by focusing on how cells are directed into distinct tissue fates with consequent morphogenetic outcomes that include coordinated invagination and cell movement.

It should be emphasized at this point that the terminal gene pathway does not act in isolation: the anterior terminal region gives rise to structures and tissues (e.g. the brain) distinct from those that form posteriorly. This is accomplished through an interaction of the terminal, the anterior and the dorso-ventral pathways in the anterior terminal domain (see IV(C) below). Similarly, the dorsal domains of the anterior and posterior termini have different fates from the ventral domains and this is accomplished through interaction of the terminal and dorso-ventral pathways at the termini (see IV(B) below).

The zygotic genes that implement terminal cell fates have been defined using two, related strategies. First, genetic screens and genetic-interaction tests have been used to

define zygotically active genes with phenotypes that are similar to (or subsets of) those produced by the maternal terminal class genes (reviewed in Lipshitz, 1991). Where possible, it has been useful to complement such analyses with tests for possible genetic interaction with defined maternal components [e.g. by tests for interaction with *torso*^{gof} mutations (Strecker et al., 1989; Strecker et al., 1991; Strecker et al., 1992)]. Second, the definition of cloned genes that are expressed in the embryonic termini and examination of their expression patterns in terminal pathway mutants, have also made it possible to position many of these genes in the terminal regulatory hierarchy. Taken together, these approaches have led to the definition of over 50 zygotic genes that are involved (or are likely to be involved) in specifying aspects of cell fate in the anterior and posterior termini (Table 1) (for a recent review, see Jürgens and Hartenstein, 1993).

B. Subdivision into Endoderm versus Ectoderm

Two genes function to subdivide the termini into distinct tissues: *huckebein* and *tailless* (Table 1, Figure 3). *tailless* is required to program the development of much of the ectoderm and its derivatives at the termini, including the brain at the anterior (Jürgens et al., 1984; Strecker et al., 1986; Strecker et al., 1988), while *huckebein* is required for the establishment of the endoderm at both poles and also the intestinal ectoderm and labrum anteriorly (Weigel et al., 1990; Brönner and Jäckle, 1991; Brönner et al., 1994; Reuter and Leptin, 1994).

tailless was the first of the zygotic genes shown by genetic interaction tests to reside in the terminal hierarchy: *torso*^{gof} phenotypes are suppressed by loss of function *tailless* mutations (Klingler et al., 1988; Strecker et al., 1989). The *tailless* gene encodes an 'orphan' receptor belonging to the steroid-receptor superfamily to which it shows homology in both its putative ligand-binding and its DNA-binding domains (Pignoni et al., 1990). Transcription of the *tailless* gene is activated directly by the TORSO-mediated terminal signal and occurs in those regions of the termini in which it is known to function

(Table 1, Figure 3). The anterior terminal expression of TAILLESS undergoes more dramatic changes than does its posterior expression; these are dependent on input from the BICOID-mediated anterior system and the dorso-ventral pathways (see section IV for more detailed discussion). Genetic analyses (Strecker et al., 1989; Strecker and Lipshitz, 1990) led to the conclusion, later supported by molecular studies (Steingrímsson et al., 1991), that TAILLESS has dual functions in the termini: to promote terminal cell identities and to suppress central cell identities in the terminal region. Ectopic expression of TAILLESS in embryos using a heat-inducible promoter can phenocopy *torso*^{gof} phenotypes. This is achieved through activation of genes such as *hunchback* and repression of genes such as *Krüppel* and *knirps* (Steingrímsson et al., 1991).

Due to the absence of the posterior midgut anlagen, *huckebein* mutant embryos fail to undergo posterior midgut invagination and they also show defects in germband extension. Similarly, the anterior midgut and stomodeal invaginations are abnormal. The formation of the anterior gut structures requires synergistic interactions between *huckebein* and the dorso-ventral genes, *snail* and *twist* (Reuter and Leptin, 1994). *huckebein* encodes a protein with similarity to the SP1/EGR family of zinc-finger transcription factors. As in the case of *tailless*, mutations in *huckebein* suppress *torso*^{gof} phenotypes (Weigel et al., 1990). Ectopic expression of HUCKEBEIN perturbs segmentation in the ectoderm and suppresses the segregation of mesoderm (Brönner and Jäckle, 1991; Brönner et al., 1994).

huckebein RNA and HUCKEBEIN protein are expressed at the syncytial blastoderm stage in polar caps that overlap with the zones of TAILLESS expression. Unlike the gap genes expressed in the central, segmented region, the products of the *huckebein* and *tailless* genes do not cross-regulate each other's expression (Brönner and Jäckle, 1991). Embryos that are homozygous for both *huckebein* and *tailless* mutations show posterior terminal region phenotypes that are almost identical to those in embryos derived from homozygous *torso*^{lof} mothers (Weigel et al., 1990), suggesting that much of

terminal development - particularly at the posterior - is programmed through the combined action of these two zygotic effector genes.

C. Genes that Program Ectodermal Development

Numerous genes function downstream of *tailless* and *huckebein* to specify the details of ectodermal development in the termini (Table 1, Figure 3), and these are summarized here.

Mutant *lines* embryos die with terminal and central region defects (Nüsslein-Volhard et al., 1984). The terminal defects are similar to those of *tailless* mutants; indeed, *lines* and *tailless* mutations show synergistic interactions (Strecker et al., 1991). In addition *lines* mutations suppress the phenotypes caused both by $torso^{gof}$ mutations and by ectopic expression of TAILLESS (Strecker et al., 1991; Strecker et al., 1992). These results suggest that *lines* acts downstream of *tailless* in the terminal pathway. *lines* has not yet been analyzed molecularly.

hunchback mutations also are able to suppress $torso^{gof}$ phenotypes (Strecker et al., 1991). The *hunchback* gene encodes a zinc-finger transcription factor that is expressed both maternally and zygotically, and it functions in the termini as well as in the central region of the embryo. Only the terminal functions of *hunchback* are considered here. Specifically, zygotic expression of HUNCHBACK in the posterior region is required for the proper formation of the seventh and eighth abdominal segments, the most centrally located part of the posterior terminal epidermal ectoderm (Lehmann and Nüsslein-Volhard, 1987); this expression is dependent on the terminal signal: it is activated by TAILLESS and repressed by HUCKEBEIN (Casanova, 1990; Brönner and Jäckle, 1991).

The *giant* gene encodes a transcriptional regulatory protein with characteristics of the b-ZIP family of DNA-binding proteins (Capovilla et al., 1992); and functions to specify fates in the termini and the central region of the embryo. Mutations in *giant* suppress $torso^{gof}$ phenotypes (Strecker et al., 1991). The major terminal pathway function of *giant*

is in the anterior where it is required for the development of the labrum (Petschek et al., 1987; Mohler et al., 1989; Petschek and Mahowald, 1990). In *torso*^{lof} embryos, the most anterior stripe of GIANT expression (stripe 1) is missing while in *torso*^{gof} embryos this stripe expands centrally. The combined activities of TAILLESS and HUCKEBEIN are required to activate stripe 1 GIANT expression (Eldon and Pirrotta, 1991).

The *empty spiracles* gene encodes a homeodomain-containing transcription factor that functions both inside and outside the terminal developmental domain. Within the posterior domain it programs the formation of the filzkörper that reside inside the posterior spiracles (Dalton et al., 1989) and anteriorly it functions in optic lobe development (Walldorf and Gehring, 1992). *empty spiracles* mutations suppress phenotypes induced by ectopic expression of TAILLESS, suggesting that it acts downstream of TAILLESS in the terminal pathway (Strecker et al., 1992).

The *tramtrack* gene also functions in both terminal and central embryonic development, as well as postembryonically. It was originally identified based on TRAMTRACK protein binding *cis*-regulatory sequences of two pair-rule genes, *fushi tarazu* and *even-skipped*. *tramtrack* encodes two related proteins, p69 and p88, that differ in their carboxy-terminal domains, and possess different pairs of C_2H_2 zinc-fingers (Harrison and Travers, 1990; Brown et al., 1991; Read and Manley, 1992). Null alleles of *tramtrack* are embryonic lethal: mutant embryos show severe cuticular defects and fail to form filzkörper posteriorly and certain head structures anteriorly. These terminal defects suggest that *tramtrack* may reside in the terminal pathway (Xiong and Montell, 1993).

The *spalt* mutation causes incomplete transformation of the labial segment into a prothorax-like segment, and the tail into structures that resemble the eighth abdominal segment. These have been interpreted as homeotic transformations towards more central identities, indicating that *spalt* functions to confer posterior terminal identities in parasegments 14 and 15 (Jürgens, 1988). *spalt* encodes a zinc-finger-containing protein and is likely to be a transcriptional regulator (Kühnlein et al., 1994). The posterior

expression domain of SPALT coincides with the TAILLESS expression domain; *spalt* expression is absent in *tailless* mutant embryos (R. Schuh and H. Jäeckle, cited in Jürgens and Hartenstein, 1993).

The *Abdominal-B* gene of the bithorax complex encodes two homeodomain transcription factors referred to as ABDOMINAL-BI and ABDOMINAL-BII (also called M-ABDB and R-ABDB). Within the termini, ABDOMINAL-BI specifies cell identity in the epidermal ectodermal derivatives of parasegment 13 while ABDOMINAL-BII specifies cell identity in the epidermal ectodermal derivatives of parasegments 14 and 15 of the embryo (Celniker et al., 1990). TAILLESS activates ABDOMINAL-BI expression in parasegment 13 (Reinitz and Levine, 1990). Activation of ABDOMINAL-BII expression in parasegments 14 and 15 is absent in embryos from $torso^{lof}$ females and in *tailless* mutant embryos, and ABDOMINAL-BII is ectopically expressed in embryos from $torso^{gof}$ females (Casanova, 1990). These data indicate that both ABDOMINAL-BI and ABDOMINAL-BII function in the terminal hierarchy.

The *forkhead* gene is under the dual control of HUCKEBEIN and TAILLESS and functions in both midgut (endodermal) and hindgut (ectodermal) development, including the Malpighian tubules (Jürgens and Weigel, 1988; Weigel et al., 1989a). *forkhead* encodes a transcription factor (Weigel et al., 1989b). With regard to its ectodermal functions, in *forkhead* mutants hindgut and foregut are replaced by more centrally derived structures, suggesting that FORKHEAD is required to confer terminal versus central cell fates in these regions.

The *Drosophila* homolog of the mouse *Brachyury* (*T*) gene, *T-related gene* (*Trg*), is required for the formation of the hindgut and the anal pads (Kispert et al., 1994). The T gene product of mice has been shown to bind DNA and possibly to function as a transcriptional regulator (Kispert and Herrmann, 1993; Herrmann and Kispert, 1994). Expression of the *Drosophila Trg* gene is dependent on TAILLESS but not on FORKHEAD. Repression of *Trg* expression in the posterior midgut primordium is

dependent on HUCKEBEIN. Antibodies directed against the mouse T protein cross-react with the *Drosophila* TRG protein as well as putative T homologs in the gut primordia of embryos of the short germband insects, *Locusta* and *Tribolium*, suggesting possible evolutionary conservation of the mechanisms used to establish hindgut identity (Kispert et al., 1994).

The Malpighian tubules derive from the hindgut and form at the junction of the hindgut and the midgut. They are specified by TAILLESS and HUCKEBEIN, which overlap in expression in this region and function jointly there. Four genes are required for the proper formation of the Malpighian tubules (Harbecke and Janning, 1989; Gaul and Weigel, 1991; Liu and Jack, 1992): *cut*, which encodes a homeodomain-containing protein (Blochlinger et al., 1988); *Krüppel*, which encodes a Zn-finger transcription factor (Rosenberg et al., 1986); *caudal*, which encodes a homeodomain-containing transcription factor (Macdonald and Struhl, 1986); and *HNF-4*, a *Drosophila* homolog of hepatocyte nuclear factor-4 (Zhong et al., 1993). In *cut*, *Krüppel* and *caudal* mutants the cells that would normally give rise to the Malpighian tubules take on hindgut characteristics, indicating that the wildtype functions of these genes is to convert the identity of these progenitor cells from a 'ground' hindgut state to that of Malpighian tubule. *forkhead* expression is activated by TAILLESS and HUCKEBEIN. FORKHEAD then activates *Krüppel* expression, and KRÜPPEL activates expression of *caudal* and *cut*. The *caudal* and *cut* genes are expressed independently of each other. HNF-4 is expressed in the primordia of the Malpighian tubules and mutant embryos lacking zygotic HNF-4 activity fail to form the Malpighian tubules. Detailed phenotypic analyses of HNF-4 mutants have not yet been reported.

Besides its function in Malpighian tubule development, zygotic expression of CAUDAL is also required for the formation of the anal pads, structures derived from the most terminal region of the posterior cuticular ectoderm (Macdonald and Struhl, 1986).

Zygotic expression of the *caudal* gene in this region is dependent on TAILLESS (Mlodzik and Gehring, 1987b).

The *cnc* (*cap'n'collar*) gene encodes a potential leucine zipper transcription factor and is expressed in the anterior of the embryo, including the labral primordium in the anterior terminal domain (Mohler et al., 1991). An anterior cap of *cnc* gene expression is activated by the BICOID and TORSO-mediated pathways at nuclear cycle thirteen (see also IV(C) below). Later its posterior extent is refined by TAILLESS and SPALT, its anterior extent by GIANT, and its ventral extent by the dorso-ventral hierarchy; all of these interactions repress *cnc* and so restrict its domain of expression to part of the labral primordium (Mohler, 1993). Mutations in *cnc* have not been reported, thus its developmental functions are not known.

The 'pair-rule' and 'segment polarity' genes were initially identified on the basis of their role in the development of the central, segmented region of the embryo: the pair-rule genes function to subdivide this region into segmentally repeated units known as parasegments while the segment polarity genes are required for maintenance of the parasegmental borders. Most of the pair rule and segment polarity genes are also expressed in the embryonic termini. Their phenotypes and functions there are less well defined (Table 1), and their positions in the terminal gene hierarchy remain to be studied in detail. We consider these genes briefly below.

There are eleven pair-rule genes: *hairy*, *even-skipped*, *fushi tarazu*, *odd-paired*, *odd-skipped*, *ten^{m}*/*odd Oz*, *paired*, *hopscotch*, *runt*, *sloppy paired* and *unpaired*.

hairy encodes a helix-loop-helix family transcription factor (Ish-Horowicz et al., 1985; Rushlow et al., 1989b) that is expressed in a patch of cells in the dorsal head region in the anterior terminal domain while, posteriorly, the seventh stripe of hairy expression falls within the terminal domain (Ingham et al., 1985). Expression of this posterior stripe is strongly reduced or absent in *tailless* mutant embryos, placing *hairy* in the terminal gene hierarchy downstream of *tailless* (Mahoney and Lengyel, 1987; Hooper et al., 1989). In

the termini, *hairy* mutants shows defects in structures that derive from these regions (Jürgens, 1987).

The *even-skipped* gene encodes a homeodomain-containing transcription factor (Nüsslein-Volhard et al., 1985; Macdonald and Struhl, 1986; Frasch et al., 1987). *even-skipped* mutants exhibit posterior terminal defects (Nüsslein-Volhard et al., 1985) coincident with the seventh stripe of EVENSKIPPED expression. This stripe is absent in *tailless* mutant embryos (Frasch and Levine, 1987; Goto et al., 1989).

The *fushi tarazu* gene encodes a homeodomain-containing transcription factor (Laughon and Scott, 1984). *fushi tarazu* mutants exhibit defects in structures derived from the posterior terminal region (Wakimoto et al., 1984; Jürgens, 1987) and coinciding with its seventh stripe of expression. This stripe is absent in embryos derived from $torso^{lof}$ females and in *tailless* mutant embryos, and is expanded in embryos produced by $torso^{gof}$ mothers (Mahoney and Lengyel, 1987; Strecker et al., 1989; Strecker and Lipshitz, 1990; Strecker et al., 1991).

Several additional pair rule genes are expressed in the termini and mutants exhibit defects in the development of structures derived from these regions. Presumably these reside in the terminal gene hierarchy, but analyses of their expression in terminal pathway mutants have not been reported. These genes are *odd-paired*, which encodes a zinc-finger containing protein (Jürgens, 1987; Benedyk et al., 1994); *odd-skipped*, which also encodes a zinc finger protein (Coulter and Wieschaus, 1988; Coulter et al., 1990); ten^{m}/*odz*, which encodes a protein homologous to TENASCIN, with eight EGF and eleven fibronectin III repeats (Baumgartner et al., 1994; Levine et al., 1994); *paired*, which encodes a transcription factor with two highly conserved domains: a *paired* box and a homeodomain (Nüsslein-Volhard et al., 1985; Bopp et al., 1986; Frigerio et al., 1986; Kilchherr et al., 1986); and *hopscotch*, which encodes a JAK-family tyrosine kinase (Perrimon and Mahowald, 1986; Binari and Perrimon, 1994).

No analyses of terminal mutant phenotypes have been reported for the remaining pair-rule genes: *runt*, *sloppy paired* and *unpaired.* Two of these have been analyzed molecularly: *runt* encodes a *runt* domain-containing transcription factor (Gergen and Butler, 1988) and *sloppy paired* encodes two *forkhead* domain-containing transcriptional regulatory proteins (Grossniklaus et al., 1992). Both of these are expressed within the terminal domains and so are likely to function in the terminal gene hierarchy.

The segment polarity genes are involved in the establishment and maintenance of the parasegments through control of cell-cell interactions (reviewed in Martinez Arias, 1993), and twelve of these are known to be expressed in the termini: *patched*, *engrailed*, *wingless*, *dishevelled*, *shaggy/zeste-white 3*, *armadillo*, *naked*, *porcupine*, *hedgehog*, *cubitus interruptus*, *gooseberry* and *fused.* While expression within the terminal domain as well as terminal mutant phenotypes have been reported for several of the segment polarity genes, no detailed analyses have been conducted that would position them within the terminal hierarchy. Consequently they are only considered briefly here (Table 1): *patched* encodes a putative transmembrane protein and mutants have defects in the tail (Jürgens, 1987); *wingless* encodes a protein homologous to vertebrate INT-1 and mutant embryos lack structures derived from both termini (Perrimon and Mahowald, 1987); *engrailed* encodes a homeodomain-containing transcription factor and mutant embryos have posterior terminal defects (Jürgens, 1987); *dishevelled* encodes a novel maternally synthesized protein that is uniformly distributed in the embryo (Klingensmith et al., 1994), and embryos derived from germline clones lack certain posterior terminal derivatives (Perrimon and Mahowald, 1987); *shaggy/zeste-white 3* also encodes a maternally synthesized, uniformly distributed protein - in this case a serine/threonine kinase homologous to glucose synthetase kinase 3 (Bourouis et al., 1990) - and embryos derived from germline clones have defects in the anterior and posterior termini (Perrimon and Smouse, 1989); *armadillo* encodes a protein homologous to vertebrate plakoglobin and mutant embryos exhibit terminal defects (Peifer and Wieschaus, 1990); *hedgehog* encodes a transmembrane protein

that is expressed in the termini, which exhibit defects (Mohler, 1988; Lee et al., 1992; Mohler and Vani, 1992); *cubitis interruptus* encodes a zinc-finger protein that is expressed in the termini, and mutant embryos exhibit terminal defects (Orenic et al., 1987; Orenic et al., 1990); *gooseberry* encodes a *paired* domain- and homeodomain-containing protein that is expressed in the termini (Baumgartner et al., 1987; Perrimon and Mahowald, 1987); *fused* encodes a serine/threonine kinase that is maternally expressed, and embryos from mutant females or germline clones exhibit posterior terminal defects (Preat et al., 1990). Finally *naked* mutant embryos (Perrimon and Smouse, 1989), and *porcupine* embryos derived from germline clones have defects in the head and tail (Perrimon et al., 1989; Siegfried et al., 1994). As discussed above (section II(C)), the four maternally expressed segment polarity genes may reside in the maternally encoded signal transduction pathway.

D. Genes that Program Endodermal Development

The list of genes that function in endodermal development is much shorter than the list of those that function in ectodermal development (Table 1). This is likely to be an artefact with two origins. First, most mutant screens have focused on the cuticle (which is an ectodermal derivative) and thus have biased the identification of loci towards the ectoderm. Second, cuticle phenotypes are easier to identify and study than those in the gut, thus biasing phenotypic analyses away from the endoderm. It is likely that many of the genes that function in ectodermal development also function in endodermal development but phenotypes in the endoderm have not yet been defined.

The *serpent* gene functions to specify midgut as distinct from foregut/hindgut identity: in *serpent* mutants endodermal midgut is transformed into ectodermal foregut/hindgut (Reuter, 1994). *serpent* mutant embryos also fail to undergo germband retraction; this may be an indirect consequence of incorrect specification of midgut cell identity. There is as yet no information on the nature of the *serpent* gene product.

As mentioned above, the FORKHEAD transcription factor functions in programming both midgut (endodermal) and hindgut (ectodermal) development. The anterior and posterior midgut primordia of *forkhead* mutant embryos invaginate, but fail to undergo migration and disintegrate. This defect has been interpreted as evidence that the midgut primordia cells fail to adopt appropriate fates in the absence of FORKHEAD function and thus die, indicating that continued expression of FORKHEAD is necessary for the cells of the midgut primordia to differentiate (Weigel et al., 1989a)

Apart from its expression and function in Malpighian tubule development (III(C) above) HNF-4 is expressed in the primordia of the midgut. Mutant embryos lacking zygotic HNF-4 activity fail to form the midgut (Zhong et al., 1993).

E. Genes that Control Morphogenetic Movement

During normal *Drosophila* embryogenesis, the posterior midgut and hindgut primordia are brought internally by the combined processes of midgut and hindgut invagination, and germband extension. Anteriorly, invaginations move the anterior midgut and the foregut primordia internally. All of these processes are accomplished by a combination of local cell rearrangement and cell shape alterations (Campos-Ortega and Hartenstein, 1985; Sweeton et al., 1991; Costa et al., 1993; Costa et al., 1994). The germband subsequently retracts bringing the body parts into their final locations along the antero-posterior axis; this process involves cell shape changes rather than cell rearrangements (Campos-Ortega and Hartenstein, 1985). Subsequently, the anterior and posterior midgut continue growing towards each other and finally fuse to form one continuous intestinal structure.

In addition to exhibiting defects in the specification of positional and tissue identity in the termini, embryos derived from homozygous $torso^{lof}$ mutant mothers show defects in morphogenesis (Schüpbach and Wieschaus, 1986b; Strecker et al., 1989; Strecker et al., 1991; Strecker et al., 1992). Embryos derived from homozygous $torso^{lof}$ mothers lack

cells with midgut and hindgut identities. They do not undergo midgut invagination, and they also have defects in germband extension: instead of moving dorsally around the posterior pole and then anteriorly toward the head, the tip of the germband remains at the posterior end of the embryo(Schüpbach and Wieschaus, 1986b). The germband is eventually thrown into deep folds or forms spirals as it extends. These spiralled embryos are reminiscent of those derived from *corkscrew* mothers (see II(C) above). It is difficult to distinguish whether these defects in morphogenetic movement are a secondary consequence of misspecification of gut identity, or whether the genes that program these movements are regulated independently of the gut identity genes by the TORSO-signaling pathway.

Genetic analyses indicate that the zygotic gastrulation mutant, *folded gastrulation*, can suppress $torso^{gof}$ mutant phenotypes (Strecker et al., 1991; Strecker et al., 1992). This may be taken as circumstantial evidence for direct regulation of *folded gastrulation* by the TORSO-mediated pathway (Table 1, Figure 3). Hemizygous *folded gastrulation* mutant embryos are defective in ventral furrow formation in the central region and in posterior midgut invagination in the posterior terminal domain (Zusman and Wieschaus, 1985; Costa et al., 1994). Only the latter derives from the posterior terminal region and will be discussed here. During normal gastrulation, at the site of posterior midgut invagination, somatic cells immediately dorsal to the pole cell cluster initiate apical constriction. Subsequently, such constrictions commence in cells located further dorsally, then in those positioned laterally and finally in cells on the ventral side of the pole cell cluster. In *folded gastrulation* mutant embryos, initiation of apical constriction in the dorsal cells is normal but the subsequent propagation of apical constrictions to other cells is defective; as a result, posterior midgut invagination does not occur (Costa et al., 1994).

Genetic analysis indicates that the *folded gastrulation* gene product acts locally and that over-expression of FOLDED GASTRULATION protein can induce ectopic cell shape changes (Costa et al., 1994). The FOLDED GASTRULATION protein exhibits no

obvious homology to any known protein (Costa et al., 1994); however, since it contains a potential amino-terminal signal sequence, it may be secreted. Expression of FOLDED GASTRULATION commences in the ventral furrow and the posterior midgut primordium about thirty minutes before the first apical constrictions of cells in these regions (Costa et al., 1994). It has been speculated that FOLDED GASTRULATION functions as a local signal that coordinates cell shape change (Costa et al., 1994). Posterior expression of FOLDED GASTRULATION is dependent on the terminal pathway; it is reduced by *huckebein*, *tailless* and *forkhead* mutations individually and is completely abolished in *huckebein tailless* double mutant embryos (Costa et al., 1994). These data suggest that the *folded gastrulation* gene resides downstream of the *tailless* and *huckebein* genes in the gut development hierarchy, but they do not resolve the issue of whether the morphogenetic defects are a primary or a secondary effect.

It was shown some time ago that, if embryos from *torso* mutant females do manage to undergo germband extension, there are later defects in the process of germband retraction which is delayed and/or incomplete (Strecker et al., 1989; Strecker and Lipshitz, 1990; Strecker et al., 1991). Several zygotic mutants exhibit a similar failure of germband retraction, including *hindsight*, *tailup* and *u-shaped* (Table 1, Figure 3) (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984). These act as suppressors of $torso^{gof}$ phenotypes, suggesting that they might reside in the terminal gene hierarchy (Strecker et al., 1991). Among the three, *hindsight* mutations are the strongest suppressors of $torso^{gof}$ phenotypes. Studies of *hindsight* mutant embryos using time-lapse video microscopy have indicated that germband extension is normal (M.L.R. Yip and H.D. Lipshitz, in preparation), thus focusing attention on the specificity of the germband retraction defect. This is further supported by the fact that the midgut and hindgut form normally in *hindsight* mutant embryos (M.L.R. Yip and H.D. Lipshitz, in preparation), excluding a possible secondary effect of misspecification of the identity of these tissues (in contrast to *serpent*; see III(D) above), and supporting the possibility of primary control of the germband retraction

process by the TORSO-mediated signal acting through *hindsight*. The *hindsight* gene encodes a putative transcription factor with fourteen widely spaced C_2H_2 Zn-fingers, that is transcribed in the posterior terminal region in the presumptive posterior midgut primordium (M.L.R. Yip and H. Lipshitz, in preparation). Activation of *hindsight* gene transcription in this region fails in embryos from *torso* mutant mothers, suggesting that it is controlled in response to the terminal signaling pathway. *hindsight* transcription occurs normally in *tailless* mutant embryos but does not occur in *huckebein* mutant embryos (M.L.R. Yip and H.D. Lipshitz, in preparation), consistent with *hindsight* residing downstream of *huckebein* in the midgut cell fate specification hierarchy. Expression of HINDSIGHT persists in the posterior midgut through the end of germband extension (M.L.R. Yip and H.D. Lipshitz, in preparation). Since HINDSIGHT is expressed in the midgut but not in the mesoderm or the epidermal ectoderm which undergo cell shape changes during germband retraction (M.L.R. Yip and H.D. Lipshitz, in preparation), it must program a cell-cell signaling pathway that initiates or coordinates the process of germband retraction.

The *u-shaped* gene has been cloned and shown to encode a zinc-finger protein (P. Simpson and W. Gelbart, pers. comm.), while the *tailup* gene has not yet been analyzed molecularly (Table 1). It is not yet known whether *tailup* and/or *u-shaped* are transcriptionally regulated by HINDSIGHT.

IV. Mechanisms for Establishing Distinct Cell Fates Within the Termini

While we have outlined many of the downstream effector genes that function in the termini, we have yet to address issues relating to how distinct cell fates are specified within the termini with respect to (1) more central versus more terminal, (2) dorsal versus ventral, and (3) anterior versus posterior. Such differences are likely to derive from several mechanisms. First, there is evidence that the TORSO-mediated signal or the response to this signal is graded within the termini (Casanova and Struhl, 1989; Casanova and Struhl, 1993), and this is likely to be relayed into the initial differential regulation of effector genes. Second, while many of the same zygotic effector genes are turned on at both termini (see Figure 3 and III above), their domains of expression are modulated by the dorso-ventral hierarchy in both termini as well as by the anterior gene hierarchy at the anterior, thus leading to distinct outcomes in cell fate specification. Such a mechanism relies on spatial control of zygotic effector gene transcription by the maternal axis specification pathways. Third, while several of the same zygotic effector genes are turned on at both termini, their regulatory capacities are modulated by the dorso-ventral hierarchy and also at the anterior by the anterior gene hierarchy, resulting in the control of at least partially distinct subsets of target genes in different regions (see below). Fourth, there are differences in the battery of effector genes turned on at the anterior versus the posterior, and along the dorso-ventral axis (see III above, and IV(B) below), again brought about by the combined action of the terminal and the other axis-specifying hierarchies. This last mechanism differs from the third in that the integration of terminal and other axial information occurs at the level of the transcriptional control of the zygotic effector genes, rather than further downstream at the level of effector gene action on their target genes.

A. More Central versus More Terminal Fates

In addition to differences between the anterior and posterior termini (see IV(C) below), both termini must acquire differences along their antero-posterior and their dorso-

ventral axes. We have considered at length above (III) the various effector genes that are differentially expressed along the antero-posterior axis of the termini. How the more central cells in the termini are first specified to become distinct from the more terminal ones is not clear. Experiments have suggested that the TORSO receptor tyrosine kinase is activated to different extents, with highest levels of activation at the poles and lower levels in the more central regions of the termini (Casanova and Struhl, 1989). There is additional evidence suggesting that this differential activation of the receptor might be a consequence of a non-uniform distribution (or activation) of the ligand for the TORSO receptor which, again, is likely to be highest at the poles (Casanova and Struhl, 1993). Just what this differential activation means at the molecular level is unclear: presumably either more receptors bind ligand and thus transduce more signal per unit area of membrane in the more terminal region than more centrally; or the occupancy rate for the ligand-receptor complex is higher in one region than the other. Either way, since the cytoplasmic signal transduction pathway appears to be conserved, it is plausible that the read-out of differential receptor activity is differential phosphorylation of transcription factors such as those in the JUN- and/or ETS-families (see II(D) above). Presumably this results in differential activity of these transcriptional regulators and thus differential activation of downstream effector genes in distinct regions of the termini.

B. Dorsal versus Ventral Fates

Next we consider the dorso-ventral axis specification genes and their possible role in specifying dorso-ventral differences within the termini. All of the so-called dorso-ventral pathway genes were identified based on genetic screens that examined the central, segmented region of the embryo. Thus, it does not follow *a priori* that they are also expressed in the termini or, if expressed there, that they are necessarily involved in dorso-ventral axis specification in the termini. That dorso-ventral axis specification in the termini is controlled, at least in part, by the terminal pathway was revealed by the fact that both

torso^{lof} and *torso*^{gof} mutations result in abnormalities in the dorso-ventral axis: *torso*^{lof} mutations result in ventralization of terminal region cells while *torso*^{gof} mutations result in dorsalization of central region cells (Strecker et al., 1991; Strecker et al., 1992). Several of the dorso-ventral pathway genes (both maternal and zygotic) are expressed in the termini, and transcriptional control of zygotic dorso-ventral genes in the termini is regulated by the terminal pathway (see below) (Table 1). Using the *torso*^{gof} phenotypes as the starting point, six zygotic and two maternal dorso-ventral mutations were identified as either suppressors or enhancers (zygotic: *decapentaplegic*, *tolloid*, *short gastrulation*, *twisted gastrulation*, *zerknüllt* and *pointed*; maternal: *dorsal* and *cactus* (Strecker et al., 1991)). Many of these genes have been shown to be required for normal terminal development, although it is not always clear that the mutant phenotypes can most readily be explained in terms of a dorso-ventral axis defect in the termini rather than a more general requirement. We outline below what is known about several of these genes, their expression and function with particular reference to the termini. It should be remembered that they also function in the central region of the embryo but those functions, as well as their expression there, are not considered in detail here.

The *decapentaplegic* gene encodes a protein with homology to a family of mammalian secreted proteins that includes transforming growth factor-β, inhibin, Müllerian inhibiting substance and bone morphogenetic proteins (BMPs) (Derynk et al., 1985; Mason et al., 1985; Cate et al., 1986; Padgett et al., 1987; Wozney et al., 1988). It is believed that these proteins exert their influence on cells that express the corresponding receptors. Null mutations of *decapentaplegic* (the *decapentaplegic* ^{Hin} alleles) are zygotic lethal and result in the dorsal and dorsolateral cells of mutant embryos adopting more ventral cell fates (Irish and Gelbart, 1987). At the anterior end, a hole is present because little or no cuticle is formed and head structures are missing; posteriorly, there are defects in the development of dorsally-derived terminal structures. Initial expression of DECAPENTAPLEGIC in the termini extends around the two poles to include more ventral cells; by the end of germband

extension, this terminal expression refines into patches (St Johnston and Gelbart, 1987; Ray et al., 1991).

The *tolloid* gene encodes a product homologous to mammalian bone morphogenetic protein-1 (BMP-1) (Shimell et al., 1991). BMPs were initially identified in mammalian cells as critical components of protein extracts that can direct cartilage and bone formation (Wozney et al., 1988). Among the seven characterized BMPs, all except BMP-1 show sequence similarity to the TGF-β superfamily (see DECAPENTAPLEGIC above). It has been postulated that BMP-1 acts as metalloprotease and is involved in activating the latent forms of the other BMPs (Wozney et al., 1988; Dumermuth et al., 1991; Shimell et al., 1991). *tolloid* mutant embryos show defects in the dorsal 40% of the segmented region and are slightly ventralized there (Jürgens et al., 1984; Shimell et al., 1991). In the head and tail, dorsally derived structures are missing (Jürgens et al., 1984; Shimell et al., 1991). Null phenotypes of *tolloid* can be suppressed by increasing the dosage of the wildtype *decapentaplegic* gene, suggesting that TOLLOID acts upstream of DECAPENTAPLEGIC (Ferguson and Anderson, 1992). This has since been confirmed at the molecular level: the protease domain of TOLLOID is required for the activation of DECAPENTAPLEGIC (Riddihough and Ish-Horowicz, 1991; Finelli et al., 1994). Since *decapentaplegic* and *tolloid* encode products homologous to different components of the BMP complex in mammals, the fact that *decapentaplegic* and *tolloid* interact genetically to specify the dorso-ventral axis of the *Drosophila* embryo suggests evolutionarily conservation of this cell-cell interaction pathway. Initial expression of TOLLOID at the syncytial blastoderm stage is very similar to that of DECAPENTAPLEGIC; however, in contrast to DECAPENTAPLEGIC, during cellularization TOLLOID expression disappears from the poles (Shimell et al., 1991).

The *zerknüllt* gene encodes a homeodomain transcriptional regulatory protein. It is required for the formation of dorsal tissues, including the amnioserosa in the central region and the optic lobes in the anterior terminal domain (Wakimoto et al., 1984). Initial

expression of ZERKNÜLLT in the termini at the syncytial blastoderm stage is very similar to that of DECAPENTAPLEGIC and TOLLOID. By the cellular blastoderm stage, however, terminal ZERKNÜLLT expression becomes restricted to two dorsal patches in the head (Doyle et al., 1986; Rushlow et al., 1987b).

In the central region of the embryo, initial expression of these zygotic genes is under the control of the maternal dorso-ventral genes (e.g. see Rushlow et al., 1987a). Specifically, nuclear localization of DORSAL, a REL/NFκB-related transcription factor, on the ventral side of the embryo represses expression of these zygotic genes thus restricting their initial expression to the dorsal side (Ray et al., 1991). Subsequent refinements of *decapentaplegic*, *tolloid* and *zerknüllt* expression are probably a result of regulatory interactions within the zygotic component of the dorso-ventral hierarchy.

In contrast to the central region, the expression of the *decapentaplegic*, *tolloid* and *zerknüllt* genes in the two termini is controlled by the TORSO-mediated terminal pathway, which also overrides repression by DORSAL on the ventral side of the termini (Ray et al., 1991; Rusch and Levine, 1994). Control by the TORSO-mediated terminal pathway is consistent with the observed genetic interactions between mutations in these three loci and $torso^{gof}$ alleles (Strecker et al., 1991; Strecker et al., 1992). For example, mutations in *decapentaplegic*, *tolloid* and *cactus* [a maternally encoded negative regulator of DORSAL, which is homologous to IκB (Geisler et al., 1992; Kidd, 1992)] are enhancers of $torso^{gof}$ alleles, while *dorsal* mutations function as suppressors (Strecker et al., 1991; Strecker et al., 1992).

Mutations in three additional zygotic dorso-ventral loci interact genetically with $torso^{gof}$ alleles: *twisted gastrulation* and *short gastrulation* mutations behave as suppressors while *pointed* mutations act as enhancers (Strecker et al., 1991; Strecker et al., 1992). Embryos hemizygous for *twisted gastrulation* or *short gastrulation* exhibit defects during gastrulation as a result of the dorsal amnioserosa cells undergoing abnormal cell shape changes (Zusman and Wieschaus, 1985; Zusman et al., 1988). In the termini, *twisted*

gastrulation mutant embryos show head and tail defects (Lindsley and Zimm, 1992). Mosaic analysis indicates that, while *twisted gastrulation* activity is required on the dorsal side of the embryo, *short gastrulation* functions in the termini and ventrally (Zusman and Wieschaus, 1985; Zusman et al., 1988). Genetically, *twisted gastrulation* probably lies downstream of, or parallel to, *decapentaplegic* while *short gastrulation* is required to repress *decapentaplegic* activity ventrally in the central region of the embryo (Ferguson and Anderson, 1992). The TWISTED GASTRULATION protein shows limited homology to human connective tissue growth factor (Mason et al., 1994) while the product of the *short gastrulation* gene is unknown at present.

pointed belongs to the so called *spitz*-group of genes that was initially identified based on their functions in the ventral ectoderm of the embryo (Mayer and Nüsslein-Volhard, 1988). *pointed* mutant embryos have defects in the anterior and posterior termini (Mayer and Nüsslein-Volhard, 1988; Klämbt, 1993). The *pointed* gene encodes two overlapping transcripts, P1 and P2, that share 3'-sequence (Klämbt, 1993). This common 3'-sequence encodes an ETS domain, which has been shown to be important for DNA-binding of other ETS-family transcription factors (Karim et al., 1990). The 5'-region of the longer transcript, P2, encodes an additional domain of homology to a subset of ETS-like proteins. P1 and P2 show differential expression patterns and activities during embryogenesis (Klämbt, 1993; Scholz et al., 1993). Recent data indicate that the POINTED^{P2} protein is a target of MAP kinase in the SEVENLESS-mediated signaling pathway in the eye (Brunner et al., 1994a; O'Neill et al., 1994). It is possible that the expression and/or function of one (or both) of the POINTED proteins is regulated similarly by the TORSO-mediated pathway in the embryonic termini.

C. Anterior versus Posterior Fates

While both the anterior and posterior termini give rise to endodermal and ectodermal tissues, there are also distinct differences in the tissues formed at the two termini.

Specifically, the brain is derived from the acron within the anterior domain of TORSO function and the development of epidermal and intestinal ectodermal derivatives is quite different in the two termini. Several effector genes function at both termini: these include *tailless*, *huckebein*, *lines*, *empty spiracles*, *tramtrack*, *forkhead*, *spalt*, *serpent*, *HNF-4*, *hairy* and *wingless* (see III above). However, the details of their spatial expression patterns differ between the two termini. In addition, several effector genes function in only one of the two termini: for example, *hunchback*, *Abdominal-B*, *T-related gene*, *Krüppel*, *cut*, *caudal*, *folded gastrulation* and *hindsight* function only in the posterior terminal region and *giant* functions only in the anterior terminal region (see III above).

How zygotic effector genes might be differentially controlled at the two termini is exemplified by analysis of transcriptional regulation of the *tailless* gene (Figure 4). In the posterior terminal domain, TAILLESS is expressed as a symmetrical cap at both the syncytial and the cellular blastoderm stages (Pignoni et al., 1990). In contrast, while TAILLESS is also initially expressed as a symmetrical cap in the anterior terminal region at the syncytial blastoderm stage, TAILLESS expression subsequently retracts from the most anterior and ventral regions and becomes restricted to the acron by the cellular blastoderm stage (Pignoni et al., 1990). The initial, symmetrical expression of TAILLESS at the anterior is programmed largely by the TORSO-mediated pathway, while the subsequent restriction of TAILLESS expression to the acron is accomplished through combined action of the anterior pathway (probably direct regulation of *tailless* gene transcription by the BICOID homeodomain protein) and the dorso-ventral pathway (repression of *tailless* transcription ventrally) (Liaw and Lengyel, 1992; Pignoni et al., 1992).

In addition to control of zygotic terminal pathway effector gene transcription by the anterior and dorso-ventral hierarchies, recent experiments have demonstrated a reciprocal action of the TORSO-mediated phosphorylation cascade upon the BICOID homeodomain protein (Ronchi et al., 1994). Specifically, it has been shown that certain zygotic target genes that reside in the anterior gene hierarchy are initially transcriptionally activated by

BICOID, but later become repressed in the most anterior terminal region of the embryo (Figure 4). This repression is dependent on the function of the TORSO receptor tyrosine kinase and its downstream genes in the cytoplasmic signal transduction pathway such as D-RAF, which were shown to result in phosphorylation of the BICOID protein (Ronchi et al., 1994). Repression did not, however, require either TAILLESS or HUCKEBEIN.

Thus, cross-regulation among the axis-specifying hierarchies can occur both among the maternally-encoded proteins and at the level of transcriptional control of zygotic effector genes. Further, these analyses indicate that the cross-regulation is bidirectional; the above examples demonstrate that the anterior pathway can regulate the terminal pathway and *vice versa.* Clearly this lends power and flexibility to the differential cell fate specification machinery.

V. Conclusions

Analysis of the terminal pathway in *Drosophila* has begun to provide us with insights into how localized activation of a generally expressed receptor can be used to provide spatial cues during development. Many components of the cytoplasmic signal transduction cascade are used at other times and in other places during *Drosophila* development. There is increasing evidence that these signal transduction cascades have been conserved among metazoa. Numerous zygotically expressed terminal pathway effector genes have been identified. These control the specification of terminal positional identity, the development of terminal tissues and terminal morphogenetic cell shape changes and movements. Several mechanisms appear to be used to modulate the general cytoplasmic signal into varied patterns of effector gene expression. These include differential activation of the transmembrane receptor as well as combinatorial action of the terminal, anterior and dorso-ventral genetic pathways in distinct regions within the termini. This likely results in activation of overlapping batteries of effector genes in spatially distinct patterns within the termini, resulting in subdivision of the terminal domains into groups of

cells with distinct fates. Understanding of the link between the maternally encoded cytoplasmic signal transduction cascade and the control of zygotic effector genes remains poor. In addition, the details of how the effector genes are differentially regulated and how they in turn specify the details of cell and tissue fates, remain key areas for future analysis.

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Table 1: Terminal Hierarchy Members - Genes Known or Likely to Reside in the Pathway

Gene (abbreviation)	Molecular details	Mutant Phenotypes (alleles)	References
Abdominal-B (*Abd-B*)	1. Homeodomain transcription factors (ABD-BI, ABD-BII). 2. Zygotic. 3. ABD-BII in parasegments 14 and 15; ABD-BI in parasegments 13-15.	lof: Deletion of posterior spiracles and filzkörper. Homeotic transformation of A8 toward A4/A5.	Sanchez-Herrero et al., 1985; Casanova, 1990; Celniker et al., 1990
armadillo (*arm*)	1. Homolog of human plakoglobin. 2. Zygotic. 3. Uniformly distributed in embryos.	lof: Head and tail defects.	Riggleman et al., 1989; Peifer and Wieschaus, 1990; Riggleman et al., 1990

cactus (*cact*)	1. Homolog of IκB. 2. Maternal. 3. Uniformly distributed in embryos.	lof: Dependent on the allelic combination, variable degree of ventralization including deletion of anal plates and filzkörper. Enhance $torso^{gof}$ phenotypes. gof: Dependent on the allelic combination, variable degree of dorsalization. In most extreme case anal plates and filzkörper are deleted.	Schüpbach and Wieschaus, 1989; Roth et al., 1991; Strecker et al., 1991; Geisler et al., 1992; Kidd, 1992
cap'n'collar (*cnc*)	1. Leucine zipper transcription factor. 2. Zygotic. 3. Terminal expression in an anterior cap activated by TORSO and BICOID at nuclear cycle thirteen. Subsequent repression by GIANT anteriorly, TAILLESS posteriorly and the dorso-ventral genes ventrally, restrict expression to part of the labral primordium.	No mutations reported.	Mohler et al., 1991; Mohler, 1993

caudal (*cad*)	1. Homeodomain transcription factor. 2. Maternal and zygotic. 3. Maternal product distributed in a posterior-to-anterior gradient in early embryo. Zygotic expression includes the primordia of midgut, hindgut and A9, A10, telson primordia.	lof: No anal tuft, abnormal anal pads, terminal sense organs lost or abnormal. No maternal and no zygotic: Deletion of A9, A10 and telson; abnormal Malphigian tubules.	Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987a; Liu and Jack, 1992
corkscrew (*csw*)	1. Putative tyrosine phosphatase with two SH2 domains. 2. Maternal and zygotic. 3. Uniformly distributed in embryo.	No maternal: Deletion of acron and labrum in the anterior; posterior midgut and Malpighian tubules posteriorly. Defects in morphogenetic movements. Suppress *torso*^{gof} phenotypes.	Perrimon et al., 1989; Perkins et al., 1992
cubitus interruptus/Cell (*ci/Ce*)	1. Zinc-finger protein. 2. Zygotic. 3. At cellular blastoderm stage, uniformly expressed from 20-90% EL; later resolves into fifteen broad stripes (the last two stripes in posterior terminal domain).	Head and tail defects.	Orenic et al., 1987; Orenic et al., 1990

cut (*ct*)	1. Homeodomain transcription factor. 2. Zygotic. 3. Expressed in Malpighian tubule anlagen.	lof: Abnormal Malphigian tubules.	Blochlinger et al., 1988; Liu et al., 1991; Liu and Jack, 1992
decapentaplegic (*dpp*)	1. TGF-β family growth factor. 2. Zygotic. 3. Initial expression occurs in the dorsal 40% of the central region of the embryo but extends around the two poles to include more ventral cells within the termini. Expression at the termini is dependent on *torso* function.	Null: Loss of acron, labral structures, posterior spiraclular hair, filzkörper, anal tuft and pads. Fail to complete germband extension. lof: Rudimentary and uneverted posterior spiracles and filzkörper. Enhance $torso^{gof}$ phenotypes.	Irish and Gelbart, 1987; Padgett et al., 1987; St Johnston and Gelbart, 1987; St Johnston et al., 1990; Casanova, 1991; Strecker et al., 1991; Ferguson and Anderson, 1992; Wharton et al., 1993
dishevelled (*dsh*)	1. Novel protein. 2. Maternal and zygotic. 3. Uniformly distributed in early embryos.	No maternal: Lack posterior spiracles and filzkörper.	Perrimon and Mahowald, 1987; Perrimon et al., 1989; Klingensmith et al., 1994; Noordermeer et al., 1994; Siegfried et al., 1994

dorsal (*dl*)	1. Homolog of REL/NFκB transcription factor. 2. Maternal. 3. Protein is translocated into the nuclei on the ventral side and in the termini of the embryo.	No maternal: Loss of endodermal gut and Malpighian tubules. Suppress $torso^{gof}$ phenotypes.	Nüsslein-Volhard et al., 1980; Steward, 1987; Steward et al., 1988; Roth et al., 1989; Rushlow et al., 1989a; Steward, 1989; Strecker et al., 1991
downstream of receptor kinases (*drk*)	1. Protein with two SH2 domains and one SH3 domain. 2. Maternal (?) and zygotic. 3. ?	lof: Suppress $torso^{gof}$ phenotypes.	Doyle and Bishop, 1993; Olivier et al., 1993; Simon et al., 1993
Dsor1	1. MAP kinase activator (MEK homolog). 2. Maternal and zygotic. 3. Expressed throughout development.	No maternal: typical terminal phenotypes. No maternal and zygotic: poor cuticular development. Dominant maternal suppressor of *tor*, *trk*, *fs(1)ph*, *tsl*, *l(1)ph* loss of function mutations and dominant maternal enhancer of tor^{gof} phenotypes.	Tsuda et al., 1993

Dsrc	1. Homologous to C-SRC. 2. Maternal and zygotic. 3. Uniformly distributed at cellularization and gastrulation.	Ectopic expression of wildtype or mutant SRC at high level causes germband retraction defects.	Simon et al., 1983; Simon et al., 1985; Kussick et al., 1993
empty spiracles (*ems*)	1. Homeodomain transcription factor. 2. Zygotic. 3. Expressed in procephalic lobe anteriorly, filzkörper anlagen posteriorly.	lof: Optic lobes and filzkörper absent. Suppress HS-*tailless* phenotypes.	Dalton et al., 1989; Strecker et al., 1992; Walldorf and Gehring, 1992
engrailed (*en*)	1. Homeodomain transcription factor. 2. Zygotic. 3. Expression is seen in parasegments 14 and 15. Later, complex expression in the head including spots in the brain and clypeolabrum.	lof: Reduced posterior spiracles.	DiNardo et al., 1985; Kornberg et al., 1985; Poole et al., 1985; Jürgens, 1987; Diederich et al., 1991; Schmidt-Ott and Technau, 1992

even-skipped (*eve*)	1. Homeodomain transcription factor. 2. Zygotic. 3. Expression in the seventh stripe is dependent on *tailless*.	lof: Reduced posterior spiracles, tuft and filzkörper.	Nüsslein-Volhard et al., 1985; Macdonald et al., 1986; Frasch et al., 1987; Frasch and Levine, 1987; Goto et al., 1989
folded gastrulation (*fog*)	1. Putative secreted, novel protein. 2. Zygotic. 3. Expressed in a posterior polar cap (0-10% EL).	lof: Failure in anterior and posterior midgut invagination. Suppress $torso^{gof}$ phenotypes.	Wieschaus et al., 1984; Zusman and Wieschaus, 1985; Strecker et al., 1991; Costa et al., 1994
forkhead (*frk*)	1. Transcription factor with *forkhead* domain. 2. Zygotic. 3. Midgut, foregut and hindgut.	lof: Anterior and posterior midgut primordia fail to undergo migration in order to fuse into a unit structure, instead they disintegrate.	Jürgens and Weigel, 1988; Weigel et al., 1989a; Weigel et al., 1989b

fs(1)Nasrat (*fs(1)N*)	1. ? 2. Maternal. 3. ?	lof: Collapsed egg and deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. 211: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. DH1: Temperature-sensitive, collapsed egg plus posterior terminal defect (18°C) or central deletion (25°C).	Degelmann et al., 1986; Degelmann et al., 1990
fs(1)polehole (*fs(1)ph*)	1. ? 2. Maternal. 3. ?	lof: Collapsed egg. 1901: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment.	Degelmann et al., 1990
fused (*fu*)	1. serine/threonine kinase. 2. maternal and zygotic. 3. Maternal transcript uniform in embryo through germband extension.	No or reduced maternal: Defects in A8.	Perrimon and Mahowald, 1987; Preat et al., 1990; Therond et al., 1993

fushi tarazu (*ftz*)	1. Homeodomain transcription factor. 2. Zygotic. 3. The seventh stripe is dependent on *torso* and *tailless*.	lof: Defects in structures derived from several posterior terminal domain segments: reduced filzkörper and spiracular hair (A8), posterior lateral sense organs (A9), anal sense organs (A10) and telson. The posterior spiracles remain on the dorso-lateral surface.	Laughon and Scott, 1984; Wakimoto et al., 1984; Jürgens, 1987
Gap1	1. Homologous to GTPase activating protein. 2. Maternal (?) and zygotic. 3. ?	lof: Enhances tor^{gof} phenotypes.	Buckles et al., 1992; Gaul et al., 1992; Rogge et al., 1992; Doyle and Bishop, 1993
giant (*gt*)	1. Transcription factor with leucine zipper. 2. Zygotic. 3. At cellular blastoderm, horseshoe-shaped domain (stripe 1) at 88-95% EL.	lof: Missing the labrum, epistomal sclerite and dorsal bridge. Suppress $torso^{gof}$ phenotypes.	Mohler et al., 1989; Eldon and Pirrotta, 1991; Strecker et al., 1991; Capovilla et al., 1992

gooseberry (*gsb*)	1. Two *paired* domain- and homeodomain-containing proteins. 2. Zygotic. 3. Striped expression in parasegments 14 and 15; later, additional expression occurs more posteriorly.	No details reported.	Baumgartner et al., 1987; Perrimon and Mahowald, 1987
hairy (*h*)	1. b-HLH transcription factor. 2. Zygotic. 3. Dorsal head patch at 85-95% EL and the seventh stripe fall within the terminal domain. Expression in the seventh stripe is dependent on *tailless*.	lof: Defects in the anterior lateral and dorso-medial sense organs, filzkörper and fell posteriorly, and median tooth of the labrum anteriorly.	Ingham et al., 1985; Ish-Horowicz et al., 1985; Jürgens, 1987; Mahoney and Lengyel, 1987; Hooper et al., 1989; Rushlow et al., 1989b
hedgehog (*hh*)	1. Transmembrane protein. 2. Zygotic. 3. Early gastrula: expressed in dorsal anterior spot at 97% EL; posterior 3 stripes in terminal domain.	Head skeleton and tail abnormal.	Mohler, 1988; Lee et al., 1992; Mohler and Vani, 1992

hindsight (*hnt*)	1. Putative zinc-finger transcription factor. 2. Zygotic. 3. Midgut.	lof: Failure of germband retraction. Suppress $torso^{gof}$ phenotypes.	Wieschaus et al., 1984; Strecker et al., 1991; M.L.R. Yip & H.D. Lipshitz, in prep.
HNF-4	1. Zinc-finger transcription factor. 2. Maternal and zygotic. 3. Maternal transcripts distributed uniformly. Zygotic transcripts appear in anterior and posterior midgut.	lof: Midgut and Malpighian tubules fail to form.	Zhong et al., 1993
hopscotch (*hop*)	1. JAK family tyrosine kinase. 2. Maternal and zygotic. 3. Uniformly distributed in embryos.	No maternal and zygotic: Deleted or reduced A8 segment and defects in posterior spiracles.	Perrimon and Mahowald, 1986; Binari and Perrimon, 1994
huckebein (*hkb*)	1. SP-1/EGR-like zinc-finger transcription factor. 2. Zygotic. 3. Two polar caps (0-12% EL and 90-100% EL).	lof: Deletion of endodermal midgut. Abnormal morphogenetic movements. Suppress $torso^{gof}$ phenotypes.	Weigel et al., 1990; Brönner and Jäckle, 1991; Brönner et al., 1994

hunchback (*hb*)	1. Zinc-finger transcription factor. 2. Maternal and zygotic. 3. At cellular blastoderm, posterior stripe from 10-25% EL.	lof: Deletion of the A8 segment. Suppress $torso^{gof}$ phenotypes.	Lehmann and Nüsslein-Volhard, 1987; Tautz et al., 1987; Strecker et al., 1991
Krüppel (*Kr*)	1. Zinc-finger transcription factor. 2. Zygotic. 3. Malphigian tubule anlagen.	lof: Malphigian tubules missing.	Gaul et al., 1987; Harbecke and Janning, 1989; Liu and Jack, 1992
l(1)pole hole (*l(1)ph*)	1. RAF serine/threonine kinase homolog. 2. Maternal and zygotic. 3. Uniformly distributed in embryo.	No maternal: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. Defects in morphogenetic movements. No maternal and zygotic: embryos degenerate 7 hr after fertilization. Suppress $torso^{gof}$ phenotypes.	Nishida et al., 1988; Ambrosio et al., 1989b; Melnick et al., 1993
lines (*lin*)	1. ? 2. Zygotic. 3. ?	lof: Head defects; missing A8, posterior spiracles and anal pads. Synergistic interactions with *tailless* alleles. Suppress $torso^{gof}$ and HS-*tailless*phenotypes.	Nüsslein-Volhard et al., 1984; Strecker et al., 1991; Strecker et al., 1992

naked (*nkd*)	1. ? 2. Zygotic. 3. ?	lof: Defective head and abnormal posterior spiracles.	Jürgens et al., 1984; Martinez Arias et al., 1988; Perrimon and Smouse, 1989
odd-paired (*opa*)	1. Zinc-finger protein. 2. Zygotic. 3. Single broad domain at 20-80% EL.	lof: Defects in multiple posterior structures: the anterior lateral sense organs, dorso-medial sense organs, anal sense organs, spiracular hair, fell and filzkörper.	Jürgens, 1987; Benedyk et al., 1994
odd-skipped (*odd*)	1. Zinc-finger protein. 2. Zygotic. 3. At early gastrulation stages, anterior pole and stripes fourteen and fifteen fall within the terminal domain.	lof: Ectopically located median tooth that is frequently misshapen; defective anal tuft and pads.	Coulter and Wieschaus, 1988; Coulter et al., 1990
paired (*prd*)	1. Paired domain and homeodomain-containing transcription factor. 2. Zygotic. 3. At cellular blastoderm, anterior head patch (87-93% EL) and thirteenth and fourteenth stripes fall within the terminal domains.	lof: Lack the telson and posterior sense organs.	Nüsslein-Volhard et al., 1985; Bopp et al., 1986; Frigerio et al., 1986; Kilchherr et al., 1986

patched (*ptc*)	1. Putative transmembrane protein. 2. Zygotic. 3. Expression includes parasegments 13 and 14, the labrum, stomodeum and hindgut anlagen (where it is eventually restricted to the Malpighian tubules).	lof: Enlarged anal tuft. Defects in all tail sense organs.	Jürgens, 1987; Hooper and Scott, 1989; Nakano et al., 1989
porcupine (*porc*)	1. ? 2. Maternal and Zygotic. 3. ?	No maternal: Head and tail defects.	Perrimon et al., 1989; Siegfried et al., 1994
Ras1	1. RAS, GTPase protein 2. Maternal and zygotic. 3. Expressed throughout development.	lof: Suppress $torso^{gof}$ phenotypes. gof: Phenocopy $torso^{gof}$ phenotypes.	Lev et al., 1985; Simon et al., 1991; Doyle and Bishop, 1993; Lu et al., 1993
rolled (*rl*)	1. MAP kinase. 2. Maternal and zygotic.. 3. Expressed throughout development.	lof: Suppress $torso^{gof}$ phenotypes. gof (Sem): Deletion of central structures similar to $torso^{gof}$ phenotypes.	Biggs III and Zipursky, 1992; Biggs III et al., 1994; Brunner et al., 1994b

runt (*run*)	1. *runt* domain-containing transcription factor. 2. Zygotic. 3. At cellular blastoderm, anterior head patch (75-85% EL) and the seventh stripe fall within the terminal domain. Later, an additional stripe and proctodeal expression occur posterior to the original seventh stripe.	Phenotypes not examined in termini.	Gergen and Wieschaus, 1985; Gergen and Butler, 1988; Kania et al., 1990
serpent (*srp*)	1. ? 2. Zygotic. 3. ?	lof: Homeotic transfomation of endodermal midgut to ectodermal foregut/hindgut.	Jürgens et al., 1984; Reuter, 1994
shaggy/zeste white 3 (*sgg/zw3*)	1. Serine/threonine kinase homologous to glucose synthetase kinase 3. 2. Maternal and Zygotic. 3. Uniformly distributed in early embryos.	No maternal: Defective head and abnormal posterior spiracles.	Perrimon et al., 1989; Perrimon and Smouse, 1989; Bourouis et al., 1990

short gastrulation (*sog*)	1. ?. 2. Zygotic. 3. ?	lof: Delayed formation and closure of anterior and posterior midgut invaginations. Germband extension incomplete. Suppress *torso*^{gof} phenotypes.	Zusman and Wieschaus, 1985; Zusman et al., 1988; Strecker et al., 1991
sloppy paired (*slp*)	1. Two *forkhead* domain-containing transcription factors. 2. Zygotic. 3. At syncytial blastoderm, anterior polar cap (70-100% EL) that undergoes rapid changes. Later, complex expression occurs in the procephalon. At germband extended stage, the last two stripes (in A8 and A9) fall within the terminal domain.	Phenotypes not examined in termini.	Grossniklaus et al., 1992; Cadigan et al., 1994
Son of sevenless (*Sos*)	1. Homologous to RAS guanine nucleotide exchange factor. 2. Maternal and zygotic. 3. ?	No maternal: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. lof: Suppress *torso*^{gof} phenotypes.	Rogge et al., 1991; Simon et al., 1991; Bonfini et al., 1992; Doyle and Bishop, 1993

spalt (*sal*)	1. Zinc-finger transcription factor. 2. Zygotic. 3. Anterior horseshoe-shaped domain at 80-86% EL. Posterior stripe at 12-20% EL is dependent on *tailless*.	lof: Partial homeotic transformation of A9 and A10 toward A8 segment.	Jürgens, 1988; Kühnlein et al., 1994; R. Schuh and H. Jäeckle, cited in Jürgens and Hartenstein, 1993
T-related gene (*Trg*)	1. Homologous to mammalian *Brackyury* (*T*) gene. 2. Zygotic. 3. Hindgut and anal pad primordia.	lof: Deletion of hindgut and anal pads.	Kispert et al., 1994
tailless (*tll*)	1. Homologous to steroid hormone receptor superfamily. 2. Zygotic. 3. Initially two polar caps (0-20% EL and 80-100% EL). The anterior cap is refined into a dorsal horseshoe-shaped domain (76-89% EL).	lof: Abnormal clypeolabrum, optic lobes and procephalic lobe. Missing segments A8, A9, A10, hindgut and Malpighian tubules. Suppress $tor so^{gof}$ phenotypes.	Strecker et al., 1986; Klingler et al., 1988; Strecker et al., 1988; Strecker et al., 1989; Pignoni et al., 1990
tailup (*tup*)	1. Zinc-finger transcription factor. 2. Zygotic. 3. ?	lof: Failure of germband retraction. Suppress tor^{gof} phenotypes.	Nüsslein-Volhard et al., 1984; Strecker et al., 1991

tenascin^{m}/odd Oz (*ten^{m}/odz*)	1. Protein homologous to TENASCIN with 8 EGF repeats and 10 fibronectin III repeats. 2. Zygotic. 3. At cellular blastoderm stage, protein present in an antero-dorsal head patch and in the posterior midgut primodium. Later, protein also accumulates in stomodeum.	lof: Abnormal telson.	Baumgartner et al., 1994; Levine et al., 1994
tolloid (*tld*)	1. Homolog of bone morphogenetic protein-1 (BMP1), a putative metallo-endopeptidase. 2. Zygotic. 3. Initial expression occurs in the dorsal 40% of the central region of the embryo but extends around the two poles to include more ventral cells within the termini.	lof: Missing pharyngeal skeleton anteriorly and filzkörper posteriorly. Enhance *torso^{gof}* phenotypes.	Shimell et al., 1991; Strecker et al., 1991; Ferguson and Anderson, 1992; Childs and O'Connor, 1994

torso (*tor*)	1. PDGF family receptor tyrosine kinase. 2. Maternal. 3. Germline expression. Translated in embryo after fertilization. RNA and protein uniformly distributed in embryo.	lof: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. No midgut or hindgut formed; reduced foregut. Defects in morphogenetic movements. Expansion of central, segmented fate map into termini. gof: Expansion of terminal fate map into central, segmented region. Deletion of central segmental trunk structures.	Schüpbach and Wieschaus, 1986b; Schüpbach and Wieschaus, 1986a; Klingler et al., 1988; Casanova and Struhl, 1989; Schüpbach and Wieschaus, 1989; Sprenger et al., 1989; Strecker et al., 1989
torso-like (*tsl*)	1. Putative secreted novel protein. 2. Maternal and zygotic. 3. Maternally expressed in the somatic follicle cells adjacent to the two poles of the oocyte.	lof: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. No midgut or hindgut formed; reduced foregut. Defects in morphogenetic movements. Expansion of central, segmented fate map into termini. ee: Deletion of central trunk structures.	Stevens et al., 1990; Savant-Bhonsale and Montell, 1993; Martin et al., 1994

tramtrack (*ttk*)	1. Zinc-finger transcription factor. 2. Maternal and zygotic. 3. Maternal transcripts distributed uniformly. Zygotic transcripts appear in anterior and posterior midgut.	lof: Abnormal head and tail (filzkörper absent).	Read and Manley, 1992; Brown and Wu, 1993; Xiong and Montell, 1993
trunk (*trk*)	1. Probable ligand for TORSO receptor tyrosine kinase. 2. Maternal. 3. ?	lof: Deletion of acron and labrum in the anterior and tail structures posterior to the A7 segment. No midgut or hindgut formed; reduced foregut. Defects in morphogenetic movements. Expansion of central, segmented fate map into termini.	Schüpbach and Wieschaus, 1986b; Schüpbach and Wieschaus, 1986a; Schüpbach and Wieschaus, 1989; Casanova and Struhl, 1993
twisted gastrulation (*tsg*)	1. Limited homology to human connective tissue growth factor. 2. Zygotic. 3. Anterior dorsal cap at syncytial blastoderm stage.	lof: Abnormal morphogenetic movements, head defects and condensed, retracted spiracles. Suppress $torso^{gof}$ phenotypes.	Zusman and Wieschaus, 1985; Zusman et al., 1988; Strecker et al., 1991; Mason et al., 1994
u-shaped (*ush*)	1. ? 2. Zygotic. 3. ?	lof: Failure of germband retraction. Suppress $torso^{gof}$ phenotypes.	Nüsslein-Volhard et al., 1984; Strecker et al., 1991

unpaired (*upd*)	1. ? 2. Zygotic. 3. ?	lof: Abnormal posterior spiracles; filzkörper altered in appearance or absent.	Wieschaus et al., 1984; Gergen and Wieschaus, 1986
wingless (*wg*)	1. Vertebrate INT-1 proto-oncogene homolog. 2. Zygotic. 3. At early blastoderm, initial expression at the stomodeum and proctodeum. Subsequently expression includes parasegments 13 and 14. Later, complex expression in the head including a spot in the labral region.	lof: Lack head structures and filzkörper.	Baker, 1987; Perrimon and Mahowald, 1987; Rijsewijk et al., 1987; Baker, 1988a; Baker, 1988b; Schmidt-Ott and Technau, 1992
zerknüllt (*zen*)	1. Homeodomain transcription factor. 2. Zygotic. 3. Initial expression occurs in the dorsal 40% of the central region of the embryo but extends around the two poles to include more ventral cells within the termini. Expression at the termini is dependent on *torso* function.	lof: Deletion of optic lobes in the anterior terminus. Suppress $torso^{gof}$ phenotypes.	Wakimoto et al., 1984; Doyle et al., 1986; Rushlow et al., 1987a; Strecker et al., 1992

Molecular details: 1. = nature of the gene product; 2. = maternal and/or zygotic expression; 3. = expression patterns. Abbreviations: A# = abdominal segment #; ee = ectopic expression; EL = egg length, with 0% representing the posterior tip and 100% representing the anterior tip; gof = gain of function; lof = loss of function or hypomorphic phenotype; ? = unknown/unreported.

FIGURE LEGENDS

Figure 1:

The TORSO receptor tyrosine kinase: (A) structure; (B) mutant phenotypes. (A) All gain of function *torso* alleles map to the extra-cellular domain, which is shown cross-hatched; while all loss of function *torso* alleles map to the cytoplasmic kinase domain (stippled) (Sprenger and Nüsslein-Volhard, 1992). The transmembrane domain is shaded black. (B) Schematic representation of the effects of *torso* mutations on the fate map of the embryo. The terminal domains of the fate map are shaded; the central, segmented region is unshaded; anterior is to the left and dorsal towards the top of the page. In embryos from *torso* loss of function females, the terminal portions of the fate map are lost and the cells in the termini adopt central, segmented fates (Schüpbach and Wieschaus, 1986b). In embryos from *torso* gain of function females, the central portions of the fate map are lost and the cells in the central region adopt terminal fates (Klingler et al., 1988; Schüpbach and Wieschaus, 1989; Strecker et al., 1989).

Figure 2:

The TORSO-mediated signal transduction pathway. The TORSO receptor (TOR) is shown interacting with its ligand, TRUNK (TRK), which is converted into its active form by the TORSO-LIKE protein (TSL). Two cytoplasmic signal transduction pathways are shown, one to the right and one to the left of the TOR receptor. To the right, the signal is transduced through DRK, SOS, RAS, GAP, RAF, DSOR1, RL and affects the phosphorylation state of transcription factors X and Y that then control nuclear transcription of the downstream effector genes *tailless* (tll) and *huckebein* (hkb). To the left, possible additional participants, SRC and CSW, are shown. For details of the functions and phenotypes of each component, see the text. Symbols: Mickey Mouse ear = SH2/SH3

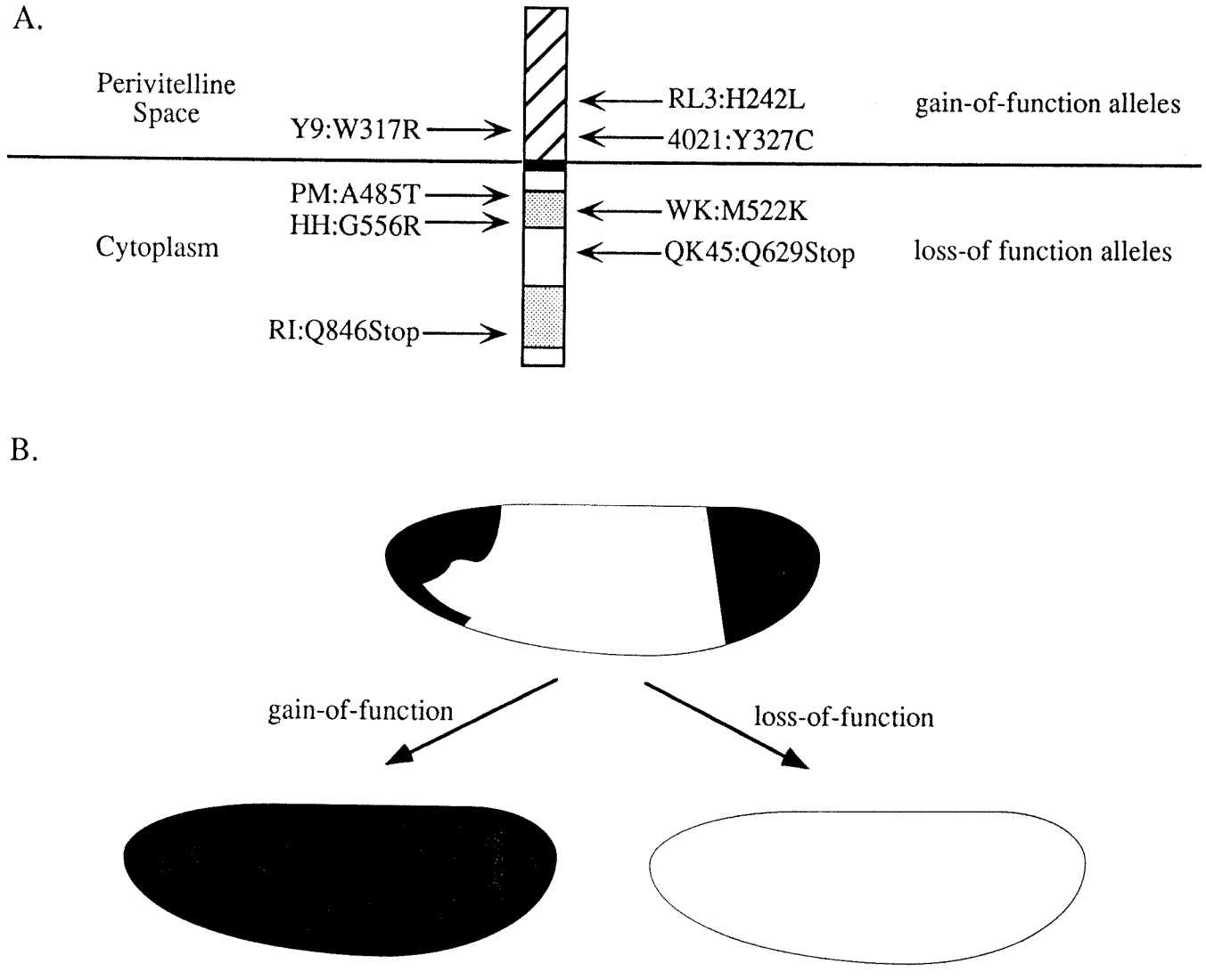
domains; rectangle = phosphatase domain; ellipsoids in the cytoplasm = kinase domain; ? = uncertain step or player in the pathway.

Figure 3:

Fate maps of the anterior and posterior termini (A) and expression/functional domains of selected terminal pathway genes (B). (A) The anterior terminal domain extends from roughly 70 - 100% egg length (EL), while the posterior terminal domain extends from 0 - 25% EL. Abbreviations of structures on the fate map: A# = abdominal segment #; Ac = acron; Amg = anterior midgut; Ap = anal pads; f = fell; fk = filzkörper; Hg = hindgut; Lr = labrum (clypeolabrum); Mp = Malpighian tubules; Pmg = posterior midgut; Te = telson; Δt = tuft. Small ovals in the anterior represent regions that will contribute to the head skeleton: dbr = dorsal bridge; da = dorsal arms; lr = labrum; vp = vertical plates. Sensory organs are symbolized by filled squares or asterisks: anteriorly, the labral sense organ (lrso), and posteriorly the dorsal medial sense organ hair (asterisk in A8), antero-lateral sense organ hair (asterisk in A9, anterior), posterior-lateral sense organ hair (asterisk in A9, posterior), anal sense organ hair (asterisk in A10), antero-lateral sense organ peg sensillum (square in A8), posterior-lateral sense organ peg sensillum (square in A9), anal sense organ peg sensillum (square in A10), dorsal medial sense organ peg sensillum (square in f-fk region). (Drawings are after: Jürgens et al., 1986; Jürgens, 1987; Kuhn et al., 1992; Jürgens and Hartenstein, 1993). (B) Bars represent expression and/or functional domains of selected terminal hierarchy genes. Cross-hatching represents uncertainty in the extent of the expression or functional domain. (After: Jürgens and Hartenstein, 1993).

Figure 4:

Mechanisms by which the anterior and the dorso-ventral pathways restrict expression of the *tailless* terminal pathway effector gene to the acronal region of the anterior terminal domain. Initially (left diagram), *tailless* expression (shaded) is activated throughout the anterior terminus through the combined action of BICOID at the anterior tip and the TORSO-mediated pathway (Pignoni et al., 1992). Subsequently (right diagram), the TORSO-mediated pathway results in the phosphorylation of BICOID, which now represses the *tailless* gene at the anterior tip; while DORSAL represses the tailless gene ventrally. Together, these result in restriction of *tailless* expression to the acron (shaded). The postulated repressive action of phosphorylated BICOID on *tailless* transcription is highly speculative and is an attempt to resolve published results (Pignoni et al., 1992; Ronchi et al., 1994) in a simple manner. To date, no *in vitro* evidence for such repression has been reported. Positive regulation is indicated by arrows; negative regulation by the 'T's. In the right diagram, although in principle the TORSO-mediated pathway could positively regulate *tailless* expression at the anterior tip and in the ventral region, only the final outcome - negative control of *tailless* expression by BICOID and DORSAL - is shown.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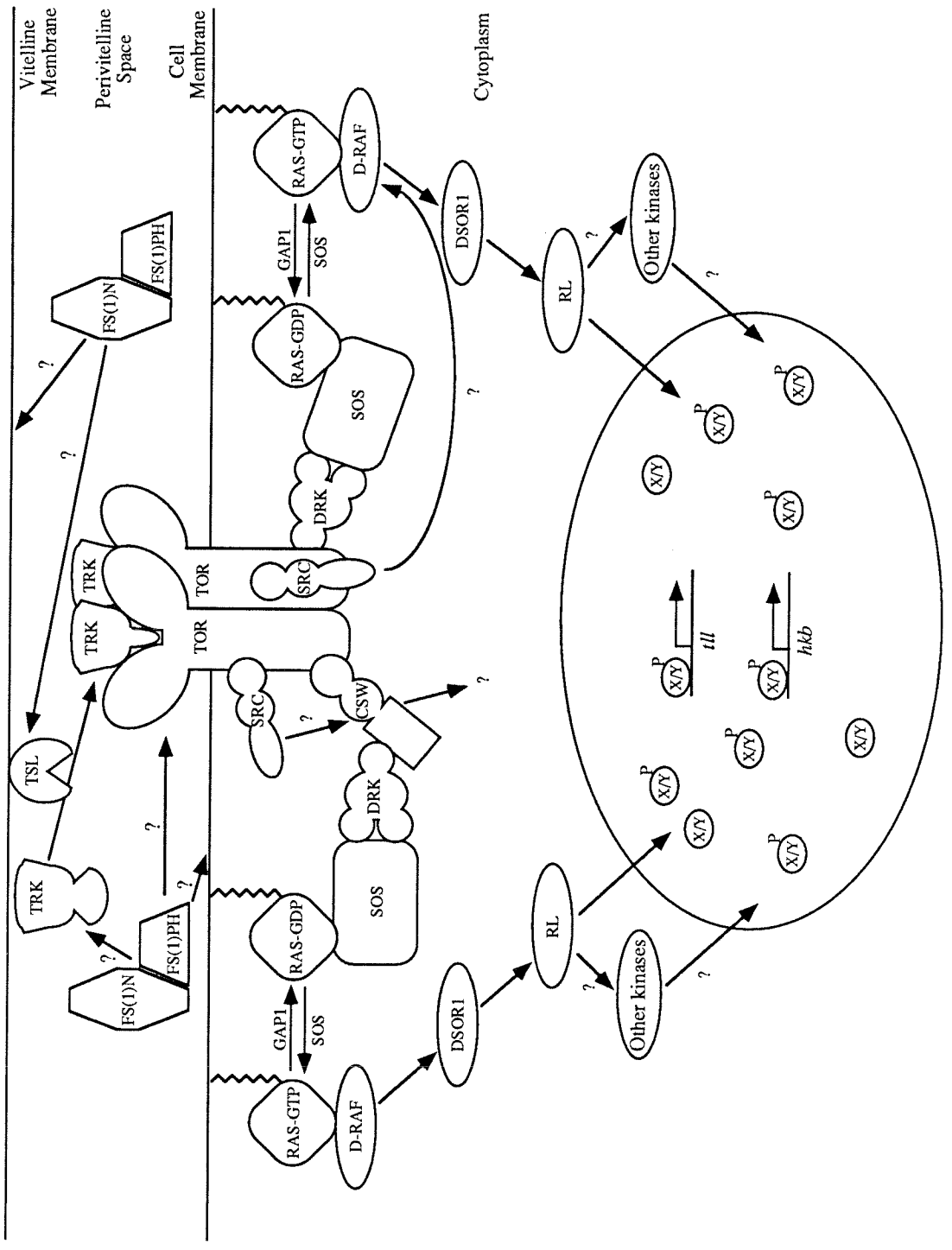
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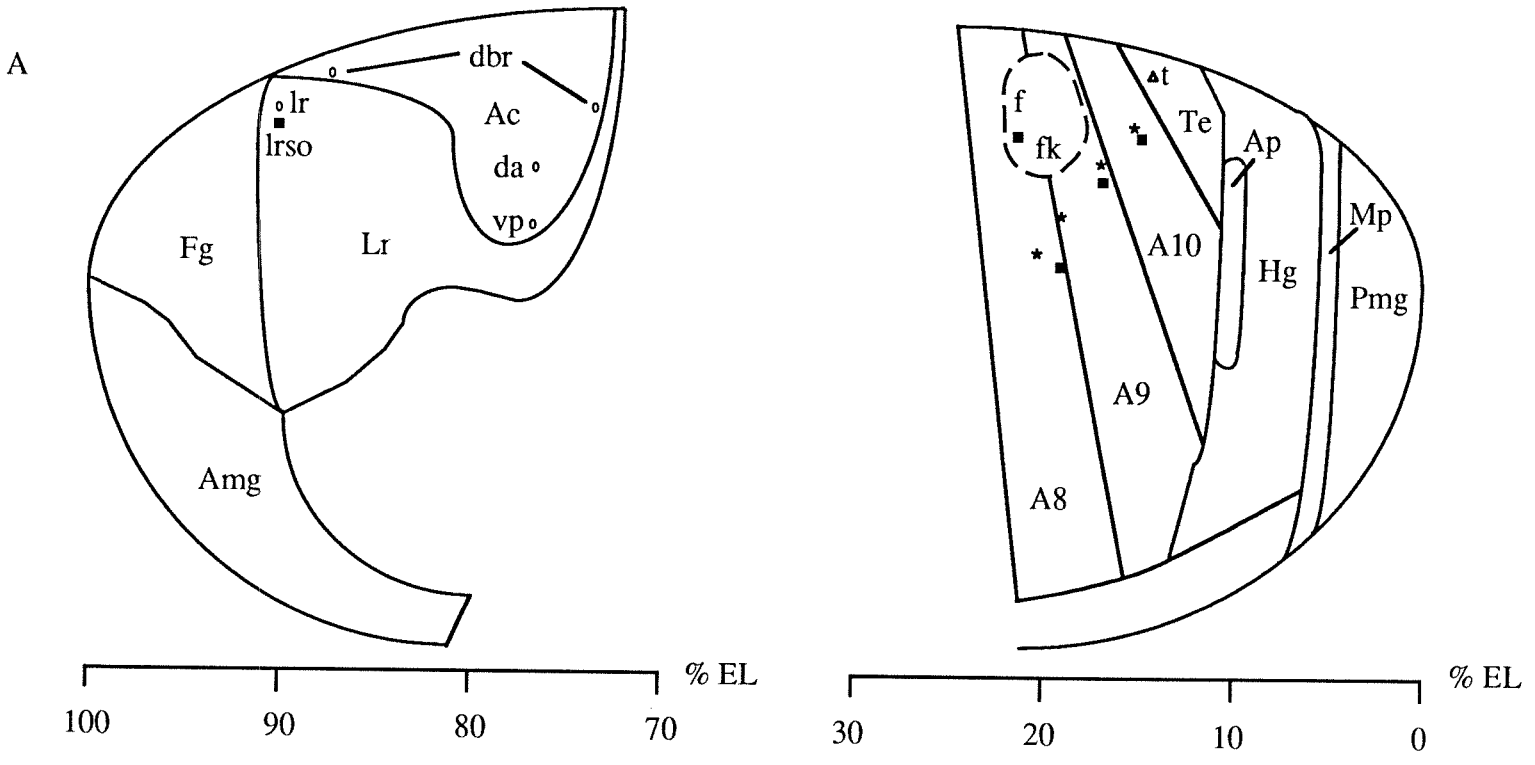
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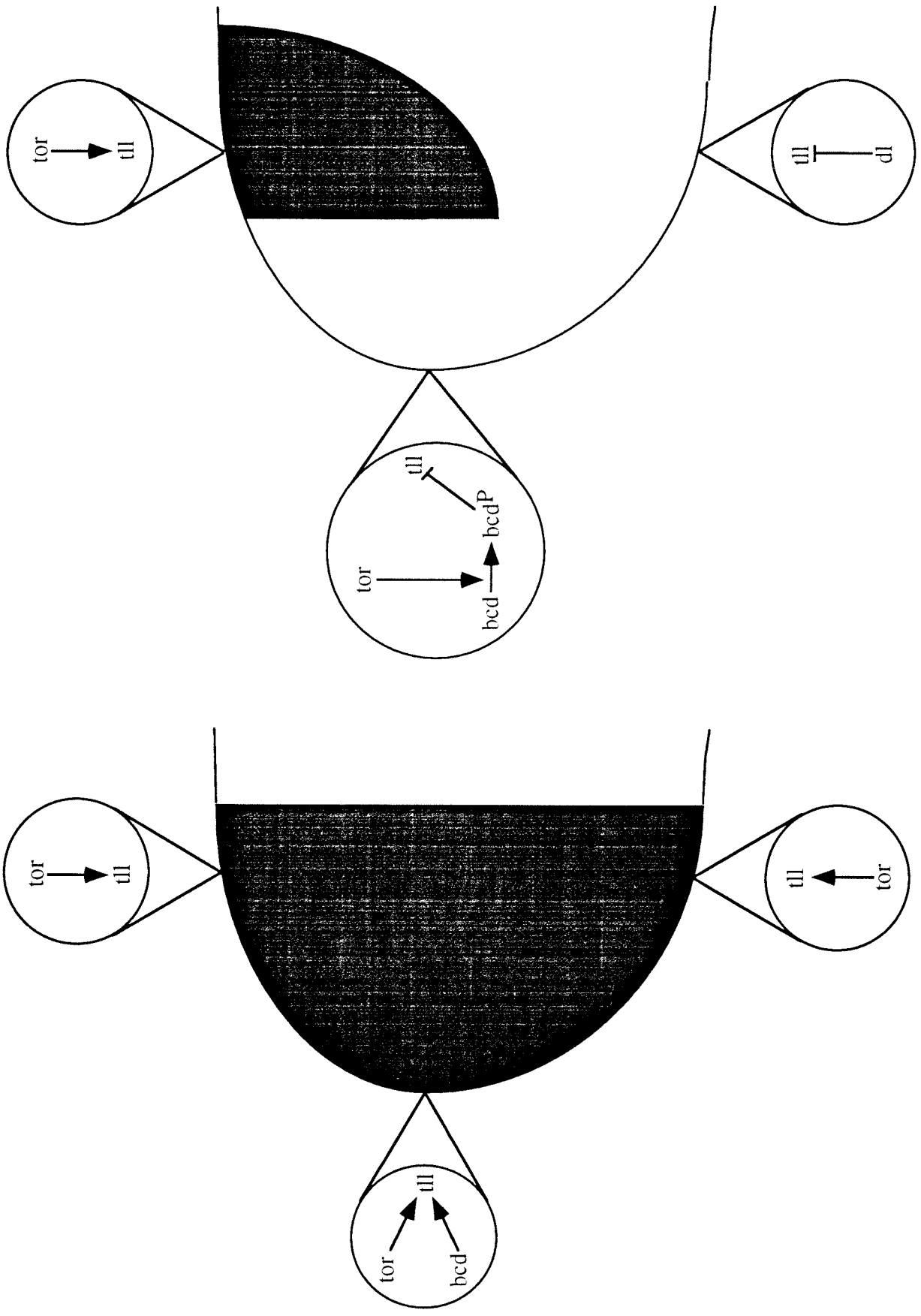
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Chapter 2

Proc. Natl. Acad. Sci. USA **88**, 5824-5828 (1991)

I contributed to all aspects of the research, except for the initial *tor*^{4021} observations, reported here.

Classification: Genetics

Zygotic Genes that Mediate *torso* Receptor Tyrosine Kinase Functions in the *Drosophila melanogaster* Embryo

(terminal genes/insect evolution/maternal effect genes/pattern formation/morphogenesis)

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Abbreviations: RTK, receptor tyrosine kinase.

Running head: *Drosophila* terminal pathway genes

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ABSTRACT The developmental signal that specifies the fates of cells at the anterior and posterior termini of the *Drosophila* embryo is transmitted by the *torso* receptor tyrosine kinase. This paper presents the results of a genetic interaction test for zygotic loci that act downstream of *torso* in the terminal genetic hierarchy. Tests of 26 zygotic mutants with defects in terminal development indicate that at least 14 reside in this hierarchy. The phenotypes associated with these genes fall into three classes, each of which represents a distinct aspect of terminal development and evolution. Four of the genes have been molecularly cloned and their products include an intercellular communication factor as well as three kinds of transcription factors.

The development of the anterior and posterior termini of the *Drosophila* embryo is programmed by a family of six maternally expressed genes collectively referred to as the "terminal" genes (1-5). One of these, *torso* (*tor*), encodes a transmembrane receptor tyrosine kinase (RTK) that is present throughout both terminal and central portions of the embryo (6, 7) but is activated only in the termini (2, 8, 9). The resulting signal is then transmitted through a cytoplasmic cascade that includes the *D-raf* serine threonine kinase (5) into the nucleus where downstream zygotic loci are transcriptionally activated or repressed.

Previous studies of the embryonic termini have emphasized the role of the terminal genes in distinguishing terminal cell fates from central ones along the antero-posterior axis of the embryo. The development of the termini, however, entails a complex series of patterning and morphogenetic events, a large subset of which are programmed by *torso* (1, 8). We previously reported that loci mediating *torso* functions could be identified by their genetic interaction with a hypermorphic *torso* gain-of-function allele that behaves like a constitutively active RTK (8). This enabled us and others to demonstrate that the zygotic locus, *tailless* (*tll*), acts downstream of *torso* in the terminal developmental pathway (8, 9), a conclusion since verified molecularly (10). While *tailless* clearly comprises one of the zygotic mediators of this terminal developmental signal, it directs only a subset of terminal development (11, 12).

Here we report that at least 13 additional zygotically active loci mediate the complex terminal developmental program. These mutants fall into three phenotypic classes that reflect distinct aspects of terminal development and evolution. The molecular biology of the terminal signal transduction pathway is considered in the light of these data.

MATERIALS AND METHODS

Loci tested in this study. *torso* (*tor*) (1, 8, 9); *tailless* (*tll*) (11, 12); *folded gastrulation* (*fog*), *twisted gastrulation* (*tsg*) (13); *faint little ball* (*flb*), *hindsight* (*hnt*), *stardust* (*sdt*), *u-shaped* (*ush*) (14); *hunchback* (*hb*) (15); *lines* (*lin*), *snail* (*sna*), *tailup* (*tup*), *twist* (*twi*) (16); *short gastrulation* (*sog*) (17); *giant* (*gt*) (18); *pointed* (*pnt*), *rhomboid* (*rho*), *Star* (*S*) (19); *decapentaplegic* (*dpp*) (20); *grain* (*grn*), *knickkopf* (*knk*), *krotzkopf verkherdt* (*kkv*), *tolloid* (*tld*) (21); *knirps* (*kni*) (22); *Krüppel* (*Kr*) (23); *spalt* (*sal*) (24); *fork head* (*fkh*) (25).

Screen for suppression/enhancement of tor^{4021} and tor^{splc} by zygotic segmentation mutants. Females that were (i) heterozygous for tor^{4021}, (ii) homozygous for tor^{splc} or (iii) tor^{splc}/tor^{+}; $Dp(2;3)P32(tor^{+})/+$, and also heterozygous for a given zygotic mutation were mated to males that were heterozygous for the same zygotic mutation. In each cross of type (i) over 50 embryos were scored, and in types (ii) and (iii) approximately 100 embryos were scored, with respect to their cuticle pattern 24 hours following fertilization. The change in the abdominal segment number was calculated relative to the wildtype controls. In crosses of types (ii) and (iii), for zygotic mutations that result in a deletion of abdominal segments, the change in the average number of abdominal segments was normalized in light of the defined zygotic mutant phenotype. For example, *tll/tll* embryos lack abdominal segment eight, so the rescued embryos can exhibit a maximum of seven abdominal segments. This result was normalized to represent a net gain of one abdominal segment more than the actual average recorded. Similarly, *gt*, *hb* and *lin* result in abdominal segment deletions (loss of three, one and one segment, respectively). This was factored into the normalized change in the number of abdominal segments (respectively the addition of 3, 1 and 1 to the observed average).

Demonstration of suppression/enhancement at the cellular blastoderm stage. Females that were homozygous for *tor*^{sp1c} and heterozygous for a given zygotic mutation were mated to males that were heterozygous for the same zygotic mutation. Their embryos were allowed to develop at 25°C (suppressors) or 21°C (enhancers) for 1-4 hours (suppressors) or 2-5 hours (enhancers) and were then scored for *fushi tarazu* (*ftz*) stripes after visualization with anti-*ftz* antibody according to standard procedures (26). In each case approximately 100 embryos were scored.

Statistical analysis. In all but one of the 29 comparisons listed in Table 1, the enhancement (En) or suppression (Su) of the cuticular or *ftz* stripe phenotypes is significant at better than or equal to the P = 0.02% level using the nonparametric Wilcoxon rank sum test (8, 27). The exceptional case is *dpp* cuticle, which is significant at the P = 0.06% level. Where no effect (NE) is recorded, the differences are not significant (P > 0.2%).

RESULTS

The *torso* phenotype. Loss of maternal terminal gene function results in the deletion of the most anterior and posterior derivatives of the embryonic fate map and the respecification of the fates of cells in the terminal regions to form more central structures (1-5). Germband movement is also abnormal in loss-of-function *torso* embryos (1, 8).

We report here that, in addition, there is a ventralization of the fate map in the anterior and posterior terminal and subterminal regions of embryos from mothers homozygous for loss-of-function mutations in the maternal terminal genes, *torso* and *trunk*. This is apparent from an examination of the number of denticles per abdominal denticle belt in wildtype embryos and embryos from maternal terminal mutant mothers. For example, in the first row of the sixth abdominal denticle belt from wildtype embryos, an average of 23.6 ± 2.5 denticles was found (range: 20 - 27; n = 5), while embryos from tor^{PM51}/tor^{PM51} mothers had an average of 39.1 ± 6.6 (range: 28 - 47, n = 10) and those from trk^{1}/trk^{1} mothers had an average of 33.8 ± 4.4 (range: 29 - 41, n = 5). In embryos from trk^{1}/trk^{1} mothers, the previously reported loss of the supraesophageal ganglion and the dorsal shift in the position of the subesophageal ganglion (12) is consistent with a similar ventralization anteriorly. Thus, in addition to specifying antero-posterior pattern and morphogenesis, maternal terminal gene activity is required to specify dorsal cell fates in the termini of the embryo.

Identification of zygotic genes that mediate *torso* RTK functions. We previously demonstrated that mutation of the *tailless* gene in embryos derived from constitutive, hypermorphic tor^{sp1c} gain-of-function mothers, restored central segments by interrupting the ectopically programmed terminal developmental pathway in the central region of the embryo (8, 28). We have extended this genetic interaction screen by testing an additional 25 loci, for suppression or enhancement of the central, segment-loss

phenotype of *tor* gain-of-function alleles (Table 1). Although mutations in these loci have been described previously as affecting terminal structures and/or morphogenesis (13-25), the loci have not been genetically associated with the terminal developmental hierarchy.

Mutations in 23 of these genes were initially tested for their effect on central development in embryos from females heterozygous for the most extreme, dominant gain-of-function allele, tor^{4021} (9). Embryos from tor^{4021} mothers can be classified into three phenotypic groups: 38% are empty sacs that exhibit remnants of the proventriculus; 56% form cuticle but no denticles; 6% form cuticle and some denticles corresponding to the anal tuft and/or eighth abdominal denticle belt (Table 1). The zygotic genes could be classified into four categories (Table 1). *Type A suppressors* are defined as those mutations which led to an increase in the proportion of embryos that formed cuticle with denticles ($29 \pm 16\%$ versus 6% of control embryos) and a decrease in the fraction of embryos that formed empty sacs ($4 \pm 3\%$ versus 38% of control embryos). A similar, but less dramatic, suppression was observed for *type B suppressors* that are defined as those mutations that led to a decrease in the proportion of embryos that formed empty sacs ($10 \pm 7\%$ versus 38% of control embryos) and an increase in the proportion of embryos that formed cuticle ($88 \pm 7\%$ versus 56% of control embryos). *Enhancers* are defined as mutations that led to a decrease in the proportion of embryos that formed cuticle or cuticle with denticles ($7 \pm 7\%$ versus 62% of control embryos) and an increase in the proportion of embryos that formed empty sacs ($92 \pm 5\%$ versus 38% of control embryos). The remaining ten loci tested with tor^{4021} did not show significant shifts in phenotype and thus are classified as *non-interacting*.

Confirmation that the zygotic loci mediate *torso* functions. In order to exclude the possibility that the observed interactions were allele-specific with respect to the tor^{4021}, we repeated our tests using tor^{splc} a weaker, semi-dominant, hypermorphic gain-of-function allele that results in embryos in which the loss or gain of segments *per se* can be assayed

(8, 28). In the present study, mutations in *tll*, five additional suppressor loci and three enhancer loci identified in the tor^{4021} screen were retested in embryos from tor^{splc}/tor^{splc} females. Seven showed significant interaction (Table 1, Figure 1); for the other two the number of abdominal segments could not be determined accurately due to abnormal cuticle formation. For two of the interacting loci, additional alleles not listed in Table 1 (hnt^{EH275a} and sog^{YP01}) were tested to confirm that the interactions were not allele-specific (data not shown). Two added loci (*fkh, grn*) were tested with tor^{splc} and neither showed significant interaction (Table 1).

Further tests were conducted using a "weak" constitutive *torso* activity level [tor^{splc}/tor^{+}; *Dp(2;3)P32* tor^{+}/+ [8, 28]). Seven suppressor, four enhancer and two non-interacting loci were retested (Table 1). As expected, all of these loci retested as interacting with the exception of those two previously classified as non-interacting.

Hence, 13 of the 14 interacting loci identified using the "high" constitutive activity allele (tor^{4021}), retested positively in our cuticular analyses using "intermediate" (tor^{splc}) and "low" (tor^{splc}/tor^{+}; *Dp* tor^{+}) *torso* activity levels. The remaining interacting locus, *fog*, retested positively in the *fushi tarazu* (*ftz*) assay (below).

The zygotic loci act in the terminal pathway prior to cell fate determination. The analyses described to this point focused on alterations in the pattern of the late embryonic cuticle as a marker of interaction with *tor* gain-of-function alleles. However, it is known from genetic (8, 9, 28) and molecular (6, 7) analyses that *tor* RTK action occurs between one and four hours of embryogenesis. Thus interacting loci must also act during, or shortly after, these stages. This was confirmed for six of the interacting loci by analysis of an early marker of the embryonic fate map, namely the expression pattern of the *ftz* segmentation gene (29) at the cellular blastoderm stage (Table 1, Figure 1). These results prove the action of these genes in the terminal developmental pathway prior to cell fate determination.

Interacting zygotic loci identify three distinct classes of developmental functions associated with the termini. The fourteen interacting loci can be classified into three phenotypic classes that together account for the *torso* loss-of-function phenotype.

Class I - Terminal-versus-central specification. Two loci - *tll* and *lin* - are required for the formation of anterior and posterior terminal structures, as well as for specifying terminal as distinct from central cell fates along the anterior-posterior embryonic axis (11, 12, 16). The latter conclusion is based on the observation that, with loss of terminal pattern in *tll* (11, 12) or *lin* embryos (this study), there is an expansion of the central pattern into the terminal regions. Thus, in these mutants, the terminal-versus-central distinction is not maintained and terminally located cells are misspecified to be central in fate.

Class II - Pattern specification within the termini. Three additional zygotic loci - *gt, hb* and *knk* - are required for pattern specification along the antero-posterior axis of the termini (15, 18, 21) while three loci - *dpp, pnt* and *tld* - are required for the establishment of dorso-ventral polarity in the termini of the embryo (19, 20, 21). Pattern defects occur without concomitant adoption of central fates by terminal cells; thus, these loci are not involved in the terminal-versus-central cell fate decision.

Class III - Morphogenesis of the termini. Two of the zygotic suppressor mutations - *sog* and *fog* - are required for the normal formation of the posterior midgut (endoderm) (13, 17). Four loci - *hnt, tsg, tup* and *ush* - are required for normal terminal morphogenetic movements (13, 14, 16). Embryos mutant for *tsg* fail to extend their germbands and lack some telson structures. In contrast, mutation of *hnt, tup* or *ush* leads to the failure of germband retraction with no obvious pattern defects.

Genetic interactions among the three classes of zygotic loci. To document interactions among the three classes of terminal pathway genes, double mutant

combinations were made between *tll* (class I) and each of *lin* (class I), *sog* (class III - endoderm) and *hnt* (class III - germband). Double mutants of *tll* with either *sog* or *hnt* exhibited additive effects, suggesting spatial or biochemical independence of class I and III gene action. Although mutation of the *tll* gene results in the strongest known suppression of tor^{4021}, it does not completely suppress the effects (Table 1). The triple mutant combination of tor^{4021} maternally with this strong zygotic suppressor (*tll*) as well as a weaker one (*hnt*), leads to even greater suppression of tor^{4021} (data not shown). Again, this suggests additive effects of *tll* and *hnt*, emphasizing their separate functions in the terminal pathway.

In contrast, there is a synergistic interaction of the two class I genes, *tll* and *lin*. Embryos mutant for both *lin* and *tll* consistently exhibited five to six abdominal segments, one to two fewer than the seven abdominal segments exhibited by each mutant separately. Furthermore, each abdominal segment appeared expanded along the antero-posterior axis, with partial fusions among these central segments. There was also evidence of a partial failure in germband retraction in these double mutant embryos, a phenotype not observed in embryos singly mutant for either of these class I loci. The *tll-lin* synergism likely relates to the joint functions of these two genes in specifying terminal versus central cell fates. The novel germband retraction phenotype suggests that there is likely to be cross-talk between the different parts of the terminal hierarchy.

DISCUSSION

Maternal *torso* activity is mediated by multiple zygotic genes. The *torso* loss-of-function phenotype suggested that several zygotic genes were likely to be involved in mediating *torso* functions in the termini of the *Drosophila* embryo. The first zygotic gene associated with the terminal hierarchy was *tailless* (8, 9, 12). Recently, additional zygotic loci that reside in the terminal developmental hierarchy have been reported (30-32). Here we have identified a further 13 loci that mediate the *torso* developmental signal in the termini of the embryo.

Mutations in the 14 zygotic terminal pathway loci studied here differ in the extent to which they suppress or enhance the central abdominal deletions resulting from constitutive *torso* activity. In general, there is a positive correlation between the number of terminal structures/functions affected by mutations in a particular locus, and the strength of its interactions with *torso* gain-of-function alleles. For example, *tll* and *tld*, respectively the strongest of the suppressors and enhancers, are required for the formation of the largest subdomains within the anterior and posterior termini.

The morphogenetic defects observed in embryos produced by *tor* loss-of-function mothers are less extreme than those observed in the zygotic loci that we have classified into Class III. The most plausible explanation for this is that, while the Class III genes (or their products) are regulated in part by the *tor* RTK-mediated pathway, they are also regulated by other intracellular signalling pathways (33). The localized focus in the posterior terminal region of the blastoderm fate map, for a "tail-up" phenotype (34) resembling that exhibited by three of the class III mutants (*hnt*, *tup* and *ush*), is consistent with our conclusion that control of germband extension is, in part, a terminal pathway function.

Evolution of terminal functions in insects and arthropods. Among the developmental processes specified by the three classes of zygotic terminal genes presented

above, the establishment of asegmental termini as separate from the central trunk region of the germband is the most evolutionarily ancient (conserved in both annelids and arthropods) (35). We have shown here that this process is mediated through *tll* and *lin* in the *Drosophila* embryo. The termini of ancestral embryos are derived from the very anterior and posterior tips of the embryonic primordium, while the regions of the *Drosophila* embryo most closely associated with these domains (acron anteriorly and telson posteriorly) have been shown to arise from the dorsal aspect of the anterior and posterior ends of the *Drosophila* blastoderm fate map (34, 36). Thus, it is not surprising that, during insect evolution, genes required to specify dorsal positional values in the *Drosophila* embryos, such as *tld* and *dpp*, have come under the regulation of the terminal developmental hierarchy. Interestingly, there are also interactions between maternal dorso-ventral pattern mutations and a *torso* gain-of-function allele (*dorsal* mutations suppress and deletion of *cactus* enhances the tor^{4021} phenotype; data not shown). Another relatively ancient association has been between posterior midgut formation and the proctodeum (telson). In more primitive insects the posterior midgut is formed from ventral, invaginating cells that lie adjacent to the proctodeum (37). This ancestral mode of development may be reflected in *Drosophila* by the fact that the presumptive posterior midgut and proctodeum lie adjacent to one another on the blastoderm fate map (34, 36), and that *sog* gene activity is required in ventral tissues as well as in the region of the presumptive posterior midgut (17). In contrast, germband retraction is a recent event in the evolution of insect development, since an analogous process does not occur during the development of primitive insects or other arthropod embryos. Consistent with this, mutations in the *Drosophila* genes *hnt*, *tup* and *ush* do not result in an alteration of pattern specification but specifically affect this particular morphogenetic movement.

Molecular biology of the zygotic terminal pathway genes. Four of the identified zygotic terminal pathway loci (*dpp, gt, hb* and *tll)* have been molecularly cloned

and their expression has been assayed in *tor* mutants (10, 38, 39, R. Ray, K. Arora, C. Nüsslein-Volhard and W. Gelbart, manuscript submitted). In all four cases it has been shown that zygotic transcription of these loci in the termini of the embryo is activated or repressed in response to *torso* activity in the early embryo. This provides direct molecular evidence supporting our conclusions that these genes function in the terminal genetic hierarchy.

These gene products represent four distinct molecular species whose functions include intercellular communication by a TGFß-family homolog (*dpp*) (40) and transcriptional regulation (*gt, hb, tll*). The latter represent three families of transcription factors: *gt* belongs to the leucine-zipper family (M. Capovilla, E. Eldon and V. Pirrotta, manuscript in preparation), *hb* belongs to the zinc-finger family (41), and *tll* belongs to the steroid hormone receptor family (10). The three transcription factor genes are regulated in response to the *torso* signal and, in turn, control the expression of downstream genes that direct the detailed development of the termini. In contrast, *dpp* participates in cell-cell communication that is involved in refining terminal pattern.

The remaining loci are likely to encode additional components of the inter- or intracellular signalling pathways or the gene regulatory apparatus that programs terminal development. Molecular analyses of these genes will lead to insights into the mechanisms by which a transmembrane receptor tyrosine kinase and its multiple downstream genes convert a simple extracellular signal into a series of complex developmental processes.

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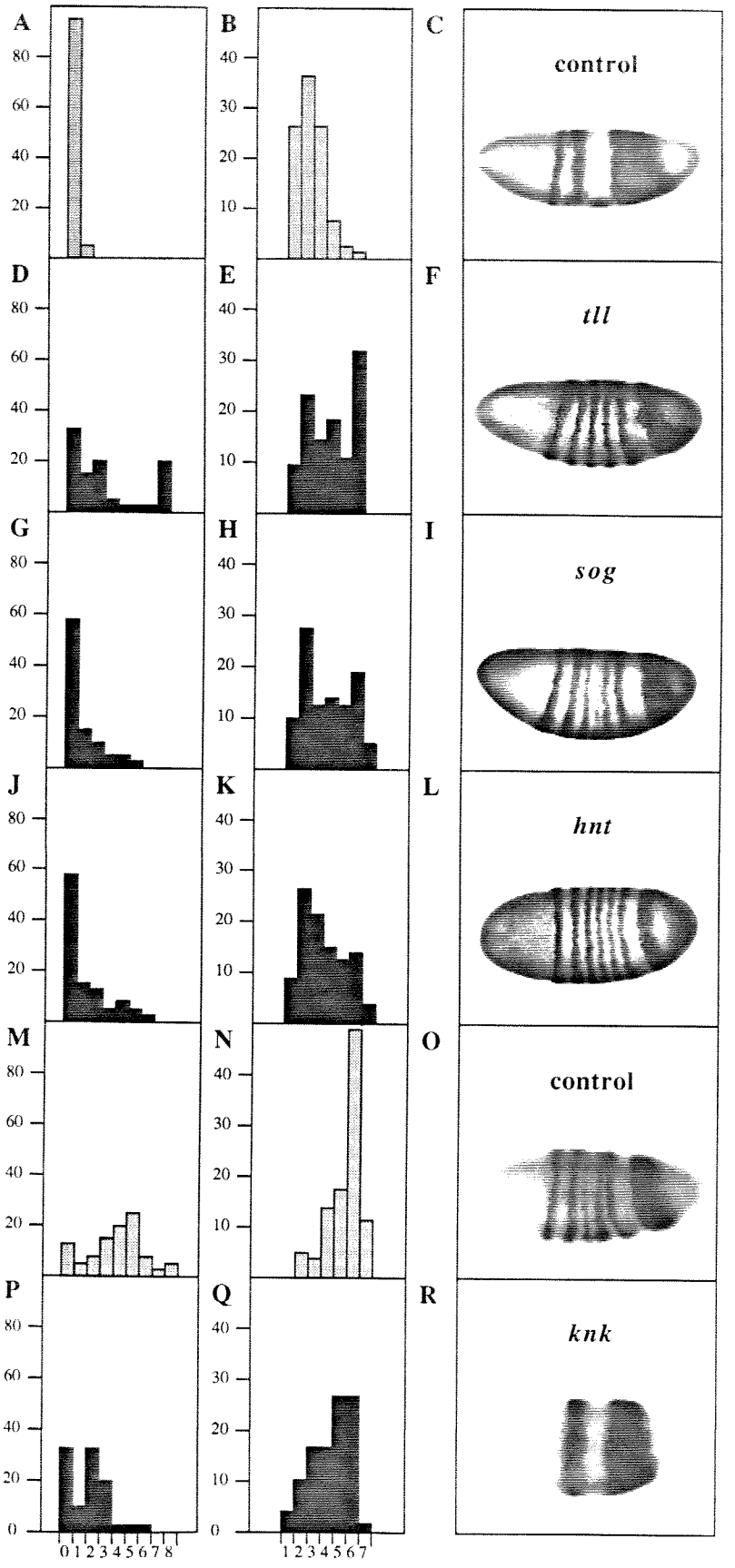
FIGURE LEGENDS

Figure 1: Suppression or enhancement of central defects by zygotic terminal mutations in embryos derived from tor^{splc}/tor^{splc} females. (A, D, G, J, M, P): The distribution of abdominal segment number in cuticle preparations of mature embryos. The percentage of embryos exhibiting a given number of abdominal segments (ordinate) is plotted against the abdominal segment number (abscissa). In grey in (A) and (M) are the distributions in control embryos. In black in (D), (G), (J) and (P) are shown the distributions in progeny from crosses of tor^{splc}/tor^{splc}; *mutation*/+ mothers to *mutation*/+ fathers (or +/*Y* fathers in the case of X-linked loci). In (D), (G) and (J) the embryos developed at 25°C, in (P) at 21°C. (B, E, H, K, N and Q): The distribution of *ftz* stripes in whole mount preparations of cellular blastoderm stage embryos. Control distributions are shown in grey, experimental ones in black. (C, F, I, L, O and R): Examples of expression patterns of *ftz* in whole mount preparations of cellular blastoderm stage embryos, visualized with anti-*ftz* antibody and photographed under bright field optics. (A-C) Control embryos developed at 25°C, from tor^{splc}/tor^{splc} females. In (C) three stripes are visible. (D-F) Embryos from tor^{splc}/tor^{splc}; *tll*/+ females that were mated to *tll/TM3* males. The maximum number of segments is 7 (D); the maximum number of stripes is six (E-F). (G-I) Embryos from *sog/FM7*; tor^{splc}/tor^{splc} females that were mated to *FM7/Y* males. In (I), seven stripes are visible. (J-L) Embryos from *hnt/FM7*; tor^{splc}/tor^{splc} mothers that were mated to *FM7/Y* males. In (L), seven stripes are present. (M-O) Control embryos developed at 21°C, from tor^{splc}/tor^{splc} females. In (O), six stripes are visible. (P-R) Embryos from tor^{splc}/tor^{splc}; *knk*/+ females mated to *knk/TM3* males. In (R), two stripes are visible. In all photographs, anterior is to the left. Views in (C), (F), (I) and (R) are lateral (dorsal towards the top of the page); that in (L) is of the dorsal side of the embryo, and that in (O) is ventro-lateral.


A
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1 2 3 4 5 6 7

Table 1. Results of double mutant analysis between *torso* gain-of-function mutations and zygotic mutants.

			Maternal *torso* Genotype											
			$tor^{4021}/+$			tor^{splc}/tor^{splc}						tor^{splc}/tor^{+}; *Dp* $tor^{+}/+$		
			Empty sacs	Cuticle only	Cuticle+ denticles	Cuticle analysis	Normalized change in # of abdominal segments	Actual number of abdominal segments ± SD	*ftz* analysis	Normalized change in # of *ftz* stripes	Actual number of *ftz* stripes ± SD	Cuticle analysis	Normalized change in # of abdominal segments	Actual number of abdominal segments ± SD
Zygotic Locus		**Allele**												
wildtype	25°C	NA	38	56	6	NA	0.0	0.0 ± 0.0	NA	0.0	2.3 ± 1.1	NA	0.0	4.9 ± 1.4
	21°C	NA	.	.	.	NA	0.0	3.7 ± 2.1	NA	0.0	5.4 ± 1.2	NA	.	.
Suppressors: type A														
folded gastrulation (*fog*)		S4	0	75	25	*	.	.	Su	+ 1.0	3.1 ± 1.6	*	.	.
hindsight (*hnt*)		E8	3	76	21	Su	+ 1.0	1.0 ± 1.5	Su	+ 1.0	3.5 ± 1.7	*	+ 1.0	5.8 ± 1.0
hunchback (*hb*)		14F21	4	70	26	†	.	.	.	.	.	Su	+ 0.5	4.5 ± 1.0
lines (*lin*)		IIF103	6	78	16	,	.	.	.	.	.	Su	+ 2.0	5.9 ± 1.1
short gastrulation (*sog*)		S6	4	56	41	Su	+ 1.0	0.9 ± 1.4	Su	+ 1.5	3.7 ± 1.8	.	.	.
tailless (*tll*)		L10	8	32	60	Su	+ 3.5	2.5 ± 1.5	Su	+ 2.5	3.8 ± 1.9	Su	+ 3.0	7.0 ± 0.1

Suppressors: type B													
giant (*gt*)	A8	3	95	2	.	.	.	.	.	.	Su	+ 2.0	3.7 ± 0.8
tailup (*tup*)	IIIB29	18	79	3	,	.	.	.	.	.	Su	+ 1.0	6.0 ± 1.5
twist gastrulation (*tsg*)	H9	1	96	3	Su	+ 1.5	1.6 ± 1.4	Su	+ 1.0	3.2 ± 1.2	.	.	.
u-shaped (*ush*)	IIA102	15	81	4	.	.	.	.	.	.	Su	+ 1.5	6.3 ± 1.3
Enhancers													
decapentaplegic (*dpp*)	Hin-r4	95	5	0	.	.	.	.	.	.	En	- 0.5	4.3 ± 1.6
knickkopf (*knk*)	5C77	97	3	0	En[d]	- 2.0	1.6 ± 1.4	En	- 1.0	4.4 ± 1.5	En[d]	- 2.5	2.4 ± 0.9
pointed (*pnt*)	9J31	85	15	0	En[d]	- 1.0	2.7 ± 1.6	.	.	.	En[d]	- 1.0	3.7 ± 0.8
tolloid (*tld*)	9B66	92	8	0	En	- 2.0	1.7 ± 1.6	En	- 2.0	3.5 ± 1.3	En	- 2.5	2.5 ± 0.7
No Effect													
faint little ball (*flb*)	IK35	30	66	4	.	.	.	.	.	.	.	.	.
fork head (*fkh*)	XT6	.	.	.	NE	0.0	0.0 ± 0.2	.	.	.	NE	- 0.5	4.4 ± 1.2
grain (*gra*)	7L12	.	.	.	NE	0.0	0.0 ± 0.1	.	.	.	.	.	.
knirps (*kni*)	5F107	28	70	2	.	.	.	.	.	.	.	.	.
krotzkopf verkhert (*kkv*)	14C73	68	32	0	.	.	.	.	.	.	.	.	.
Krüppel (*Kr*)	2	72	28	0	.	.	.	.	.	.	.	.	.
rhomboid (*rho*)	YM43	50	50	0	.	.	.	.	.	.	.	.	.

snail (*sna*)	IIG05	56	42	0	.	.	.	.	.	.	.	.	.	
spalt (*sal*)	IIA55	27	73	0	.	.	.	.	.	.	NE	0.0	5.0 ± 1.3	
Star (*S*)	IIN23	39	61	0	.	.	.	.	.	.	.	.	.	
Stardust (*sdt*)	N5	27	64	9	.	.	.	.	.	.	.	.	.	
teist (*twi*)	ID96	35	65	0	.	.	.	.	.	.	.	.	.	

".", not tested in this particular assay; En, enhancer; NA, not applicable; NE, no effect; Su, suppressor.

* *fog/fog* embryos consistently exhibit large holes in cuticle thereby preventing an accurate count of abdominal segments in the mature embryo.

† *hb/hb* embryos consistently exhibited a fusion or lawn of abdominal denticles which prevented an accurate count of abdominal segments in these mature embryos.

‡ These numbers refer to the entire population rather than homozygotes and thus the enhancement is underestimated. In all other cuticle analyses listed, the homozygotes could be recognized and suppression/enhancement was assayed in that subpopulation only.

Chapter 3

Developmental Biology **150**,422-426 (1992)

I contributed to all aspects of the research reported here.

Genetic control of cell fate in the termini of the *Drosophila* embryo

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ABSTRACT

Cell fates in the anterior and posterior termini of the *Drosophila* embryo are programmed by multiple zygotic genes that are regulated in response to a maternally encoded signal transduction pathway. These genes specify terminal as distinct from central cell fates, program pattern along the antero-posterior and dorso-ventral axes of the termini, and also control endoderm specification and terminal morphogenetic movements. Here, we use a genetic interaction test to dissect the zygotic components of the terminal genetic hierarchy. We show that two genes, *lines* and *empty spiracles*, act downstream of *tailless* to repress central and promote terminal cell fates along the antero-posterior axis of the termini. Genes that control dorso-ventral pattern in the termini and genes that program terminal morphogenesis, act in distinct branches of the genetic hierarchy that are independent of *tailless*.

INTRODUCTION

Development of the anterior and posterior termini of the *Drosophila* embryo is programmed by maternal and zygotic genes in the terminal genetic hierarchy (for review, see Lipshitz, 1991). The product of the maternal gene, *torso* (*tor*), a PDGF-like receptor tyrosine kinase, is activated only in the terminal domains of the embryo where it promotes terminal and represses central cell fates. In embryos from mothers carrying the gain-of-function mutations, tor^{4021} and tor^{sp1c}, which result in constitutive *torso* activition throughout the terminal and central regions, centrally situated cells are programmed to adopt terminal cell fates (Klingler et al., 1988; Strecker et al., 1989; Strecker and Lipshitz, 1990). This results in a loss of segments from the central trunk region.

The zygotic terminal gene *tailless* (*tll*) (Strecker et al., 1986; 1988) and at least thirteen additional zygotic genes are involved in programming terminal development in

response to the *torso*-mediated terminal signal (Strecker et al., 1989; 1991). These conclusions derived from genetic-interaction tests that showed that mutations in these 14 zygotic genes either suppress or enhance the central segment-loss phenotype produced by constitutive *torso* activity. Recently, it has been shown that the ectopic expression of the *tailless* gene driven by the heat-inducible *hsp70* promoter (*HS-tll*), results in expanded terminal specification along with a loss of central trunk segments (Steingrimsson et al., 1991), a phenotype similar to that caused by constitutive *torso* activity. This enabled us to apply the genetic-interaction approach to determining which genes act downstream of *tailless* in the terminal hierarchy and which might act independently. In the present study, mutations in nine genes required for terminal pattern specification or morphogenesis were tested for their ability to restore central, segmental development in *HS-tll* embryos. Mutations in two of these loci suppress the *HS-tll* phenotype and thus act downstream of *tailless*.

MATERIALS AND METHODS

Loci and P-element transformants

HS-tll transformed lines (Steingrimsson et al., 1991) were provided by J. Lengyel and E. Steingrimsson (University of California, Los Angeles). The mutant alleles tested in this study are the same as those used previously (Strecker et al., 1991): *fork head* (fkh^{XT6}), *folded gastrulation* (fog^{S4}), *grain* (grn^{7L}), *hindsight* (hnt^{E8}), *lines* (lin^{IIF}), *spalt* (sal^{IIA}), *short gastrulation* (sog^{S6}). Sources of these alleles are listed in Strecker et al. (1991). Alleles of two additional loci that we have subsequently shown to interact with *tor* gain-of-function mutations, were also tested: *empty spiracles* (ems^{7D}) (Dalton et al., 1989) and zen (zen^{f62}) (Wakimoto et al., 1984). All alleles tested are hypomorphic or amorphic.

Embryo collection and treatment

Embryos were collected at 25°C for one hour on wet nitex filters lying on grape juice agar collection plates and were aged an additional one and one-half hours following

collection at this same temperature. The nitex filter containing the embryos was transferred to a pre-warmed (37°C) plate and embryos were immediately heat-shocked for 30 minutes at 37°C. Then the embryos were placed at 25°C and were allowed to develop overnight. Twenty-two hours following egg collection, all embryos and hatched larvae were mounted on slides in lactic acid: ethanol (9:1) and their cuticles were cleared overnight at 60°C.

Crosses and method of data analysis

Following a 30 minute heat-shock at the blastoderm stage, embryos from a cross of *HS-tll*/+ mothers and wildtype fathers fell into two distinct groups with respect to the extent of central development. Forty-five percent of the embryos exhibited normal segmentation in the central trunk region (6 - 8 abdominal segments) and thus did not inherit the *HS-tll* from their mothers. In contrast, the remaining 55% exhibited at most three abdominal segments, with the majority of embryos exhibiting zero or one segment, and thus received the *HS-tll* gene from their mothers.

To test the effect of zygotic mutations on the loss of central development induced by *HS-tll*, embryos from a cross of mothers that were heterozygous for both *HS-tll* and a zygotic mutation and fathers heterozygous for the same zygotic mutation, were heat-shocked and scored for the restoration of abdominal segments. Among the 25% of the embryos expected to be homozygous for the zygotic mutation, half (12.5% of the total embryos) received the *HS-tll* gene from their mothers, while the remaining half (12.5% of the total embryos) did not. Thus, if a given zygotic mutation did not suppress the central segment-loss phenotype produced in *HS-tll* embryos, at most 12.5% of the total embryos would be expected to have six or more abdominal segments as well as exhibit the homozygous mutant phenotype. On the other hand, if a given zygotic mutation did suppress the central segment-loss phenotype produced in *HS-tll* embryos, then the fraction of the embryos that exhibited 6 or more abdominal segments and were homozygous mutant would be significantly greater than 12.5%, possibly approaching a maximum of 25%. It should be noted that we had to adopt the procedure of first scoring for 6 or more segments,

then for the homozygous zygotic mutant phenotype (rather than the other way around), because the loss of segments in *HS-tll* embryos prevented reliable scoring of the zygotic phenotype in those embryos with central segment deletions.

RESULTS AND DISCUSSION

The nature of the interaction test and choice of loci for analysis

The *tailless* (*tll*) gene has dual functions in specifying antero-posterior pattern in the termini: it promotes terminal cell fates and it represses central fates (Strecker et al., 1989; Strecker and Lipshitz, 1990; Steingrimsson et al., 1991). A genetic interaction test such as the one used here, that assays for restoration of central segments in *HS-tll* embryos, is expected to identify genes that function downstream of *tll* to implement both of these dual functions. This is based on the presumption that inactivation of only the repression functions in a particular central cell would still leave that cell misexpressing products that promote terminal fates. Thus, if a gene acts downstream of *tll* to implement only its positive or only its negative functions, it would likely not test as interacting in the current assay.

Of the nine zygotic loci tested here, six are involved in terminal pattern specification, while three function largely in programming endoderm and/or terminal morphogenetic movements (Table 1). Three of the six pattern loci, *lines* (*lin*), *empty spiracles* (*ems*) and *zerknüllt* (*zen*), interact genetically with *torso* gain-of-function alleles while three, *fork head* (*fkh*), *grain* (*grn*) and *spalt* (*sal*), do not (Strecker et al., 1991; T.R.S., unpublished observations). All three "morphogenetic" loci, *folded gastrulation* (*fog*), *hindsight* (*hnt*) and *short gastrulation* (*sog*), interact with *torso* gain-of-function alleles (Strecker et al., 1991). Interaction or non-interaction with *HS-tll* provided us with a direct means of testing whether any of these genes function downstream of *tll* to implement its dual functions.

The *empty spiracles* and *lines* genes act downstream of *tailless* in the terminal genetic hierarchy

We previously proposed that *tll* and *lin* function together to repress central and promote terminal cell fates (Strecker et al., 1991). This was based on the phenotypic similarities between *tll* and *lin* mutant embryos, their similar interaction with gain of function *tor* alleles, and the synergistic *lin; tll* double mutant phenotype. The suppression of the *HS-tll* phenotype by mutations in *lin* (Table 1, Figure 1) provides direct genetic evidence supporting these conclusions and indicates that *lin* lies downstream of *tll* in this branch of the terminal hierarchy (Figure 2).

ems mutations interact with the *tor* gain-of-function alleles (T.R.S., unpublished observations; Table 1). In the posterior terminus, the *ems* phenotype and gene expression occur in a subset of the *tll* phenotypic and spatial expression domain (Dalton et al., 1989; Pignoni et al., 1991). The restoration of central, segmental development in *HS-tll* embryos carrying the *ems* mutation (Table 1, Figure 1) suggests that, in a subdomain of the posterior terminal region, *ems* functions downstream of *tll* to implement its dual functions (Figure 2).

Mutations in terminal segment identity genes and *grain* do not interact with *HS-tll*

Mutations in *fork head* (*fkh*), *grain* (*grn*), and *spalt* (*sal*) failed to interact with gain-of-function *tor* alleles (Strecker et al., 1991). In the present study, they also failed to restore central development in *HS-tll* embryos (Table 1, Figure 1), despite the fact that they program aspects of antero-posterior pattern within the *tll* phenotypic domain. One interpretation of these results is that, while these genes indeed act downstream of *tll*, they implement only positive, terminal cell fate functions and play no role in the repressive functions. As discussed above, they would then be opaque to the particular genetic interaction test used here.

Dorso-ventral positional cues in the termini are specified independent of *tailless*

Loss-of-function *tor* alleles result in an inability to specify terminal cell fates with associated alterations in both antero-posterior and dorso-ventral pattern (Strecker et al., 1991). In contrast, the *tll* product is involved only in programming antero-posterior pattern in the termini (Strecker et al., 1986; 1988). The expression of the dorso-ventral pattern gene, *zerknüllt* (*zen*), is known to be regulated in the termini of the embryo in response to the *tor* receptor tyrosine kinase-mediated terminal signal (Rushlow et al., 1987). Mutations in *zen* and three other zygotic dorso-ventral pattern specification genes, *decapentaplegic* (*dpp*), *pointed* (*pnt*) and *tolloid* (*tld*), interact with *tor* gain-of-function alleles (Strecker et al., 1991; T.R.S., unpublished observations). The interaction of *zen* mutations with *tor* gain-of-function alleles but not with *HS-tll* (Table 1, Figure 1) provides direct genetic evidence that the assignment of dorso-ventral and antero-posterior positional information to cells in the termini is programmed by distinct subsets of zygotic genes that are independently regulated in response to the *tor*-mediated terminal signal (Figure 2).

Terminal morphogenesis is controlled by genes that act independent of *tailless*

Embryos derived from mothers carrying either loss- or gain-of-function *tor* alleles exhibit defects in endoderm specification as well as in germ band extension and retraction (Schüpbach and Wieschaus, 1986; Strecker et al., 1989; 1991). *tll* mutations have little effect on the endoderm and cause no abnormalities in germband movement (Strecker et al., 1986; 1988; Pignoni et al., 1990). The three terminal morphogenesis genes tested here - *fog*, *hnt* and *sog* - interact genetically with gain-of-function *tor* alleles and thus function in the terminal genetic hierarchy (Strecker et al., 1991). The double mutant combinations *hnt; tll* and *sog; tll* exhibit additive phenotypes (Strecker et al., 1991) suggesting that distinct subsets of the terminal genetic hierarchy control terminal pattern and terminal morphogenesis. The failure of mutations in *fog*, *hnt* and *sog*, to restore central development in *HS-tll* embryos (Table 1, Figure 1) supports this hypothesis (Figure 2).

Conclusions

In the *Drosophila* embryo, the intracellular signalling cascade initiated by the *torso* receptor tyrosine kinase results in the transcriptional regulation of genes that program cell fates along the antero-posterior and dorso-ventral axes of the termini, endodermal specification and the morphogenetic cell movements that occur in the termini (Casanova, 1990; Strecker et al., 1991). The *tailless* gene is transcriptionally regulated in response to the *torso* receptor tyrosine kinase-mediated signal (Pignoni et al., 1990). It functions to program antero-posterior pattern in a subset of the termini by repressing central cell fates and promoting terminal fates (Strecker et al., 1989; 1991; Strecker and Lipshitz, 1990; Steingrimsson et al., 1991).

The results of the present study suggest that *lines* and *empty spiracles* act downstream of *tailless* implement these dual functions along the antero-posterior axis of the termini (Figure 2). Dorso-ventral pattern in the termini, as well as the specification of endoderm and terminal morphogenetic movement, while under the control of *torso*, are not controlled by *tailless*. Rather, they are programmed by distinct branches of the zygotic terminal gene hierarchy (Figure 2).

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TABLE 1

Genetic interaction of zygotic mutations with *tor* gain-of-function alleles and *HS-tll*

Phenotypic classification	Locus	Embryos with > 6 abdominal segments[1] (%)	Total embryos scored	Interaction with *HS-tll*	Interaction with *tor*[2]
Control[3]	NA	25[3]	NA	NA	NA
Terminal Pattern	*ems*	24	99	+	+
	fkh	7	218	-	-
	grn	13	250	-	-
	lin	19[4]	313	+	+
	sal	10	266	-	-
	zen	9	206	-	+
Terminal Morphogenesis	*fog*	12	167	-	+
	hnt	7	196	-	+
	sog	7	189	-	+

Abbreviations: +, genetic interaction; -, no genetic interaction; NA, not applicable.

[1] These are the percentages of embryos with six or more abdominal segments and that exhibit the zygotic mutant phenotype (see Materials and Methods).

[2] Reported in Strecker et al. (1991) with the exception of *ems* and *zen*, which have since been shown to interact with tor^{splc} and tor^{4021}, respectively (T.R.S., unpublished).

[3] This is the expected percentage (see Materials and Methods).

[4] Since *lin/lin* embryos lack one abdominal segment but were still scored for 6 or more segments, this number is a slight underestimate of the true percentage.

FIGURE LEGENDS

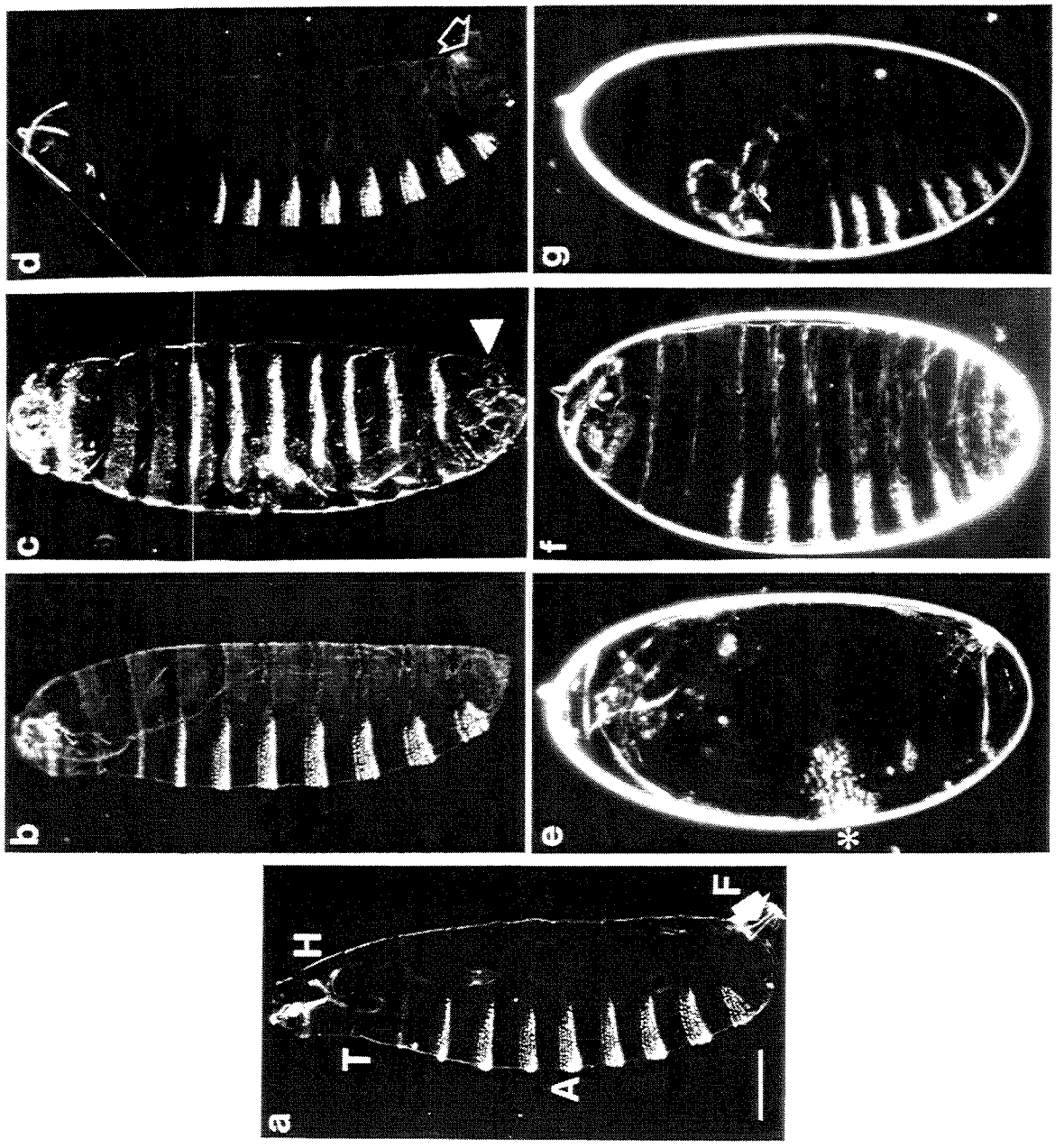
Figure 1:

Cuticle phenotypes of wildtype, zygotic mutants, *HS-tll*, and suppressed mutant combinations. (a) wildtype, (b) *tll*, (c) *lin*, (d) *ems*, (e) *HS-tll*, (f) *lin; HS-tll*, (g) *HS-tll; ems*. In (b), *tll* embryos have an abnormal head skeleton and posteriorly are lacking the eighth abdominal segment and telson structures such as the filzkörper. This can be compared to (c), where *lin* embryos are lacking derivatives of the eighth abdominal segment (white arrowhead). In (d), *ems* embryos lack the birefringent filzkörper normally found within the posterior spiracles (open arrow). In (e), *HS-tll* embryos have a small lawn of abdominal denticles (asterisk) in place of the abdominal segments. This can be compared to (f) where *lin; HS-tll* embryos have a suppressed phenotype with the restoration of seven abdominal segments, and (g) where *HS-tll; ems* embryos exhibit a similar restoration of abdominal segments. The embryos in (e) - (g) are mounted in their vitelline membrane, while (a) - (d) were devitellinized. In all cases, anterior is toward the top of the page and ventral is to the left [except in (c), which is viewed from the ventral side]. Darkfield optics were used. H, head skeleton; T, thorax, consisting of three segments with feint denticle belts; A, abdomen, consisting of eight segments with bright denticle belts; F, filzkörper (white arrow) derived from the telson and located within the posterior spiracles. Scale bar in (a): 100μm.

Figure 2:

Schematic diagram indicating the structure of the terminal genetic hierarchy as deduced from the genetic interactions. Only the genes analyzed in this study are shown in the diagram. At the bottom of each branch of the hierarchy is shown the aspect of terminal cell fate programmed by that branch: antero-posterior position, dorso-ventral position, morphogenesis (endoderm and cell shape changes/movement) Additional genes can be

assigned to likely positions within the hierarchy (Strecker et al., 1991), but are not shown in the diagram since they were not assayed using the *HS-tll* test. These are *giant*, *hunchback* and *knickkopf* (antero-posterior position); *decapentaplegic*, *pointed* and *tolloid* (dorso-ventral position); and *twisted gastrulation*, *tailup* and *u-shaped* (endoderm/morphogenesis).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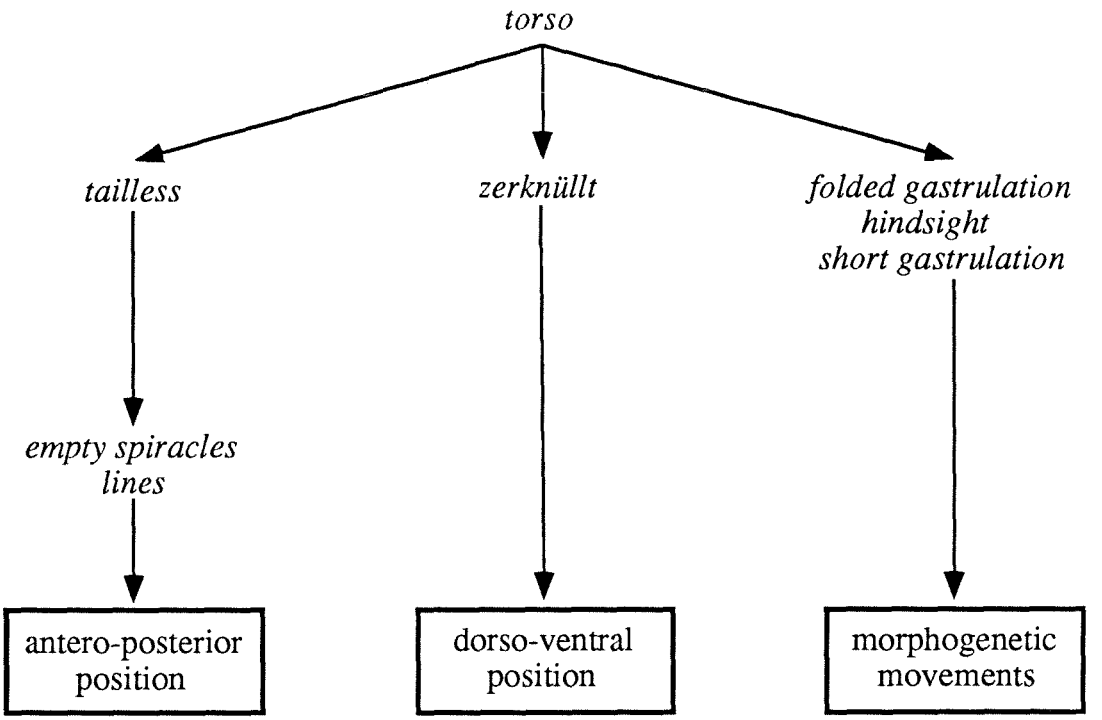
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folded gastrulation
hindsight
short gastrulation
empty spiracles
lines
antero-posterior
position
dorso-ventral
position
morphogenetic
movements

CHAPTER 4

This chapter will be submitted for publication in *Development*.

The *Drosophila hindsight* gene regulates germband retraction and encodes a putative zinc-finger transcription factor

Running title: Regulation of germband retraction by hindsight

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SUMMARY

The *Drosophila hindsight* gene function is required for germband retraction. Embryos lacking *hindsight* activity have a normal body plan and undergo normal morphogenetic movement prior to the onset of germband retraction. However, they fail to retract their germbands. *hindsight* encodes a large nuclear protein of 1920 amino acids. Sequence analysis reveals that it contains fourteen C_2H_2 type zinc-fingers, arranged in widely spaced clusters. Additional features of the HINDSIGHT protein, such as glutamine-rich and proline-rich domains, suggest it functions as a putative transcription factor. Embryonic expression of *hindsight* is complex: it is found in the endoderm (anterior and posterior midgut), amnioserosa, subsets of the central nervous system, the peripheral nervous system and the tracheal system. However, it is the expression of *hindsight* in the midgut that is important for germband retraction since mutations which abolish *hindsight* endodermal expression also affect germband retraction. Although hnt is not expressed in ectoderm, it is these cells that undergo the cell shape changes that accomplish germband retraction. We propose that *hindsight* activity regulates a signal produced by the endoderm that is responsible for the coordination of morphogenetic cell shape changes and movements in ectoderm.

INTRODUCTION

In order to form complex three-dimensional body structures, multicellular organisms have to be able to coordinate the activities of all cells involved; in metazoa the control of various morphogenetic movements during gastrulation are particularly important.

Although advances have been made in our understanding of how morphogenetic movements are controlled and implemented, this area still lags behind in comparison with our understanding of pattern formation and differentiation. Among organisms that are currently used for studying morphogenesis, *Drosophila* embryos provide an excellent model system. During *Drosophila* embryogenesis, gastrulation begins soon after blastoderm cellularization is completed (reviewed by (Costa et al., 1993). First, mesoderm segregates from ectoderm through the formation of the ventral furrow. Soon after mesoderm invagination begins, endoderm is internalized by anterior and posterior midgut invagination from both ends of the embryo. Concurrent with posterior midgut invagination is germband extension, which dramatically reorganizes the embryo into an elongated and slender shape which is folded upon itself dorsally. During germband extension, the dorsal extraembryonic tissue, the amnioserosa, moves laterally to give way to the advancing germband. About four and a half hours after germband extension, the germband starts to retract and body segments become longer dorsoventrally and shorter rostrocaudally. Germband retraction also repositions the caudal part of embryos to the posterior end. Migration of the lateral epidermis dorsally and its fusion at the dorsal midline (dorsal closure) marks the end of major external morphogenetic movements.

Recently, some of the genes and possible mechanisms that are involved in these processes have been identified. Mesoderm invagination and posterior midgut invagination share many cellular characteristics and indeed two known mutations affect both processes. Maternal activity of *concertina* (*cta*) and zygotic activity of *folded gastrulation* (*fog*) are required for the invagination of posterior midgut and mesoderm. There are two phases of mesoderm and posterior midgut invagination. The early phase is slow and stochastic in nature. Some cells within the presumptive mesoderm and endoderm begin apical constriction individually. The second phase is rapid. All the unconstricted cells constrict simultaneously (Kam et al., 1991; Sweeton et al., 1991). It is the transition between the slow and the rapid phases of constriction that is defective in both *cta* and *fog* mutants

(Zusman and Wieschaus, 1985; Sweeton et al., 1991; Parks and Wieschaus, 1991; Costa et al., 1994). *cta* encodes a G_{α}-like protein that is produced maternally and deposited into embryos. *fog* encodes a novel protein believed to be secreted. It is postulated that they constitute a signaling pathway which coordinates group behaviour of cells. Neither *cta* nor *fog* affect the specification of positional values in the embryos.

During germband extension, an embryo increases its body length about 2.5 fold while decreasing its width simultaneously. These changes occur in the absence of cell division or shape changes (Campos-Ortega and Hartenstein, 1985). Using video microscopy, Irvine and Wieschaus (1994) followed the trajectories of individual cells during germband extension; they found extensive intercalation of dorsal and ventral cells. By comparing the behaviours of cells during germband extension in different genetic backgrounds, they proposed that pair-rule genes establish stripes of cells with different adhesive properties. During germband extension, dorsal and ventral cells with the same adhesive property intercalate to maximize their contacts. The net result is transformtion of tall and narrow stripes of cells into short and wide patches of cells.

In contrast, less is known about how germband retraction is controlled and executed. Campos-Ortega and Hartenstein (1985) suggested germband retraction is "attained through shape changes of individual cells." Six genes have been reported to be required for germband retraction. These genes are *Drosophila* homolog of mammalian EGF receptor (*Egfr*) (Clifford and Schüpbach, 1989; Raz et al., 1991; Clifford and Schüpbach, 1992), *hindsight* (*hnt*) (Wieschaus, 1980; Strecker et al., 1991; Strecker et al., 1992), *tailup* (*tup*) (Nüsslein-Volhard et al., 1984), *torso* (*tor*) (Schüpbach and Wieschaus, 1986; Strecker et al., 1991; Strecker et al., 1992), *u-shaped* (*ush*) (Nüsslein-Volhard et al., 1984) and *serpent* (*srp*) (Jürgens et al., 1984; Reuter, 1994). *srp* mutant embryos have their endodermal midgut transformed into ectodermal foregut/hindgut (Reuter, 1994), suggesting that the endoderm plays an important role in the process of germband retraction. Embryos derived from *tor* loss-of-function show germband retraction defects (Strecker et

al., 1989). In addition, mutations in *hnt*, *tup* and *ush* suppress *torso* gain-of-function phenotype (Strecker et al., 1986; Strecker et al., 1991; Strecker et al., 1992). We have focused our attention on *hnt* because its effects are late and specific to germband retraction and show no overall pattern or tissue specification defects. Moreover, *hnt* is a stronger suppressor of *tor* gain-of-function phenotypes than *tup* and *ush*, suggesting a position higher in the germband retraction genetic hierarchy.

In this report, we present our analysis of *hnt* mutant phenotype. Our results indicate *hnt* does not affect pattern formation or tissue specification but regulate germband retraction specifically. Molecular cloning and sequencing of the *hnt* gene and localization of HNT protein in nuclei suggest that it functions as a transcriptional regulator. Analysis of *hnt* expression in different mutant backgrounds provide information regarding its regulation while genetic and expression analyses allow us to propose a model for the control of germband retraction.

MATERIALS AND METHODS

Drosophila genetics and manipulations

The original *hnt* alleles, *XE81* and *X001*, were isolated by Wieschaus et al. (1984). A subsequent EMS mutagenesis screen obtained three additional alleles, *EH275a*, *EH587a* and *EH704* (Ebrel and Hilliker, 1988) The temperature sensitive rough eye mutation,*pebbled*, and other mutations used in this study, if not otherwise indicated, are described in Lindsley and Zimm (1992). Unless specified, all flies were raised on standard medium at room temperature or 25^{o}C. The restrictive temperature used for *pebbled* was 28$\pm$1^{o}C.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was based on previously published protocols (Tautz and Pfeifle, 1989; Ding et al., 1993).

Histology and immunocytochemistry

Cuticles were prepared by clearing embryos in mounting media (11.5 ml saturated chloral hydrate (5g/ml), 7.6 ml Shandon immu-mount (Pittsburgh, PA 15275) and 1ml lactic acid) as described by Ashburner (1989), except that embryos were not fixed before mounting. Antibody staining of embryos was done according to MacDonald and Struhl (1986).

Time-lapse video microscopy

Short periods (1/2 hour) of embryo collection were carried out using females heterozygous for *hnt* function. These embryos were then filmed for more than 12 hours. Usually three to four embryos can be filmed simultaneously. The embryos were submerged in halocarbon oil and the filming room was humidified to prevent embryos from dehaydration.

Nucleic acid manipulations and analysis

Standard protocols were performed as described (Sambrook et al., 1989). Overlapping phage clones covering about 70 kb proximal to the proximal deficiency breakpoint of Df(1)rb^{46}were hybridized to labeled probes. Probes were made using 0-3 hour embryonic poly-A RNA as template to identify early embryonic transcription units. A 4.5 kb BamHI fragment from phage clone X.59 was identified and used to screen a cDNA library. We isolated a cDNA clone, designated E20, with a 2 kb insert, from the first embryonic cDNA library we screened (Poole et al., 1985). Using this cDNA as a probe, we screened another embryonic cDNA library to isolate longer cDNA clones (Brown and Kafatos, 1988). Repeated screening using progressively more 5' portion of cDNA inserts obtained from previous rounds of screening was required to isolate the cDNA clones with large

inserts. Sequencing of cDNA inserts was done using the Sequenase kit (US Biochemical Corp.) or the Cycle Sequencing Kit (Applied Biosystem).

Analysis of mutant alleles

Genomic DNA was isolated from *hnt* mutant embryos using, in general, at least 100 emhryos for each preparation. Polymerase Chain Reaction (PCR) was used to amplify fragments of the *hnt* coding region from the genomic DNA purified from each pool of mutant embryos. For each amplified fragment, three duplicate PCR reactions were set up and processed at the same time. Amplication procedures were modified from Williams et. al. (1992).1 1 µl of genomic DNA (30-50 ng/µl) was added to 9 µl of buffer (50 mMKCl, 10 mM Tris pH8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20 and 0.01% gelatin) and heated to 95°C for 15 minutes. 40 µl of PCR master mix was added quickly and the final conditions are: 50 pmol for each primer, 10 mMTris (pH8.3), 50 mM KCl, 1.5 mMMgCl_2, 0.001% gelatin, 0.2mM for each dNTP and 0.6 unit Taq polymerase (Perkin Elmer Cetus). After a brief microfuge spin, the reactions were cycled 30 times: 94°C for 1 minute, 55°C for 30 seconds and 72°C for 1 minute 30 seciond, and then 94°C for 3 minutes and 72°C for 7 minutes. After PCR amplification, the contents from the three duplicates were combined and the amplifed fragment was purified using QIAquick PCR Purification Kit (Qiagen). Amplified fragments were then sequenced.

Germline transformation and phenotypic rescue

A 6.3 kb SspI-NotI fragment containing the entire *hindsight* cDNA was cloned in the HpaI-NotI sites of pCaSpeR-hs vector (Thummel and Pirrotta, 1991) and transformed into flies (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Two transformant lines were obtained: hs-*hnt*:E (X chromosome) and hs-*hnt*:M (2nd chromosome). Virgins females from four *hindsight* allele stocks were crossed to males from the hs-*hnt*:M transformed lines. All progeny from such crosses carry one copy of the hs-*hnt* transgene and were

collected on yeasted apple juice agar plates of 2-2.5 hour interval. 3.5 or 4 hours after collection, embryos were heat shocked twice at 36.5°C for 0.5 hour with 0.5 hour at 25°C between heat shocks. Embryos were then allowed to develop at 25°C before mounting for analysis.

Expression of HNT protein and production of anti-HNT antibodies

A 907 bp BglII-BamHI fragment was cloned into the BamHI site of pGEX1 vector (Smith and Johnson, 1988). The GST-HNT fusion protein was expressed and purified from *E. coli* (Ausubel et al., 1987). To generate anti-HNT antibody, 50 mg of purified GST-HNT fusion protein suspended in RIBI adjuvant (RIBI Biochem) was injected into three Balb/c mice (Simenson). At day 14, they were re-injected with the fusion protein/adjuvant mixture. A week later, anti-sera from the mice were tested by ELISA against the fusion protein and on fixed *Drosophila* embryos. All three mice were injected again on day 28 and on day 42 and tested again at day 49. The mouse that produced the strongest response was re-injected once more. Four days after, this mouse was sacrificed. The blood from this mouse was saved as an anti-HNT polyclonal sera; 7.9×10^7 spleen cells from this mouse were fused with 2.8×10^7 HL-1 myeloma cells (Hycor) and grown at 2×10^5cells/well. Samples from the cultures to be cloned were collected and the concentration of viable cells determined. Samples containing approximatiely 230 hybridoma cells were suspended in 4.6 ml AT medium. A portion of this suspension was distributed in 36 wells of a 96-wells plate at 0.1 ml/well. 4 ml AT medium and 10% Hybridoma Cloning Factor (Fisher) were added to the remaining 1 ml of this suspension and another 36 wells were plated at 0.1ml/well. 1.4 ml AT medium was then added to the remaining cell suspension and the last 24 wells were plated. Six days later, single clones were noted and half of their medium was replaced with fresh medium until the clones turned yellow. Supernatants from these clones were tested by ELISA against the fusion protein and on fixed embryos.

RESULTS

Genetic characterization of *hindsight* alleles and the *hindsight* embryonic phenotype

Eight different alleles of *hnt* have been described previously (Wieschaus et al., 1984; Schalet, 1986; Ebrel and Hilliker, 1988; Lindsley and Zimm, 1992). Two of these alleles were lost, four are embryonic lethal, one is semi-lethal with occasional escapers and one is viable. The four embryonic lethal alleles, *XE81*, *X001*, *EH704a* and *EH587a*, display a similar phenotype: embryos hemizygous for these alleles fail to retract their germband (Fig. 1A). All mutants have the normal number of thoracic and abdominal segments which are patterned normally. The posterior ends of the *hnt* embryos are folded onto the dorsal side of the embryos and the heads of the embryos face the posterior of the embryo. Additionally, mutant embryos show defects in head involution and some have a severely disrupted cephalopharyngeal skeleton. Since the mutation is on the X chromosome and no useful duplication of the region is available, we were unable to make trans-heterozygotes of *hnt* alleles and deficiencies uncovering the region. However, by comparison with the phenotype of embryos homozygous for $Df(1)bi^{D3}$, a small deficiency uncovering *hnt*, we believe that these four are very strong loss-of-function, if not amorphic, alleles. This is consistent with the fact that *EH587a* maybe a small deficiency and is also defective for a proximal neighboring gene, *ovo/shaven baby* (Ebrel and Hilliker, 1988); our molecular analysis). Most of our analyses used *XE81* and *X001* but we obtained similar results when we tested *EH704a* and *EH587*.

The semi-lethal allele, *EH275a*, does not cause any detectable cuticular abnormality and germband retraction is normal in most of the embryos. Occasionally, embryos have small dorsal hole on the cuticles. Normal looking hnt^{EH275a} escaper males emerge; however, they are sterile and some have their genital disc rotated various amounts.

We have identified an additional viable *hnt* allele that was originally named *pebbled* because of its rough eyed phenotype (Lindsley and Zimm, 1992). None of the four embryonic lethal alleles complement the *pebbled* rough eye phenotype (Fig. 1B), suggesting that *hindsight* and *pebbled* are allelic. This is supported by the observation that when larvae heterozygous for embryonic lethal alleles were irradiated with X-rays to generate homozygous hnt^- somatic clones in the eyes, prominent scars were seen next to the hnt^+ twin spots in the adult eyes, suggesting that *hnt* functions during eye development (data not shown). Table 1 is a summary of *hnt* mutant phenotypes.

Molecular characterization of *hindsight* phenotype

Since the cuticle of *hnt* mutant embryos did not show any patterning defects, we stained mutant embryos with a panel of antibodies that recognize proteins/epitopes with different temporal and spatial expression patterns and functions in embryos, to determine if any defects occur in cells or structures that are not part of the cuticle. Table 2 lists the antibodies tested. With one exception, all markers we tested behaved similarly in *hnt* mutant embryos and wild-type embryos. *Krüppel* expression differed in wild-type versus *hnt* mutant embryos. In wild-type embryos, the large, flat nuclei of amnioserosa cells express *Krüppel* protein strongly. In hnt^- mutant embryos, the amnioserosa cells display similar morphology to those of the wild-type. However, these nuclei did not express *Krüppel* protein (Fig. 1C). The significance of this difference in *Krüppel* protein expression is not clear since no function of *Krüppel* protein in the amnioserosa cells has been reported.

Time-lapse video analysis of living *hindsight* embryos

Our cuticular examination and antibody survey did not reveal any obvious patterning defects or defects in tissue specification and morphology in hnt^- mutant embryos and strongly suggest that failure of germband retraction is the primary phenotype produced

by loss of *hnt* gene activity. However, it remained possible that other earlier embryonic developmental processes are affected in *hnt* mutants and that failure of germband retraction was a secondary defect. One possible scenario was that *hnt* mutant embryos have defects in the timing or spatial aspects of germband extension such that the embryos fail to receive the "retraction" signal, resulting in the mutant phenotype. To rule out such a possibily, we used time-lapse video microscopy to film the development of wild-type and *hnt* mutant embryos, in order to determine the exact time at which development of *hnt*$^-$ mutant embryos deviates from development of their heterozygous siblings. *hnt* mutant embryos were identified on the basis of their failure to retract their germband. Comparison of the developmental profile of wild-type and *hnt* mutant embryos embryos allowed us to determine that both classes extended their germbands at a similar rate. The first obvious difference appeared at the start of germband retraction. Three quarters of the embryos (presumbly *hnt*$^-$/*hnt*$^+$ or *hnt*$^+$/Y) started germband retraction while the remaining quarter of them (presumbly *hnt*$^-$/Y) did not initiate retraction or retracted at a much reduced rate. Therefore, we conclude that *hnt* does not affect embryonic pattern formation, tissue specification or earlier morphogenetic events, such as germband extension, but that it regulates germband retraction specifically.

Cloning of the *hindsight* gene

Previous studies mapped *hnt* within the 4C5,6 region (Oliver et al., 1988; Lindsley and Zimm, 1992); however, in contrast to published data (Oliver, et al., 1988), we found that the deficiency chromosome, Df(1)rb^{46}, does complement the rough eye phenotype of *hnt*^{pebbled}, and therefore placed *hnt* proximal to the proximal breakpoint of Df(1)rb^{46}. A chromosomal walk was recently conducted within this region that spanned the proximal breakpoint of Df(1)rb^{46} (Pflugfelder et al., 1990). Using overlapping phage clones (kindly provided by Dr. Pflugfelder) proximal to the breakpoint, we initiated a search for early embryonic transcripts within the region. One BamHI fragment was identified and

used for cDNA library screening. Multiple cDNAs were isolated after screening of two embryonic libraries. Two clones, denoted E20 (the first one identified) and NB701 (one of the longest cDNA), were analyzed further.

Sequence analysis of NB701 and E20 revealed that E20 was a partial cDNA and that both clones encoded the same protein. NB701 contained a single large open reading frame (ORF) of 1920 codons with approximately 250 bp 5' and 3' untranslated regions. Conceptual translation of this ORF is shown in Figure 2A. It encodes a protein of 1920 amino acids. This ORF shows characteristics of transcription factors when compared to previous described sequences using the BLAST algorithm (Altschul et al., 1990). It contains 14 C_2H_2 type zinc-fingers in widely spaced clusters (Fig. 2B). It also contains multiple glutamine-rich domains, proline-rich domains, serine/theronine-rich domains and acidic/charged domains.

We confirmed that this cDNA is encoded by the *hnt* gene in three ways. First, we identified the mutational site of an EMS induced allele, hnt^{X001}. Sequence analysis of PCR fragments amplified from hnt^{X001} genomic DNA revealed a C to T transition that introduced a stop codon at amino acid residue 348 in place of a glutamine (CAG to TAG).

Second, we made an hsp70 promoter-*hnt* cDNA transgene using the NB701 clone and transformed it into flies using P-element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Two independent transformant lines hs-hnt:E (X chromosome) and hs-hnt:M (2nd chromosome) were isolated. The transformant line hs-hnt:M rescues all four embryonic lethal alleles (Table 3). On average, 50% of the heat-shocked genetically identified y^- hnt^- embryos carrying one copy of the transgene were rescued and retracted their germbands in contrast to <5% in controls. Therefore, we conclude that we have cloned the *hnt* gene and that it encodes a putative transcription factor.

Third, none of the four hnt^- lethal alleles shows detectable immunostaining when examined with our anti-HNT antibodies (Fig 1E).

Embryonic expression of *hindsight*

The embryonic expression of *hnt*, as determined by whole-mount in situ hybridization of digoxigenin labeled RNA probes (Fig. 3) and anti-HNT antibodies (Fig. 4), is complex. We first detect *hnt* mRNA expression in cellular blastoderm (stage 5; staging as in (Campos-Ortega and Hartenstein, 1985)) in a posterior-dorsal domain corrseponding to the posterior midgut primordium. Additional staining appears as dorsal patches of expression emerge during stage 6 and expand to cover the entire dorsal region from the cephalic furrow to the end. This region eventually gives rise to the amnioserosa (Fig. 3A,B). Anterior-ventral staining, corresponding to anterior midgut invagination, begins at stage 7 (Fig. 3 C). All of these initial expression patterns continue as gastrulation proceeds. At stage 11, *hnt* expression is detected in the cells of the emerging larval peripheral nervous system and tracheal system (Fig. 3). As the peripheral nervous system and tracheal system proliferate and elaborate, *hnt* staining can be seen in most, if not all, of the cells (Fig. 3).

HNT protein is localized to the nuclei and its pattern, as judged by anti-HNT antibodies staining, is the same as that of the *hnt* mRNA. The complex expression pattern of *hnt* suggests that it may have multiple functions during embryogenesis; functions in the CNS will be reported elsewhere (M. Lamka and H.D. Lipshitz, in prep.).

Regulation of *hindsight* expression

Our previous genetic analysis of the *torso*-mediated terminal pathway suggested that *hnt* acts downstream of *torso* in regulating germband retraction (Strecker et al., 1991; Strecker et al., 1992). Consistent with this hypothesis, embryos produced by homozygous *torso* loss-of-function mutant females lack posterior *hnt* expression (i.e. in the posterior midgut primordium) which lies within the domain of *tor* function. Instead of extending their germband dorso-anteriorly, most form a spiral germband (Fig. 5C) (Yip and Lipshitz, 1995). Embryos from homozygous *tor* gain-of-function mutant mothers lack dorsal

expression (i.e. in the presumptive amnioserosa) consistent with conversion of central cell fates to more terminal ones. They also show twisted and ectopic gastrulation pattern (Fig. 5D) (Yip and Lipshitz, 1995).

Two genes, *tailless* (*tll*) and *huckebein* (*hkb*), have been identified as key components of the *torso*-mediated terminal pathway. *tll* encodes a protein homologous to the steroid hormone receptor superfamily and probably functions as a transcription factor. *tll* mutant embryos have abnormal clypeolabrums, optic lobes and procephalic lobes. They are also lacking abdominal segments A8-A10, hindgut and Malpighian tubules (Strecker et al., 1986; Klingler et al., 1988; Strecker et al., 1988; Strecker et al., 1989; Pignoni et al., 1990). *hkb* encodes a Sp-1/Egr-like zinc-finger transcription factor and is required for the formation of endodermal midgut and stomodeum as well as controlling morphogenetic movements in these tissues (Weigel et al., 1990; Brönner and Jäckle, 1991; Brönner et al., 1994). While *hnt* expression is largely unaffected by *tll* mutations (Fig. 5E), *hkb* mutant embryos lack *hnt* polar expression in the anterior midgut and almost completely lack the posterior midgut expression (Fig. FF). As we predicted based on our genetic analysis, these results indicate that the terminal pathway is branched, and that *hnt* is largely under the control of *hkb* but not *tll*. (Strecker et al., 1991; Strecker et al., 1992). This is consistent with *hnt* expression being restricted to the anterior and posterior midgut (Fig. 3, 4) and not occurring in the foregut or hindgut. Anterior and posterior midgut are largely specificed by *hkb* and there is only a minor requirement for *tll* in posterior midgut identifty (Pignoni, et al., 1990). In *hkb tll* double mutant embryos, *hnt* is only expressed by the dorsal presumptive amnioserosa (Fig. 5G).

srp acts downstream of *hkb* to establish the identity of the endodermal midgut. Loss-of-function mutations in *srp* result in transformation of the endoderm into ectoderm (Reuter, 1994). As a consequence, *srp* mutant embryos fail to undergo germband retraction. We found that endodermal expression of *hnt* is missing in *srp* mutant embryos

(Fig. 4H). Therefore, *hnt* expression in the endoderm is dependent on the functions of *hkb* and *srp* .

Besides *srp*, mutations in three other genes: *ush*, *tup* and *Egfr*, also affect germband retraction. *hnt* expression is not affected by any of these mutations (Fig. 4I). This suggests either that *hnt* is upstream of these genes in the same hierarchy or that these genes reside in a parallel pathway that is involved in regulating germband retraction.

hnt expression in the amnioserosa is regulated by the dorso-ventral pathway. Dorsal *hnt* expression is expanded in genetically ventralized embryos (Fig. 4J-L) and suppressed in dorsalized embryos (Fig. 5M). The anterior midgut expression of *hnt* is affected by the dorso-ventral pathway to a greater degree than that of the posterior midgut domain. This is probably due to the fact that anterior midgut development requires inputs from both terminal and dorso-ventral pathways (Reuter and Leptin, 1994).

The maternal gene, *cta* and the zygotic gene, *fog*, strongly affected morphogenesis: both are required for proper ventral furrow formation and posterior midgut invagination. *hnt* expression is not affected by either mutation (Fig. 5N,O). These results suggest that mesodermal and posterior midgut invagination and germband retraction are controlled separately from germband retraction.

DISCUSSION

We have shown that *hnt* gene activity is specifically required for germband retraction. Two lines of evidence support this conclusion. First, *hnt* mutant embryos have a normal body plan, as determined by cuticular analysis. They also display normal expression of a panel of gene products required for embryonic patterning and development which serve as patttern and tissue markers. Second, the temporal and spatial aspects of the morphogenetic events that precede germband retraction, as determined by time-lapse video microscopy, are the same in *hnt* mutant and in wild-type embryos.

Three lines of evidence argue that the transcript we have identified is encoded by the *hnt* gene and implements its role in germband retraction. First, we can rescue the germband retraction phenotype of all four embyonic lethal alleles using a hsp70-promoter driven cDNA in transgenic animals. Second, one of the embryonic lethal alleles, *X001*, has a C to T transition in the coding region that introduces a premature stop codon at amino acid residue 348. Third, none of the four embryonic lethal alleles show any immunostaining using our anti-HNT antibodies, which were raised against the central part of the HNT protein.

The *hnt* gene encodes a large protein with fourteen C_2H_2 type zinc fingers. This type of zinc-finger is found in many proteins, particularly transcription factors, and has been shown to function as a DNA binding domain (Pabo and Sauer, 1992). However, the spacing of zinc-fingers in *hnt* is unique. In HNT, the zinc-fingers are in widely spaced clusters. Each cluster has two or three zinc-fingers positioned in tandem. Two zinc-fingers, the ninth and twelfth, are isolated from the others and are not in clusters. Previous structural and functional studies have shown that two zinc-fingers is the minimal DNA binding unit; we do not at this point have any direct evidence that the *hnt* zinc-fingers bind DNA. In addition to the zinc-fingers, HNT protein also contains structural domains commonly found in transcriptional regulators, including multiple glutamine-rich domains, proline-rich domains, serine/theronine-rich domains and acidic/charged domains.. It has been demonstrated that glutamine-rich domains (Courey and Tjian, 1988; Tanaka and Herr, 1990; Madden et al., 1991), proline-rich domains (Mermod et al., 1989; Madden et al., 1991; Han and Manley, 1993) and acidic domains (Ptashne and Gann, 1990) (Stringer et al., 1990; Dynlacht et al., 1991; Lin and Green, 1991; Lin et al., 1991) are trans-acing domains that can mediate protein-protein interactions. Combined with the nuclear localization of HNT proteins as revealed by anti-HNT antibody staining, we postulate that HNT protein functions as a transcription factor that regulates genes which coordinate germband retraction.

The early expression of *hnt* in the dorsal amnioserosa and anterior and posterior midgut primordia are differentially affected by the dorso-ventral and terminal pathways. While posterior midgut versus dorsal amnioserosa expression of *hnt* is regulated independently by the terminal and dorso-ventral pathways, respectively, expression of *hnt* in the anterior midgut primordium requires input from both of these systems. Mutations in maternal *cta* or zygotic *fog* functions do not affect *hnt* expression, suggesting that multiple mechanisms exist to control morphogenetic movement.

Our previous genetic analysis suggested that regulation of germband retraction is part of the function of the *torso*-mediated terminal pathway (Strecker et al., 1991; Strecker et al., 1992). Consistent with this, the posterior midgut expression of *hnt* is indeed transcriptionally activated by the terminal signal and supports our genetic models. We were able to further dissect the terminal pathway by showing *hnt* expression in the midgut is lost in *hkb* and *srp* mutant embryos but is only slightly affected in *tll* mutant embryos in the portion of the posterior midgut which requires *tll* function. Thus *hnt* is positioned downstream of the genes which specify endodermal (midgut) identity.

hnt expression is normal in embryos bearing other three germband retraction failure mutations, *ush*, *tup* and *Egfr*. Furthermore, the hs-*hnt* transgene is unable to rescue any of these three mutants under experimental conditions that rescue *hnt*. These data are consistent with the idea that *ush*, *tup* and *Egfr* are either downstream of *hnt* or in a parallel pathway which regulates germband retraction.

How is germband retraction coordinated? Since mutations such as *srp* that result in the conversion of endoderm to ectoderm show germband retraction defects while embryos lacking mesoderm do not (Leptin et al., 1992), we conclude that endoderm is critical but that mesoderm is dispensable for germband retraction. Further, mutations that affect mesodermal development also disrupt the migration and morphogenesis but not specification of the endodermal midgut identity (Reuter et al., 1993; Tepass and Hartenstein, 1994). Therefore, migration and morphogenesis of the endodermal midgut

are not required for germband retraction. The most likely candidate tissue responsible for executing germband retraction is the ectoderm. Morphological and anatomical analyses of developing embryos show that ectodermal cells undergo extensive cell shape changes during germband retraction (Campos-Ortega and Hartenstein, 1985). Thus, endoderm is critical for programming germband retraction, but ectoderm accomplishes the actual process.

Among the mutations that fail to retract their germband, *hnt* and *Egfr* are the only two for which information regarding gene products and expression patterns are available. Strikingly, *hnt* is expressed in endoderm but not ectoderm, while *Egfr* is expressed in ectoderm but not endoderm. Based on these and the preceding observations, we hypothesize that *hnt* functions as a transcription regulator of genes that produce a signal orginating in the endodermal midgut that is received by ectodermal cells. This signal would cause ectodermal cells to undergo the coordinated shape changes that produce germband retraction. We speculate that *Egfr* is the receptor that receives and transduces the signal into ectodermal cells. The validity of this model can be tested by examining the expression of genes transcriptionally activated in response to the *Egfr* in *hnt* mutant embryos.

The complex expression pattern of *hnt* suggests it may have multiple functions during development. *hnt* is expressed early on in the peripheral nervous system and tracheal system as they develop and continues throughout their development. In *hnt* mutant embryos, the general organization of both systems are normal. However, some subtle defects may be obscured by the global morphological defects caused by failure of germband retraction in mutant embryos.

For example, dorsal extension and migration of cells from both systems are limited because dorsal movement is prevent by the extended germband. For the same reason, defects in dorsal closure in *hnt* mutant embryos cannot be ruled out decisively in the four strong alleles. In hnt^{EH275a} mutant embryos, the occasional dorsal closure defects suggested hnt may play a role in dorsal closure. However, given the low frequency and

small size of the dorsal holes that formed, additional analysis is required to address this possibility. Since amnioserosa is the substrate upon which the lateral epidermis migrate dorsally to complete dorsal closure, and *hnt* shows strong expression in amnioserosa, it is possible that *hnt* may influence the closure process.

The cause of head defects in *hnt* mutant is not clear. However, these phenotypes are very similar to those seen in homozygous *thick veins* or *punt* mutant embryos. *tkv* and *punt* encode type I and type II receptors, respectively, for the *decapentaplegic* signaling pathway Both *tkv* and *punt* functions are also required for dorsal closure, proper midgut and tracheal development (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Affolter et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). It would be interesting to find out if *hnt* activities may be modulated by other receptor tyrosine kinases, such as *tkv*, and *punt*.

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Table 1. *hindsight* mutant phenotypes.

Alleles	Embryonic lethality	Germband retraction	Dorsal closure	Complementation of *pebbled*
XE81	-	-	NA	-
X001	-	-	NA	-
EH704a	-	-	NA	-
EH587a	-	-	NA	-
EH275a	+/-	+	+/-	+
pebbled	+	+	+	NA

Table 2. Molecular markers used to examined *hnt*⁻ mutant embryos

Molecular markers	Expression patterns of interest in wildtype embryos	Deviation from wildtype in *hnt*⁻ mutant embryos
α-Abdominal B	Hindgut, posterior spiracles.	None
α-cut	Malphigian tubules and peripheral nervous system	None
α-forkhead	Invaginating foregut, midgut, hindgut and salivary glands	None
α-Krüppel	Malphigian tubules anlagen, amnioserosa, central nervous system and muscle precursor cells.	Loss of amnioserosa expression
α-labial	Endodermal cells around second midgut constriction	None
Mab 2A12	Developing tracheal system	General organization is normal
Mab 22C10	All neuronal cells	General organization is normal
Mab D3	Developing tracheal system	General organization is normal

Table 3. Rescue of *hindsight* failure of germband retraction using hs-*hnt*.

Maternal genotype	paternal genotype	Age of embryos at first heat-shock	Age of embryos at second heat-shock	% of y^- *hnt*$^-$ /Y embryos retract their germband
y^- *hnt*^{XE81}/FM7	w^{1118}	4-6 hours	5-7 hours	<5% (43)
y^- *hnt*^{X001}/FM7	w^{1118}	4-6 hours	5-7 hours	<5% (21)
y^- *hnt*^{XE81}/FM7	w^{1118}; hs-*hnt*:M	4-6 hours	5-7 hours	44% (70)
y^- *hnt*^{XE81}/FM7	w^{1118}; hs-*hnt*:M	3.5-6 hours	4.5-7 hours	46% (82)
y^- *hnt*^{X001}/FM7	w^{1118}; hs-*hnt*:M	4-6 hours	5-7 hours	63% (27)
y^- *hnt*^{EH587}/FM7	w^{1118}; hs-*hnt*:M	3.5-6 hours	4.5-7 hours	52% (62)
y^- *hnt*^{EH587}/FM7	w^{1118}; hs-*hnt*:M	4-6 hours	5-7 hours	51% (83)
y^- *hnt*^{EH587}/FM7	w^{1118}; hs-*hnt*:M	3.5-6 hours	4.5-7 hours	73% (56)
y^- *hnt*^{EH704a}/FM7	w^{1118}; hs-*hnt*:M	4-6 hours	5-7 hours	65% (46)
y^- *hnt*^{EH704a}/FM7	w^{1118}; hs-*hnt*:M	3.5-6 hours	4.5-7 hours	84% (67)

FIGURE LEGENDS

Fig. 1: Characterization of *hindsight* mutant phenotypes

hnt mutant embryo fail to undergo germband retraction. (A) Cuticle of an hemizygous hnt^{XE81} mutant embryo. Note the normal thoracic and abdominal segements. The head is facing posterior. (B) hnt^{X001} does not complement *pebbled* rough eye phenotype. (C) Germband extended stage hnt^{XE81} mutant embryo does not express *Krüppel* protein in amnioserosa even though other patterns of *Kr* expression is normal. (D) Germband extended stage hnt^{XE81} mutant embryo shows wild-type *forkhead* protein expression pattern. (E) hnt^{XE81} mutant embryo does not show any immunostaining when examined with α-HNT antibody.

Fig. 2: Sequence of *hindsight* cDNA

(A) Predicted amino acid sequence of the HNT protein based on cDNA sequence analysis (nucleotide sequence not shown but entered in data base). The zinc-fingers are underlined. (B) Alignment of the fourteen zinc-fingers in HNT protein. (C) Schematic representation of the HNT protein sequence. The predicted amino acids contained several domains rich in glutamine, proline, serine/threonine, acidic and charged residues.

Fig. 3: Expression of *hnt* mRNA

(A) In stage 5 embryo, *hnt* is expressed in the posterior midgut primordium and patches on the dorsal side. (B). In stage 6 embryo, *hnt* expression covers the dorsal amnioserosa and invaginating posterior midgut. (C) Anterior midgut primordium begins to express hnt at stage 7. (D) At stage 9, *hnt* expression remains strong in amnioserosa, anterior and posterior midgut. (E) Stage 11 embryo shows strong staining at the invaginating tracheal pits. (F) Expression of *hnt* is also seen in subset of cells in the central nervous system. (G) Germand retracted embryo shows strong expression of *hnt* in the

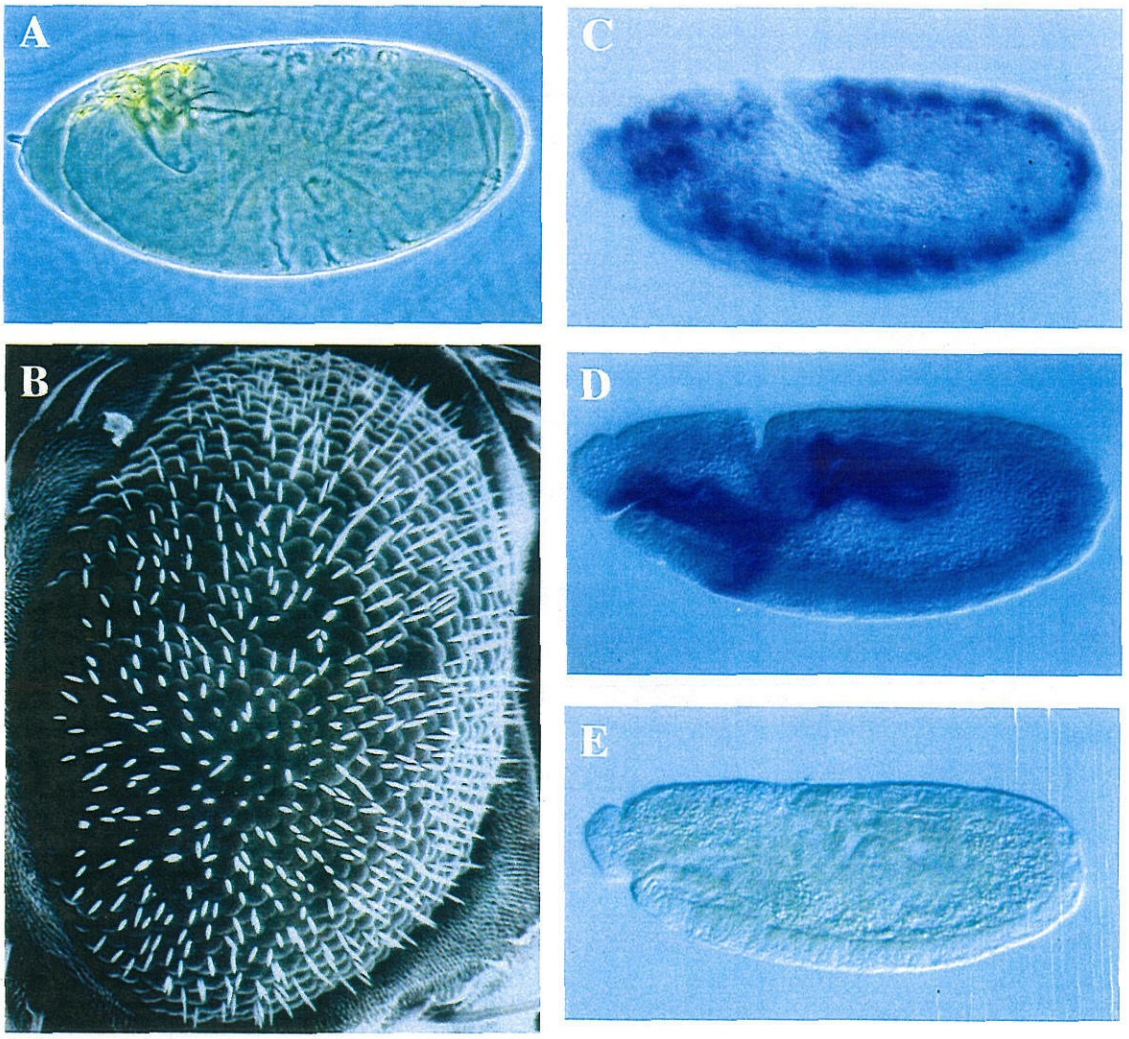
cells of peripheral nervous system. (H) Stage 14 embryo shows strong expression in the midgut, peripheral nervous system and stomatogastric nervous system.

Fig. 4: HNT protein expression

(A) HNT protein starts to appear in late stage 5 embryo at the posterior midgut primordia and patches on the dorsal side. (B) In stage 6 embryo, more cells on the dorsal side of express HNT protein. (C) At stage 8, HNT protein in seen in the anterior midgut primordium. (D) Fully germband extended embryo show strong staining in the midgut and the large nuclei of amnioserosa cells. (E) Tracheal pits and PNS precursors start to express HNT as soon as they begin to develop in stage 11 embryos. (F) Germband retracted embryo continues to express HNT in the midgut and PNS cells.

Fig. 5.Regulation of HNT protein expression.

(A,B) Wild-type HNT protein expression at stage 8. (C) Embryo from homozygous *torso* loss-of-function mother lack posterior midgut expression. (D) Embryo from homozygous *torso* gain-of-function mother lack dorsal expression. (E)HNT expression is largely unaffected in tll^{1} mutant embryo. (F), HNT expression in anterior midgut is lost in hkb^{2} mutant embryos. Only a very small posterior midgut remains in hkb^{2} mutant embryo. All endodermal expression is lost in hkb^{2} tll^{1} double mutant (G) and in srp^{9L} mutant embryos (H). (I) HNT expression is normal in $Egfr^{f1}$ mutant embryos. (J,K) Embryos derived from homozygous *cactus* or *saxophone* mother lost the dorsal amnioserosa. (L) zen^{f62} mutant embryo also lose the dorsal amnioserosa HNT expression. (M) Embryo derived from homozygous *pelle* mother has expanded dorsal expression and loss of anterior midgut expression. Neither *concertina* (N) nor *folded gastrulation* (O) affected HNT spatial expression pattern.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   121  GRHKQRRSGGATTQASIVMDYSSGDASSLRKKFRLNRSAASLSESGFVDASSTTGHSGYL
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   241  IGAGDEHMKYLCPICEVVSATPHEFTNHIRCHNYANGDTENFTCRICSKVLSSASSLDRH
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   541  SDIPAKCRDCEVIFATHRQLQSHCCRLPNALAGGLPPLLGASSSPLHNEEPEDEEHGDDE
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   661  SSSSFLRNFEQSVNTPNSSQYSLDGRDQEEEAQDAFTSEFRRMKLRGEFPCKLCTAVFPN
   721  LRALKGHNRVHLGAVGPAGPFRCNMCPYAVCDKAALVRHMRTHNGDRPYECAVCNYAFTT
   781  KANCERHLRNRHGKTSREEVKRAIVYHPAEDAGCEDSKSRLGEDLADTSFRSISPTPPPP
   841  PVNESKSQLKHMLLGENHLAPVNQQPPLKIQVKSLDQLVDKKPSAPAPQQQQQQQQQEKS
   901  GSALDFSMDVLDLSKKPTGGASLTPAVTRTPTPAAVAPVTPGGVGTPDLAAAIEQQQLLL
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  1261  LDEQPAEKEAEEEQELPKPLEQLGTKEAAQKMAETILEQAIKAGKPLSPPPTKENASPAN
  1321  PTVATTMQEPAITAPSTNPSSLKTMIAQAEYVGKSLKEVASSPFKDESQDLVPVAKLVDN
  1381  ATSQNMGFNSYFRPSDVANHMEQSDEEGLVASGSASESNNSGTEDVTSSSSSSEPKKKSA
  1441  YSLAPNRVSCPYCQRMFPWSSSLRRHILTHTGQKPFKCSHCPLLFTTKSNCDRHLLRKHG
  1501  NVESAMSVYVPTEDVSEPIPVPKSVEEIELEEQRRRQEAEREKELELERERELERERELE
  1561  RERQLEKEKERERQQLIQKLAAQMNAAATAAAVVAAASAVNGGASGGPHGPIADALAGGD
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  1741  KHSMLRHMKKHSGRAHNGDTPGSDCSDDEQVSSPPSTPHPTQPTSANNNNSCHNNNNNAN
  1801  NNNNNNNNNNNNSSSKLGLKLHDLLDKASEWRASRLGEHKENMGEATPSGATVAGSDLIG
  1861  NLLGISDQGILNKLLSSRTRRPNFWVWTTSERNSSDNRATPRAINTGVAAVLHRLTYTKA
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B

C_2H_2 Zn-finger Consensus		E[K/R]P[F/Y]x**C**xx**C**xxx**F**xxx[x/S]x**L**xx**H**xxx**H**TG
Finger 1	247	HMKYL**C**PI**C**EVVSATPHEFAN**H**IRC**H**NY
Finger 2	279	TENFT**C**RI**C**SKVLSSASS**L**DR**H**VLV**H**TG
Finger 3	307	ERPFN**C**RY**C**HLT**F**TTNGNMHR**H**MRT**H**KQ
Finger 4	513	ALLLS**C**PA**C**GASD**F**ETLPA**L**CV**H**LDAM**H**S
Finger 5	542	DIPAK**C**RD**C**EVI**F**ATHRQ**L**QS**H**CCRLPN
Finger 6	706	RGEFP**C**KL**C**TAV**F**PNLRA**L**KG**H**NRV**H**LG
Finger 7	738	AGPFR**C**NM**C**PYAVCDKAA**L**VR**H**MRT**H**NG
Finger 8	766	DRPYE**C**AV**C**NYA**F**TTKANCER**H**LRNR**H**GK
Finger 9	1056	ERPFA**C**EH**C**SAR**F**TLRSNMER**H**VKQQ**H**PQ
Finger 10	1445	PNRVS**C**PY**C**QRM**F**PWSSS**L**RR**H**ILT**H**TG
Finger 11	1473	QKPFK**C**SH**C**PLL**F**TTKSNCDR**H**LLRK**H**GN
Finger 12	1620	DLPYK**C**HL**C**EGS**F**AERLQCLE**H**IKQA**H**AH
Finger 13	1698	NRKVI**C**AF**C**LRR**F**WSTED**L**RR**H**MRT**H**SG
Finger 14	1726	ERPFQ**C**DI**C**LRK**F**TLKHSMLR**H**MKK**H**SG

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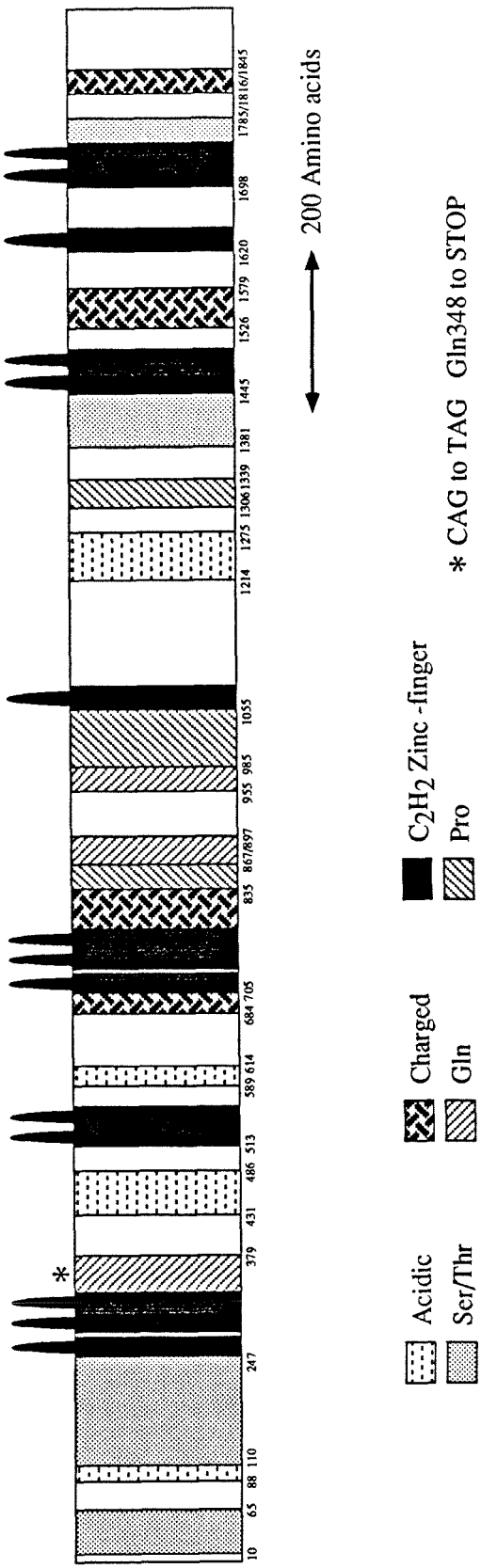
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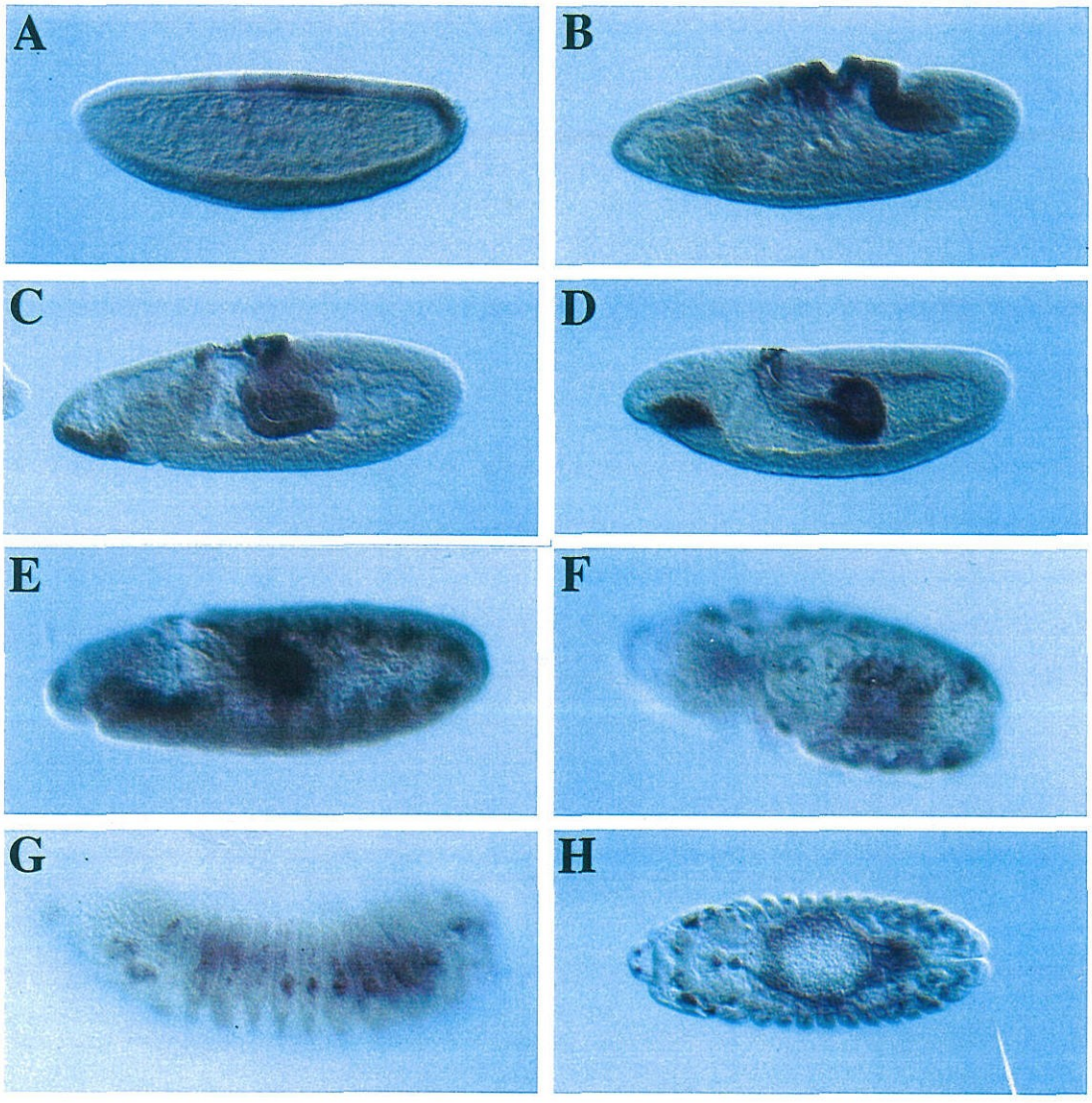
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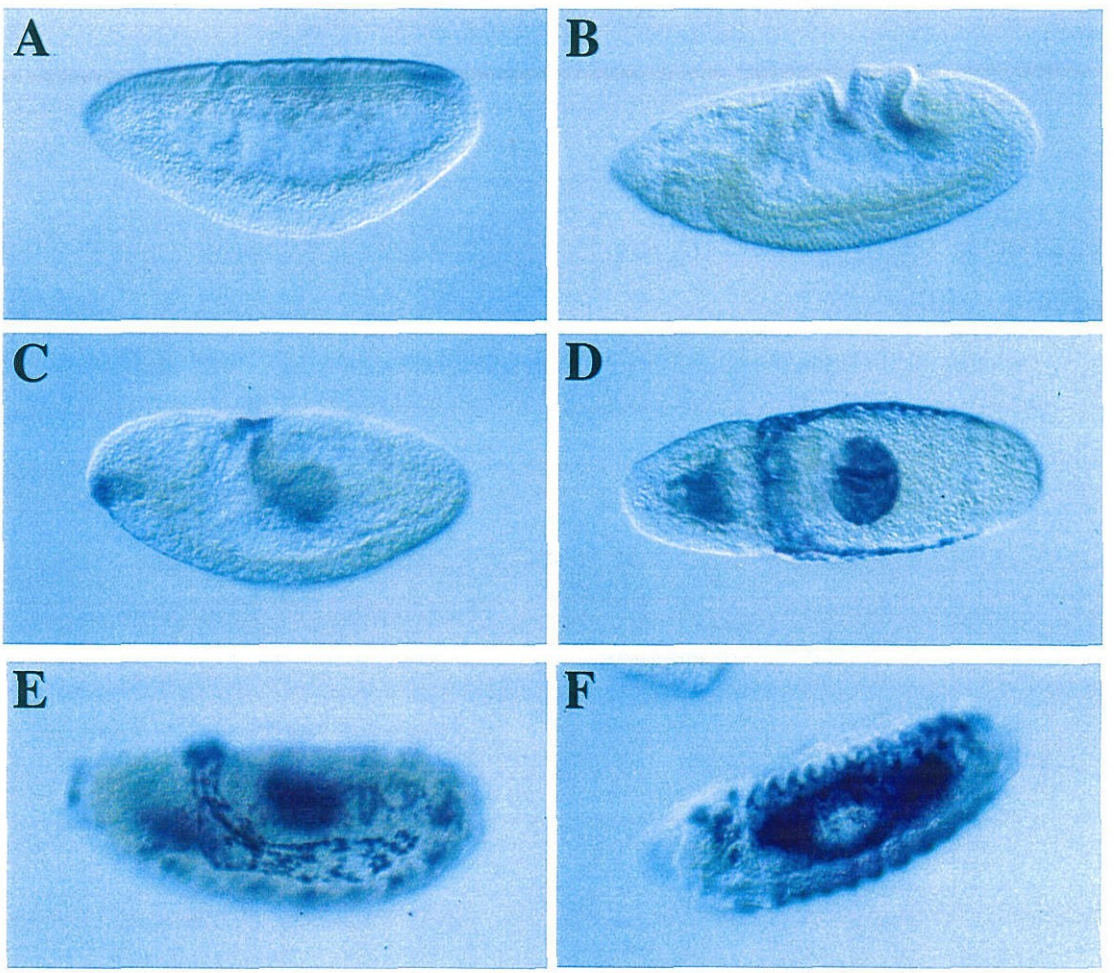
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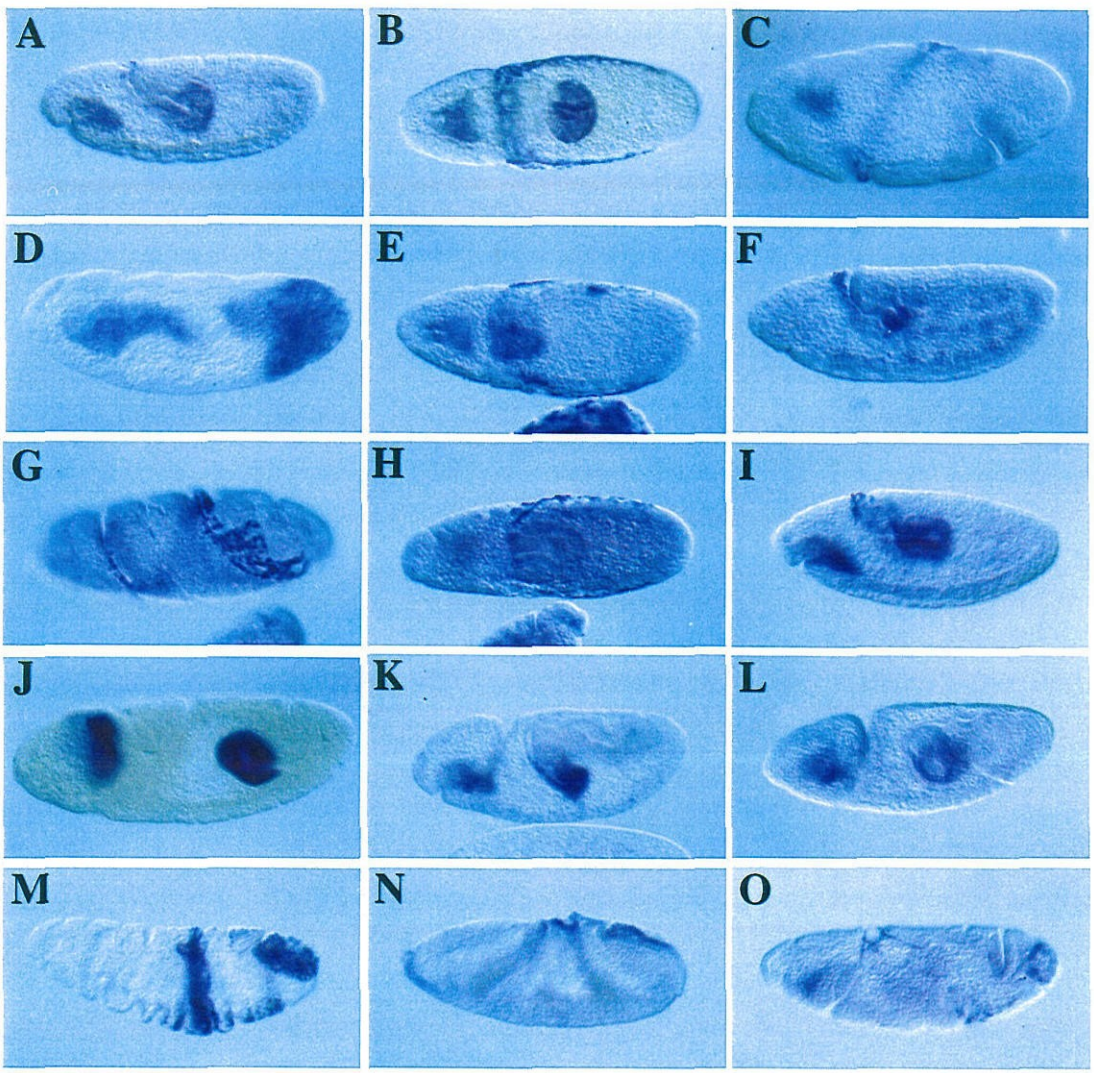
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CHAPTER 5

This chapter will be submitted for publication in *Development.*

Note: Qi Sun, Michele Lamka and I collaborated on this work. I determined *pebbled* is allelic to *hindsight.* I did the genetic interaction test between *hnt*^{pebbled} and various rough eye mutations. I constructed the strains with appropriate markers and FRT site for clonal analysis. I analyzed the phenotype of mosaic clones in adult notums. Qi did the adult eye mosaic analysis and pupal eye discs studies. Michele and Qi did the larval eye discs antibodies staining. Michele also examined *hindsight* expression in all other larval imaginal discs.

The *hindsight* gene controls pattern specification and morphogenesis during *Drosophila* eye development

Running title: hindsight in Drosophila eye development

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Key words: hindsight, zinc-finger, Drosophila, eye development, morphogenesis

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SUMMARY

hindsight, a gene that encodes a putative transcription factor with fourteen C_2H_2 zinc-fingers, is required for normal eye development. HINDSIGHT protein is initially detected in the morphogenetic furrow of the developing *Drosophila* eye. HINDSIGHT is expressed in all photoreceptor cells as they are recruited into the ommatidial cluster. Mosaic analysis of *hindsight* in the larval eye disc reveals that homozygous *hindsight* mutant patches contain regularly spaced ommatidial clusters with variable numbers of photoreceptor cells. Analysis of these photoreceptor cells using cell-specific and general developmental markers indicates that differentiation of these photoreceptor cells is abnormal. The presumptive R8 cells fail to express BOSS protein and the presumptive R2-5 cells do not express ROUGH protein. Genetically, *hindsight* shows synergistic interaction with *Star*, a gene also involved in photoreceptor specification. Taken together, these results demonstrate an early role for *hindsight* in photoreceptor development. Furthermore, *hindsight* is expressed throughout eye development and its activity is required late in pupal development when photoreceptor cells undergo morphological changes, such as apical-basal extension and rhabdomere separation. Similarities and differences between *hindsight* functions during embryonic germband retraction and postembryonic eye development are discussed.

INTRODUCTION

The wild-type *Drosophila* retina consists of a regular hexagonal array of about 750 ommatidia that develop from a monolayer epithelium, the eye imaginal disc. Surrounding

each ommatidium is a lattice of bristles and secondary and tertiary pigment cells. Internally, the photoreceptor cells (R1-8), the cone cells and the primary pigment cells are organized in stereotypical positions that can be identified unambiguously. Interactions among cells in each ommatidial cluster are critical for fate specification and pattern formation in the developing eye disc (Ready et al., 1976; Tomlinson, 1985; Tomlinson and Ready, 1987; Cagan and Ready, 1989; Wolff and Ready, 1991). Differentiation of retinal cells begins during larval development at the morphogenetic furrow, a landmark apical-basal indentation that moves across the disc in a posterior to anterior direction. Anterior to the furrow, cells are uncommitted and have the potential to become any one of the retinal cells. Within the furrow, interactions among cells result in the emergence of regularly spaced rosettes of six or seven cells. Posterior to the furrow, each rosette loses one or two cells and rearranges into a distinct five cells ommatidial precluster. Additional cells are recruited sequentially into the preclusters to assemble the ommatidia. Since the cells in an ommatidium are not related by lineage, it is believed that ommatidial assembly is guided by a series of cell-cell interactions (reviewed in (Tomlinson, 1988; Ready, 1989; Dickson and Hafen, 1993; Zipursky and Rubin, 1994).

Previous genetic and molecular studies have shown that a number of genes, including *scabrous*, *Notch* and *Egfr* (the *Drosophila* homolog of the epidermal growth factor receptor), are involved in the establishment of the regularly spaced pattern of ommatidia. Interactions among uncommitted cells in the morphogenetic furrow result in differentiation of the founding R8 photoreceptor cell, which subsequently inhibits its neighbours from adopting the R8 cell fate and directs the ommatidial assembly process. *scabrous* encodes the putative inhibitory signal expressed by the R8 cell . The mechanism that determimes which cells in the morphogenetic furrow express *scabrous* protein is not well understood. Mutations in the *Egfr* or *Notch* loci disrupt normal spacing and differentiation of the photoreceptor cells (Baker and Rubin, 1989; Clifford and Schüpbach, 1989; Xu and Rubin, 1993) and are good candidates for genes that organize the regular

spacing of ommatidia. Both genes encode large transmembrane molecules and probably function as receptors for intercellular signals (Shilo, 1992; Artavanis-Tsakonas et al., 1995)

Signals transduced by receptors like *Notch* and *Egfr* are transmitted into the nuclei of developing photoreceptor cells and regulate the expression and activity of nuclear proteins that can implement the appropriate differentiation program. Several nuclear factors critical for eye development have been identified. These include *glass* (Moses et al., 1989), *rough* (Tomlinson et al., 1988; Basler et al., 1990; Kimmel et al., 1990; Heberlein et al., 1991), *seven-up* (Mlodzik et al., 1990b), *sina* (Carthew and Rubin, 1990), yan (Lai and Rubin, 1992) and *pointed*(Klämbt, 1993; Scholz et al., 1993; Brunner et al., 1994; O'Neill et al., 1994). *glass* encodes a zinc-finger protein that is required for the development of all photoreceptor cells. *glass* mutant cells were able to become neuron-like but cannot differentiate as photoreceptor cells (Moses et al., 1989). All other genes are required in different subsets of developing photoreceptor cells for the specification of neuronal identity.

To assemble a multicellular structure as complex and yet as regular and precise as the *Drosophila* eye, coordination among the participating cells is critical. This includes not only coordinatied expression of appropriate gene products but also coordinated morphogenetic cell shape changes and movements. Although much is known about cell signaling and fate specification of retinal cells in the developing eye, coordinated shape changes and movements are poorly understood. Recently, however, a newly identified mutation, *marbles*, demonstrated that the highly orchestrated nuclear migrations seen during eye development can be uncoupled from the cell determination process (Fischer-Vize and Mosley, 1994). This suggests that parallel pathways are coordinated to ensure the formation of the crystalline-like eye structures.

We are interested in the mechanisms that regulate morphogenetic cell shape changes and movements during eye development. In this report, we analyze the functions of the

hindsight (*hnt*) gene during eye development. *hnt* encodes a nuclear protein with fourteen C_2H_2 zinc-fingers in widely spaced clusters and other features characteristic of transcription factors (Yip and Lipshitz, 1995). *hnt* function is important in controlling germband retraction during embryogenesis, possibly through the regulation of signaling between endodermal and ectodermal cells that involves the *Egfr* pathway (Yip and Lipshitz, 1995). One *hnt* allele, $hnt^{pebbled}$, is viable and shows a temperature-sensitive rough eye phenotype (Lindsley and Zimm, 1992; Yip and Lipshitz, 1995). Here we show that *hindsight* activity is required non-autonomously for photoreceptor cell development during ommatidial assembly. *hindsight* also functions autonomously in regulating subsequent morphological changes in photoreceptor cells. We suggest that *hindsight* is involved in the coordination of morphogenetic shape changes with fate specification of photoreceptor cells.

MATERIALS AND METHODS

Drosophila genetics and manipulations

The original *hindsight* alleles, *XE81* and *X001*, were isolated by Wieschaus et al. (1984). Three additional alleles, *EH275a*, *EH587a* and *EH704*, were subsequently obtained from an EMS-mutagenesis (Ebrel and Hilliker, 1988). The temperature sensitive rough eye mutation, *pebbled*, and other mutations used in this study, if not otherwise indicated, are described in Lindsley and Zimm (1992). Unless specified, all flies were raised on standard medium at room temperature or 25°C. The restrictive temperature used for $hnt^{pebbled}$ was 28 ± 1°C.

Mosaic analysis

X-ray irradiation (1000 rad) or FLP recombinase (Golic and Lindquist, 1989; Xu and Rubin, 1993) was used to induce somatic recombination and generate homozygous hnt^- clones. For X-ray induced hnt^- clones, $w\ hnt^-/w^{co}\ sn^2$ larvae between the ages of 20-44

hours were X-ray irradiated (1000 rad). Adult eyes were screened for twin spots. Twin spots were observed in approximately 1 out of 40 eyes. For FLP recombinase induced *hnt*⁻ clones, *y hnt*⁻ P[*ry*⁺; hs-neo; FRT]18A/FM7, ftz-lacZ (Kania et al., 1990) females were crossed to *w* P[*mini-w*⁺; hs-neo; FRT]5A, 10D, P[*ry*⁺; hs-neo; FRT]18A; MKRS, hs-FLP/TM6B, Tb males and allowed to lay eggs in vials for approximately 24 hours at 25°C. Twenty four hours later, vials containing first instar larvae were submerged in a 38.5°C water bath for 1 1/2 hours to induce FLP recombinase expression. Subsequently, eye discs from non-Tb, late third instar female larvae (either *y hnt*⁻ P[*ry*⁺; hs-neo; FRT]18A/*w* P[*mini-w*⁺; hs-neo; FRT]5A, 10D, P[*ry*⁺; hs-neo; FRT]18A; MKRS, hs-FLP/+ or FM7, ftz-lacZ/*w* P[*mini-w*⁺; hs-neo; FRT]5A, 10D, P[*ry*⁺; hs-neo; FRT]18A; MKRS, hs-FLP/+) were dissected and immunostained as described below.

Whole-mount in situ hybridization

Whole-mount in situ hybridization of the larval eye disc was done as described by Bonini et. al. (1994).

Histology and immunocytochemistry

Antibody staining of imaginal discs from third instar larvae was modified from the procedure described by Pattatucci and Kaufman (1992). Discs were dissected in Tri-PBS and kept on ice for no longer than 10 minutes. After removal of the Tri-PBS, 400 µl of PEMP fixative (0.1 M Pipes, pH 6.95, 1 mM $MgSO_4$, 2 mM EGTA, 4% paraformaldehyde) and 500 µl of heptane were added. The eppendorf tube was shaken by hand for 45 seconds, the PEMP/heptane mix was removed and 760 µl of PEMP fix and 40 µl dimethylsulfoxide was added. Discs were rocked for 20 minutes and then washed with methanol. To neutralize endogenous peroxidases, discs were incubated with 980 µl of methanol and 20 µl of 30% H_2O_2 for 30 minutes. Discs were then blocked with PBSBT (1X PBS, 0.5% bovine serum albumin, 0.1% Triton-X 100) for 2-3 hours at room

temperature. Antibody staining of discs was carried out using horseradish peroxidase (HRP) or fluorescent immunohistochemistry as described by Patel (Patel, 1994) except that PBSBT was used in place of PBT and normal goat serum was omitted from the incubations. HNT protein was detected using either the anti-HNT monoclonal (1:50 dilution) or polyclonal (1:2000 dilution) antibodies described in Yip and Lipshitz (1995). Mouse anti-SCABROUS monoclonal antibodies (MAbsca1; X. Hu, E. Lee, and N. Baker, unpublished) were used at a dilution of 1:300. Rabbit anti-ß-galactosidase polyclonal antibodies (Cappel) were used at a dilution of 1:1000 to detect ß-galactosidase protein from the A101 enhancer trap insertion in the *neuralized* gene (Huang et al., 1991) and the A2-6 enhancer trap insertion in the *scabrous* gene (Mlodzik et al., 1990a). To assess the phenotype of *hnt*⁻ clones in third instar mosaic eye discs, the following antibodies were used in double-labeling experiments with anti-HNT monoclonal or polyclonal antibodies: mouse anti-BOSS monoclonal (1:2000 dilution anti-boss 1 MAb ascites; Alonso and Cabrera, 1988), rat anti-ELAV polyclonal (1:500 dilution; Rabinow and White, 1991), mouse anti-ROUGH monoclonal (1:2000 dilution MAbro1 ascites; Kimmel et al., 1990), anti-HRP (1:500). For double labeling experiments with HRP-conjugated secondary antibodies, detection of the first antibody was enhanced by the addition of nickel chloride to the staining solution. In order to detect ROUGH protein, it was necessary to use a biotinylated secondary antibody and streptavidin-HRP (Kierkegard and Perry Laboratories).

Pupal eye discs were dissected and stained with antibody as described by Bruce Kimmel (Kimmel et al., 1990). Phalloidine staining was done as described by T. Wolff and D. Ready (1991).

Adult eyes with *hnt*⁻ clones were fixed in 4% paraformaldehyde, embedded in epon, and sectioned at 1 µm. For scanning electron microscopy, adult flies were preserved in 95% ethanol until use. They were critically dried and then mounted on SEM stubs. Samples were viewed and photographed on an ETEC instrument

RESULTS

Expression of *hindsight* in eye disc

Initial *hnt* mRNA expression is detected in regularly spaced groups of cells in the morphogenetic furrow in third instar larval eye imaginal discs. *hnt* mRNA continues to be expressed in cells posterior to the furrow (Fig. 1A). Similar to the distribution of *hnt* mRNA, eye discs at the same stage of development expressed HNT protein in the nuclei of groups of cells in the morphogenetic furrow. Typically, one to three nuclei from each group of cells were stained. The number of HNT expressing cells increases posteriorly. By row three, HNT is detected in three to five cells. Eventually, HNT protein is expressed in eight cells per cluster (Fig. 1B, 2B). The expression pattern of HNT protein, eight nuclei per cluster with regular spacing between each cluster, suggested these cells may be the photoreceptor cells of each ommatidium.

Double labeling experiments using anti-HNT and anti-SCA antibodies (X. Hu, E. Lee, and N. Baker, unpublished) or the A2-6 enhancer trap insertion in the *sca* gene (Mlodzik et al., 1990a) confirmed that the initial HNT expression is coincident with the earliest expression of SCA, a furrow marker (Fig. 2A-D). When tested with other general markers of neuronal development, we found the expression of HNT precedes that of the neural antigens ELAV (Fig 4C) and HRP (data not shown).

HNT protein is detected throughout eye development. Immunostaining of pupal eye discs shows that HNT protein is found in all eight photoreceptor cells (Fig. 3A). In addition, HNT protein is also expressed in the neuronal cells of the bristles (Fig. 3B) as determined by the basal location of the nuclei and double labeling with anti-HNT and anti-ELAV antibodies (Fig. 3C, D). Since ELAV protein is expressed in all neuronal cells, the identical staining patterns observed with anti-HNT and anti-ELAV antibodies indicated the additional HNT expressing cells must be the bristle neurons.

The early onset and continuous expression of *hnt* throughout eye development suggests that it plays an important role in photoreceptor development and ommatidial assembly.

hindsight function is required for early ommatidial assembly

None of the four lethal *hnt* alleles express protein that can be recognized by our anti-HNT antibody. One of the alleles, hnt^{X001}, has a stop codon at amino acids residue 348. Since the truncated polypeptide does not overlap with the fragment we used to induce our anti-HNT antibody, it should not be recognized by the antibody (Yip and Lipshitz, 1995)). The antibody provided us the tool to look at development of *hnt* mutant cells at early stages. We used the FLP recombinase system (Golic and Lindquist, 1989; Xu and Rubin, 1993) to generate homozygous hnt^- mutant clones at a high frequency. Larval eye discs were examined for mutant phenotypes. The eye discs were stained with anti-HNT antibody to identify the mutant clone and with neuronal markers to detect abnormalities in cells within the clone. Anti-ELAV antibody stains the nuclei of all 8 photoreceptors in wild-type eye discs. As shown in Figure 4, the beginning of ELAV expression in hnt^- clones is delayed by one or two rows when compared to the staining in the heterozygous neighbor, but the spacing of the ommatidial clusters is normal. A similar delay is also observed when we stained discs with another neuronal marker, anti-HRP antibody (data not shown). In addation to the delay within the clone, the number of photoreceptor cells is reduced in all clusters. In the most mature *hnt* mutant ommatidia (located in clones at most posterior region of the eye disc) there are 5 or less photoreceptor cells in each cluster, compared to 8 cells in wild-type eye discs.

Delay of expression of neuronal markers and missing photoreceptor cells in the ommatidial clusters within the hnt^- clones suggest that *hnt* may be involved in regulating the differentiation and/or interactions of the photoreceptor cells during the assembly of ommatidal clusters. To determine how *hnt* function affects photoreceptor cell

differentiation and which cells are missing from the mutant ommatidial clusters, we examined the expression of *rough* (*ro*) and *bride of sevenless* (*boss*) gene products within the *hnt*⁻ clones.

The RO homeodomain protein serves as a marker for photoreceptor cells R2, R3, R4 and R5 and is required for the proper differentiation of photoreceptor cells R2 and R5 and subsequent recruitment of photoreceptor cells to the ommatidial clusters (Tomlinson et al., 1988; Basler et al., 1990; Kimmel et al., 1990; Heberlein et al., 1991). Flies carrying a null alleles of *ro*, ro^{x63}, have reduced number of photoreceptor cells in their ommatidia (Heberlein et al., 1991). *hnt*⁻ mosaic eye discs double labeled with anti-HNT and anti-RO (Kimmel et al., 1990) antibodies revealed that *hnt*⁻ clones lack RO staining, suggesting that the decreased number of photoreceptor cells in the ommatidial clusters within the *hnt*⁻ clones may be partly caused by the loss of the RO protein expression (Fig. 5).

boss gene function is required in R8 cells for the specification of R7 cells identity in ommatidia (Reinke and Zipursky, 1988). *boss* encodes a transmembrane protein that is the ligand for the *sevenless* receptor tyrosine kinase (Hart et al., 1990). BOSS protein is normally located at the apical tip of the R8 cells. In the *hnt*⁻ clones, we generally do not detect any BOSS protein expression ; in rare occasions a few ommatidia show BOSS staining at very low level (Fig. 6). The loss of BOSS protein expression in *hnt*⁻ clone suggests the differentiation of R8 cells are aberrant..

The adult eye phenotype of *hindsight*

Since HNT protein is detected throughout eye development, we were interested in its possible functions at later stages of eye development. We examined the adult eye phenotype of flies carrying somatic *hnt*⁻ clones and of flies bearing a temperature-sensitive, viable allele of *hnt*, $hnt^{pebbled}$ (Yip and Lipshitz, 1995).

Homozygous or hemizygous $hnt^{pebbled}$ flies raised at the restrictive temperature have rough eyes (Fig. 7B) (Lindsley and Zimm, 1992). Cross sections of such eyes

showed that the normally regular ommatidial columns are curved and irregular (Fig. 7E). Internally, most ommatidia have a reduced number of photoreceptor cells. Furthermore, many pigment cells are irregular or missing, causing fusion of adjacent ommatidial clusters (Fig. 7H).

Somatic *hnt*$^-$ clones can easily be detected because they form a scar adjacent to the *hnt*$^+$ twin spots in the adult mosaic eye (Fig. 7C). In the middle of the *hnt*$^-$ clone, no ommatidia are detectable. However, individual rhabdomeres, light-collecting organelles of photoreceptor cells, can be seen (Fig. 7I). Cross section through *hnt*$^-$ clones revealed that ommatidial columns had collapsed. The basal membrane underlying the retina is missing from *hnt*$^-$ clones; individual rhabdomeres can be seen to fall through these holes in the basal membrane (Fig. 7F).

By examining normally constructed, but genetically mosaic, ommatidia along the border of the *hnt*$^-$ clone, we were able to determine which photoreceptor cells require *hnt* function in order to program normal ommatidium formation. Only 50 mosaic ommatidia were found out of a total of 52 eye clones. This number is fewer than expected, since we found many mosaic ommatidia along the *hnt*$^-$ clone when we examined similar clones at the larval stage. Table 1 summarizes the results of this analysis: any photoreceptor cells can be mutant for *hnt* function and still contribute a normal ommatidium. However, the frequency of finding a *hnt*$^-$ R8 cell, in a normal ommatidium is 2-3 fold lower than for other cells.

While the number and pattern of the photoreceptor cells in these mosaic ommatidia is normal, specific morphological defects were found in half of the *hnt* mutant photoreceptor cells in these mosaic ommatidia. In normal ommatidia, the cell bodies of the outer photoreceptor cells (R1-6) extend the full length of the ommatidial column (i.e. the full apical-basal extent of the adult retina). In contrast, the central photoreceptor cells (R7 and R8) extend only half the length of the ommatidial column; R7 is present apically and R8 basally. The apical-basal length of the photoreceptor cells increases 4 fold from 30μm to 120μm during late pupal stage (40-96 hour postpuparium formation at 25^{o}C). In each

photoreceptor cell, specialized light-harvesting organelles known as rhadomeres occupy the full length of the cell. Rhabdomeres are constructed of densely packed microvilli, with photopigment molecules embedded therein. Rhabdomere morphogenesis begins at about 48 hours postpuparium (at 25^{o}C) with the infolding of the photoreceptor cell membrane and accumulation of vesicles. The process commences near the apical surface and proceeds basally. At the same time, the infolding and packing of microvilli continue toward the center of photoreceptor cells. At 110 hours, a central cavity or interrhabdomal space occurs as rhabdomeres separate (Weddington and Perry, 1960; Cagan and Ready, 1989).

Very often, *hnt* mutant photoreceptor cells have shortened cell bodies. Figure 8A shows that one such *hnt* mutant photoreceptor cell, R5, is shorter than the heterozygous counterpart as indicated by the failure of the rhabdomere to extend the normal full length of the ommatidial column. Another common defect is that rhabdomeres fail to separate. Figure 8B shows the rhabdomere of a *hnt* mutant photoreceptor cell, R4, is well separated from its neighboring R3 rhabdomere at the apical but is fused at the basal level. Thus, *hnt* is involved in control of morphogenetic cell shape changes during pupal eye development.

These adult eye phenotypes indicate that even though *hindsight* function is not absolutely required in any one photoreceptor cell, it strongly affects the development of ommatidial clusters. No mosaic ommatidial clusters with more than 3 *hnt* mutant photoreceptor cells survive to adulthood. Moreover, *hnt* is also involved in regulating morphogenetic changes, such as apical-basal extension and rhabdomere separation, during later stages of eye development.

Genetic interaction of *hindsight* with *Star*

Given the possible functions of *hnt* during ommatidial assembly, we tested other known genes involved in eye development for possible interactions with *hnt*. To test for such interactions, we introduce mutations of interest (homozygous for viable mutations or

heterozygous for lethal mutations) into homozygous *hnt*^{pebbled} background and analysis of possible changes in retinal morphology (Table 2).

Most of the double mutants between *hnt*^{pebbled} and rough eyed mutations do not show any interaction, however, *hnt*^{pebbled} shows synergistic interaction with *Star* (Fig. 9). The interaction is most striking between *hnt*^{pebbled} and dominant alleles of *Star*: The eyes are very small and very few facets and bristles exist. *hnt*^{pebbled} also weakly interacts with recessive alleles of *Star*, known as *asteriod* (*ast*). *Star* encodes a novel protein with a putative transmembrane domain (Kolodkin et al., 1994). Its function is necessary for the development of photoreceptor cells R8, R2 and R5 (Heberlein and Rubin, 1991; Heberlein et al., 1993; Kolodkin et al., 1994) and is also involved in recruitment of R7 (Heberlein and Rubin, 1991; Heberlein et al., 1993; Kolodkin et al., 1994). The nature of STAR protein function is not known; however, *Star* mutations interacts genetically with components of signal transduction pathways that are initiated by two different receptor tyrosine kinases: the EGF and SEVENLESS receptors (Heberlein et al., 1993; Kolodkin et al., 1994). The genetic interaction between *Star* and *hnt* suggests that the functions of *hnt* include modulation of the output of signal transduction pathways that control eye development.

Expression of *hindsight* in other imaginal discs

In addition to its eye expression in the developing eye, HNT protein accumulates in the nuclei of sensory organ precursor cells in third instar wing, haltere, leg (prothoracic, mesothoracic and metathoracic) and eye-antennal discs (Fig. 10). The identity of these HNT-staining cells was confirmed in antibody double labeling experiments using the A101 enhancer trap insertion in the *neuralized* gene as a marker for sensory organ precursor cells (data not shown; (Boulianne et al., 1991; Huang et al., 1991)). Preliminary clonal analysis suggested *hnt* activity affects the development of the macrochaetae (Fig.11).

DISCUSSION

Our analysis of the *hnt* gene shows that it has multiple functions during eye development. *hnt* is required for the promotion of photoreceptor cell fate and ommaditial assembly during early photoreceptor cell development. It is also involved in the control of morphogenetic cell shape changes at the pupal stage.

Two observations support the conclusion that *hnt* is not involved in the determination of the spacing of the ommatidia per se. First, ommatidial clusters are regularly spaced within mosaic *hnt* clones. Second, the double mutant phenotype of $hnt^{pebbled}$ and *sca* (*sca* is involved in the determination of cluster spacing) is additive in nature suggesting that *hnt* and *sca* are in independent pathway.

However, ommatidial clusters within *hnt* mosaic clones have variable numbers of photoreceptors (0-5 8 in wild-type) . Staining *hnt* clones with anti-BOSS (which marks R8) and anti-RO (which marks R2, R3, R4 and R5) antibodies, indicated that neither protein is present within the *hnt* mutant patches. Normally, BOSS is expressed by R8 cells as soon as they start to differentiate. Loss of BOSS protein expression by R8 cells within hnt^{-} clones suggests that differentiation of R8 cells is abnormal. Since R8 is the first photoreceptor cell in an ommatidium to differentiate and is responsible for directing the stepwise addition of cells into the ommatidium, any defect in the differentiation of R8 will have serious consequences for ommatidial assembly. In this case, expression of RO protein is missing and development of the R2 and R5 cells as well as subsequent addition of cells to the clusters is disrupted. However, loss of *hnt* activity does not completely block neuronal differentiation as cells within hnt^{-} tissue are able to express ELAV and HRP proteins. Nonetheless, the expression of ELAV and HRP are delayed and the number of ELAV-positive and HRP positive cells are reduced. Consequently, we hypothesize that *hnt* causes defective R8 development and thus affects its ability in organizing ommatidial assembly.

Compared to other genes that act early in eye development (Renfranz and Benzer, 1989), the regular spacing of ommatidial clusters combined with delayed expression of neuronal markers in *hnt*⁻ tissue in mosaic clones is unusual. Most known mutations affecting eye development, with the exception of *glass*, prevent photoreceptor cells from taking on neuronal identity. *glass* mutant cells fail to undergo photoreceptor cell differentiation but are able to express neuronal markers with the same kinetics as wild-type cells. Therefore, *hnt* is not required for the developing photoreceptors to become neurons, however, its activity modulate the process.

Formation of normally constructed but genetically mosaic ommatidia along the border of homozygous *hnt*⁻ somatic clones allowed us to determine that *hindsight* is not absolutely required in any specific photoreceptor cells to program normal ommatidial assembly. Given the expression pattern of *hindsight* and its role in photoreceptor cell development, the simplest interpretation of this result is that *hnt*⁻ mutant cells are rescued by genetically normal neighboring cells during ommatidial assembly. Since we never see mosaic ommatidia with more than 3 genetically mutant *hnt*⁻ cells, we conclude that at least 5 cells must be genetically wild-type for this rescue to occur.

The mechanism by which this rescue operates is not clear. However, genetic interactions between $hnt^{pebbled}$ and *Star* raise an interesting possibility. It has been shown that *Star* is involved in modulating two signal transduction pathways, the *Egfr* and *sevenless* pathways . *hindsight*, as a transcription factor, might regulate production of components of a signal transduction pathway (secreted signaling molecules or cell surface molecules) that acts to recruit or instruct nearby cells to participate in ommatidial assembly. In this model, *Star* is also a component of the pathway and modulates its output. Reducing the activities of two components of the same signal transduction pathway might greatly affect the output of the pathway and thus cause a more extreme phenotype as we observe. This model is also consistent with the proposed function of *hindsight* during embryonic development. There, expression of *hindsight* in the endoderm is postulated to result in the

production of a signal that is received by ectodermal cells and instructs them to carry out the cell shape changes that drive germband retraction (Yip and Lipshitz, 1995).

hnt activity is also required autonomously in each photoreceptor cell during the pupal stage when these cells undergo extensive cell shape changes. Half of the *hnt*$^-$ mutant cells within mosaic ommatidia show morphological defects, such as failure to undergo apical-basal extension and rhabdomere separation. This late function is also reminiscent of *hnt*'s embryonic function. *hnt* mutant embryos fail to retract their germband because of the failure of the ectodermal cells to undergo the necessary morphogenetic cell shape changes. In the embryo, this is largely a non-autonomous process since *hnt* is expressed in endoderm but not ectoderm (Yip and Lipshitz, 1995). In the late pupal eye disc, *hnt* mutant cells fail to undergo the morphogenetic cell shape changes in an autonomous fashion.

Is there a common mechanism that can explain the functions of *hnt* during eye development? One attractive model is that *hnt* is involved in the regulation of morphogenetic cell shape changes during eye development. During early eye development, cell-cell interactions among photoreceptor cell precursors are important for the establishment and maintenance of the differentiated states. Defects in a pathway which regulate morphogenetic cell shape changes would certainly interfere with cell-cell interactions as well as differentiation of photoreceptors. Furthermore, *hnt* mediated regulation of cell shape would explain the morphological defects observed during pupal stage eye development. An alternative model proposes that *hnt* may regulate different target genes during different stages of eye development. Given that HNT protein has zinc-fingers arranging in widely spaced clusters, it is possible that different zinc-finger clusters have different targets. Depending on the stages of the development, differential regulation of downstream targets would allow a single transcription factor to control multiple developmental pathways. Additional experiments can be done to distinguish between these possibilities.

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Table 1. Analysis of *hnt* mosaic ommatidia

	R1	R2	R3	R4	R5	R6	R7	R8
1				x				
2			x	x	x			
3				x				
4				x				
5	x					x	x	
6						x		
7		x						x
8					x			
9		x						
10				x				
11			x	x				
12	x						x	
13		x	x					
14							x	
15	x							
16	x							
17		x						
18				x				
19								x
20						x		
21				x				
22						x		
23	x							
24		x	x					
25	x					x	x	
26				x	x			
27			x		x			
28						x	x	x
29	x					x	x	
30						x		
31						x		
32				x				
33		x						
34				x				
35			x					
36			x					
37				x				
38			x					
39			x					
40	x			x				
41	x							
42						x		
43				x				
44		x						
45		x	x					
46				x				

47				x				
48	x					x		
49	x				x			
50			x					

Score sheet of 50 normally constructed ommatidia containing both wild-type and *hnt*$^-$ cells.

A x indicates *hnt*$^-$ photoreceptor cells.

Table 2. Mutations tested for genetic interaction with *hnt^{pebbled}*

Genotype	Interaction with *hnt^{pebbled}*
sca/sca	0
S^{1}/+	+++
S^{126}/+	++
Df(2L)S^{3}/+	++
ast^{1}/ast^{1}	+
ast^{4}/ast^{4}	+
Egfr^{E1}/+	0
Egfr^{f1}/+	0
Egfr^{f2}/+	0
drk^{10626}/+	0
Gap1^{PB}/+	0
Sos^{JC2}/+	0
Sos^{x122}/+	0
ro^{1}/ro^{1}	0
pnt^{2}/+	0
spi/+	0

Symbols indicating the results are as follow: 0, no detectable interaction or additive effect; +++, strong synergistic interaction; ++, moderate synergistic interaction; +, weak synergistic interaction.

FIGURE LEGENDS

Fig. 1. Expression of *hnt* mRNA (A) and protein (B) are seen in regularly spaced groups of cells at the morphogenetic furrow in larval eye disc. Expression continues behind the furrow in the developing eye.

Fig. 2. Appearance of HNT protein begins in the morphogenetic furrow. HNT protein is readily detected in the row of cells immediatlely posterior to the first row of cells that express SCABROUS (SCA), a furrow marker. (A) A wild-type third instar eye disc double stained to show the pattern of HNT protein (black) and SCA protein (brown). (B,C.D) A third instar eye disc from the *sca* enhancer trap line A2-6 double labelled for HNT (green) and β-galactosidase (red). Arrows mark the position of the morphogenetic furrow.

Fig. 3. HNT protein is expressed in all neuronal cells (photoreceptors and bristle neurons) in the pupal eye. (A) HNT protein in the eight photoreceptors. (B, C, D) A pupal eye double labeled for HNT (green) and ELAV (red) at the basal level where bristle neurons locate.

Fig. 4. Anti-ELAV antibody staining of *hnt*$^-$ eye clones. Third instar eye discs with *hnt*$^-$ clones were double labeled for ELAV (green) and HNT (red). In *hnt*$^-$ clone, the initial detection of ELAV protein is delayed by one or two rows. In more posterior regions of clones, the number of photoreceptors is reduced.

Fig. 5. Anti-ROUGH antibody staining of *hnt*$^-$ clones. ROUGH staining is shown in brown and HNT staining is purple. (A) In wild-type third instar eye discs, ROUGH protein is normally present in R2, R3, R4 and R5 cells, but in *hnt*$^-$ clones (B, arrows), ROUGH protein cannot be detected.

Fig. 6. Anti-BOSS antibody staining of *hnt*⁻ clones. Boss staining is shown in brown and HNT staining is shown in black. (A) In wild-type third instar eye discs, BOSS protein is normally present in R8, but in *hnt*⁻ clones (B), BOSS protein cannot be detected.

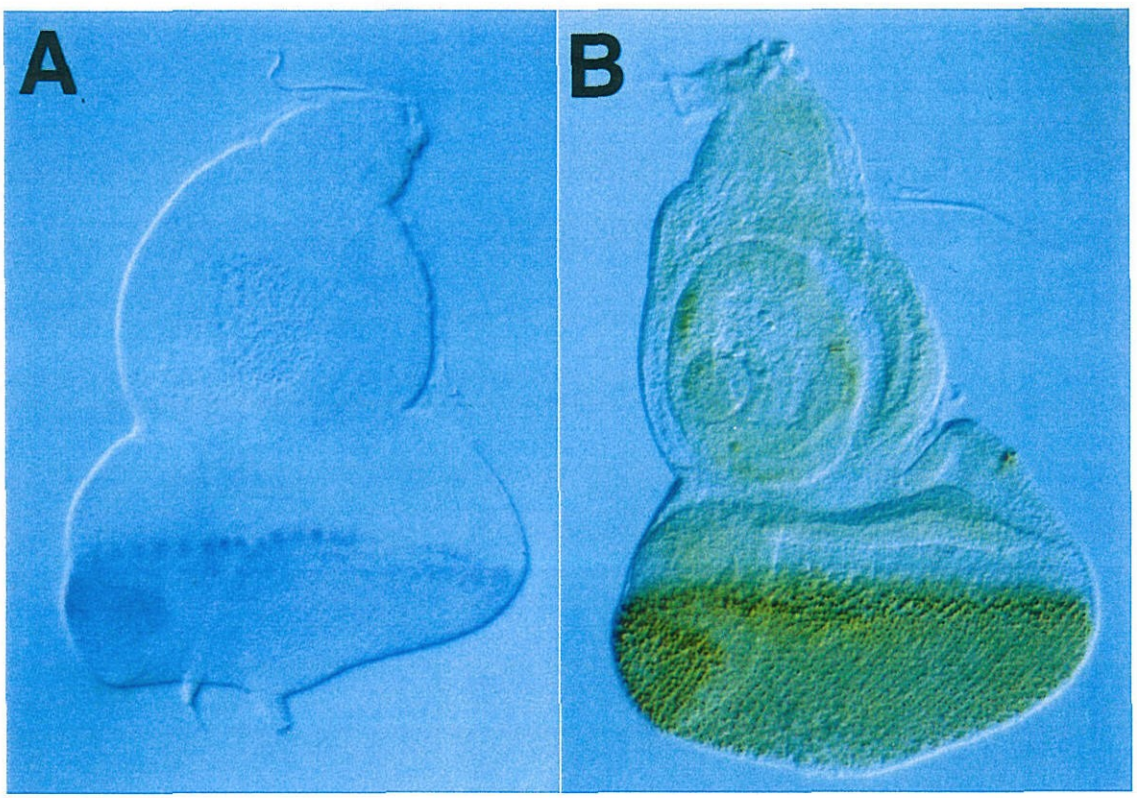
Fig. 7. The *hnt* mutant eye phenotype. (A-C) Scanning EM and (D-I) sections through wild-type eyes (A, D, G), hnt^{peb} (B, E, H) and hnt^{X001} eye clones (C, F, I). Ommatidia in hnt^{peb} eyes show irregular ommatidial columns (E) and reduction in the normal number of photoreceptors (H). In *hnt*⁻ clones, the ommatidial columns had collapsed and basal membrane underlying the retina is missing; individual rhabdomeres can be seen to fall through these holes in the basal membrane (F). Ommatidia are absent from the center of *hnt*⁻ clones (I).

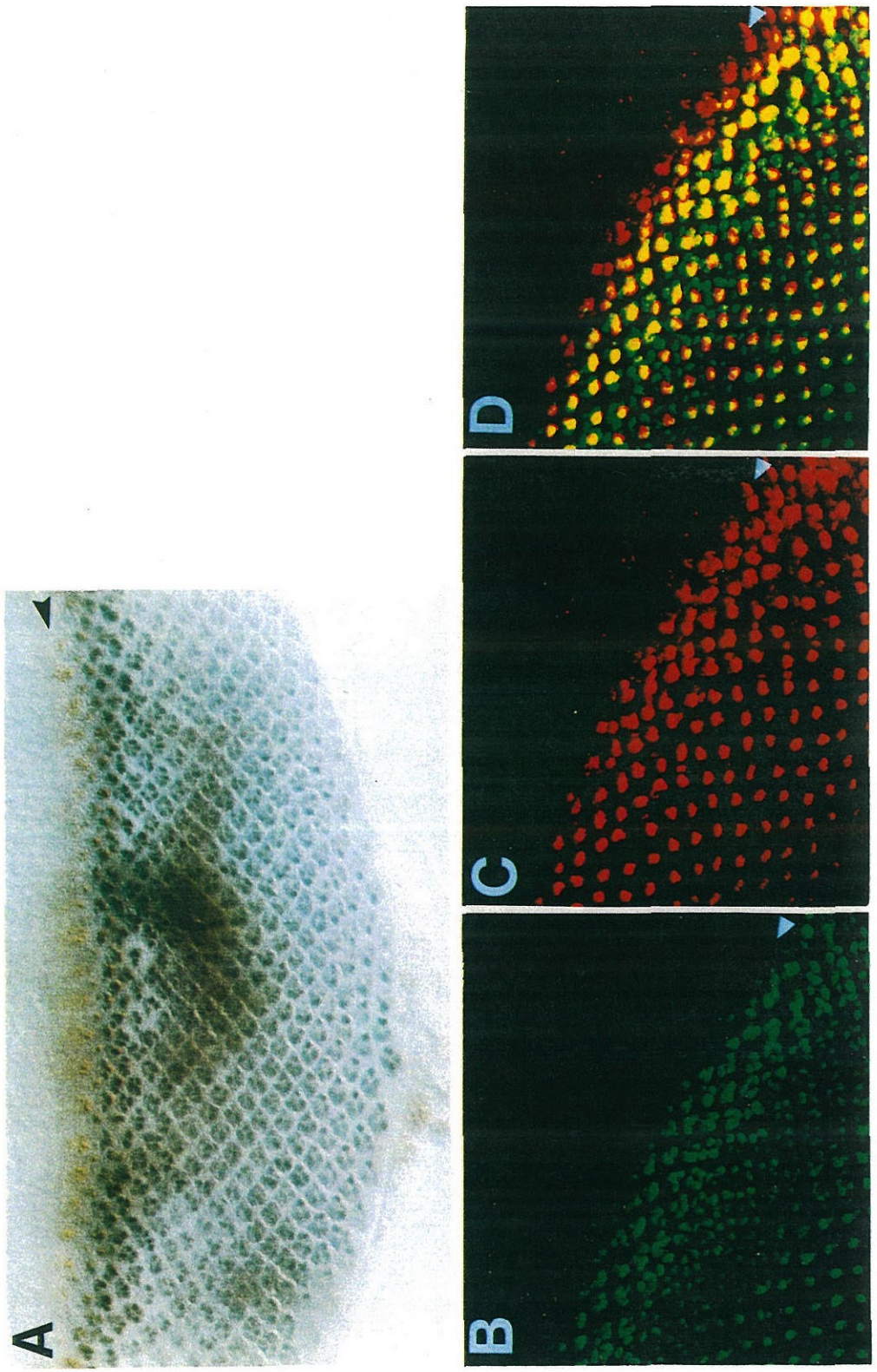
Fig. 8. Examples of *hnt* mutant cells fail to undergo cell shape changes in mosaic ommatidia. R5 is *hnt*⁻ in (A) while R4 is *hnt*⁻ in (B). In (A), R5 has a shorter cell body than its neighbors. In (B), R4 has a fused rhabdomere with its neighbor R3 at the basal level.

Fig. 9. Genetic interaction ofhnt^{peb} with *Star*. SEM of adult eyes. (A) hnt^{peb} male raised at 28°C. (B) *Star*/+ male raised at 28°C. (C) hnt^{peb} ;*Star*/+ male raised at 28°C.

Fig. 10. HNT protein is expressed in sensory organ precursor cells of the third instar wing (A), haltere (B), and leg (C-prothoracic, D-mesothoracic, E-metathoracic) discs.

Fig. 11. Mosaic *hnt*⁻ clone on adult notum shows defects in macrochaetae. One has a very small hair shaft (arrow) and the other has no hair shaft and socket (arrowhead).


A
B


A
B
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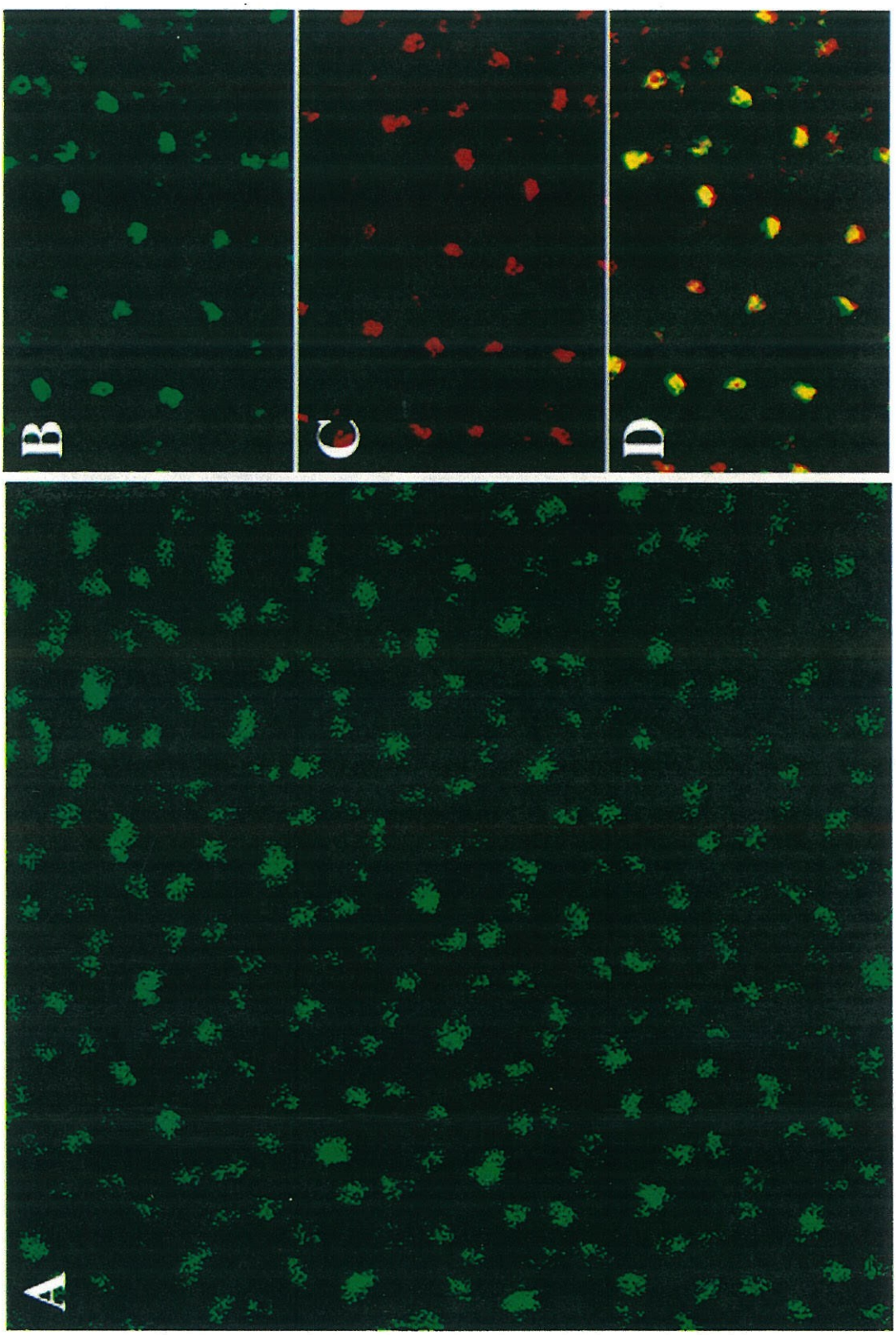
D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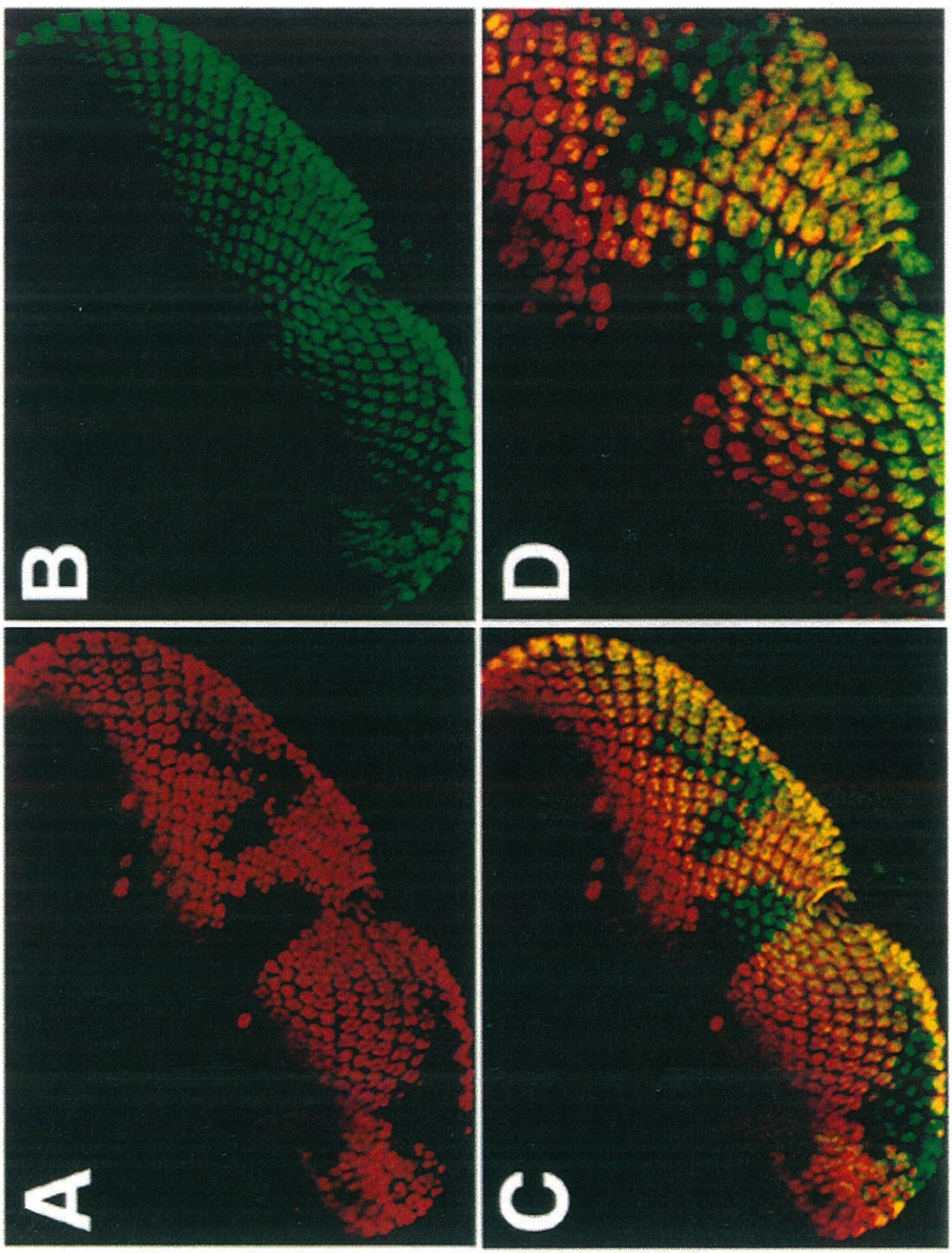
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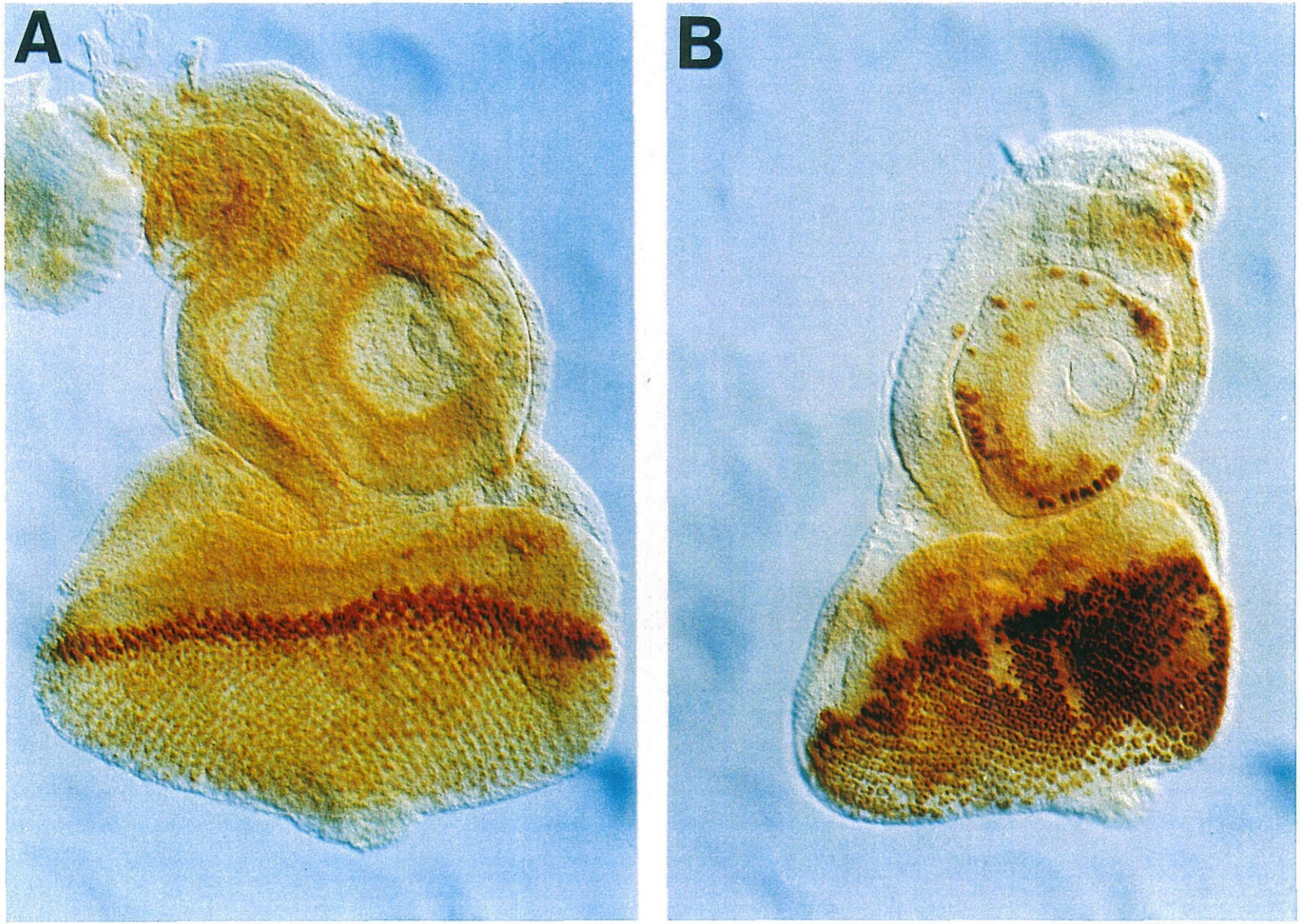
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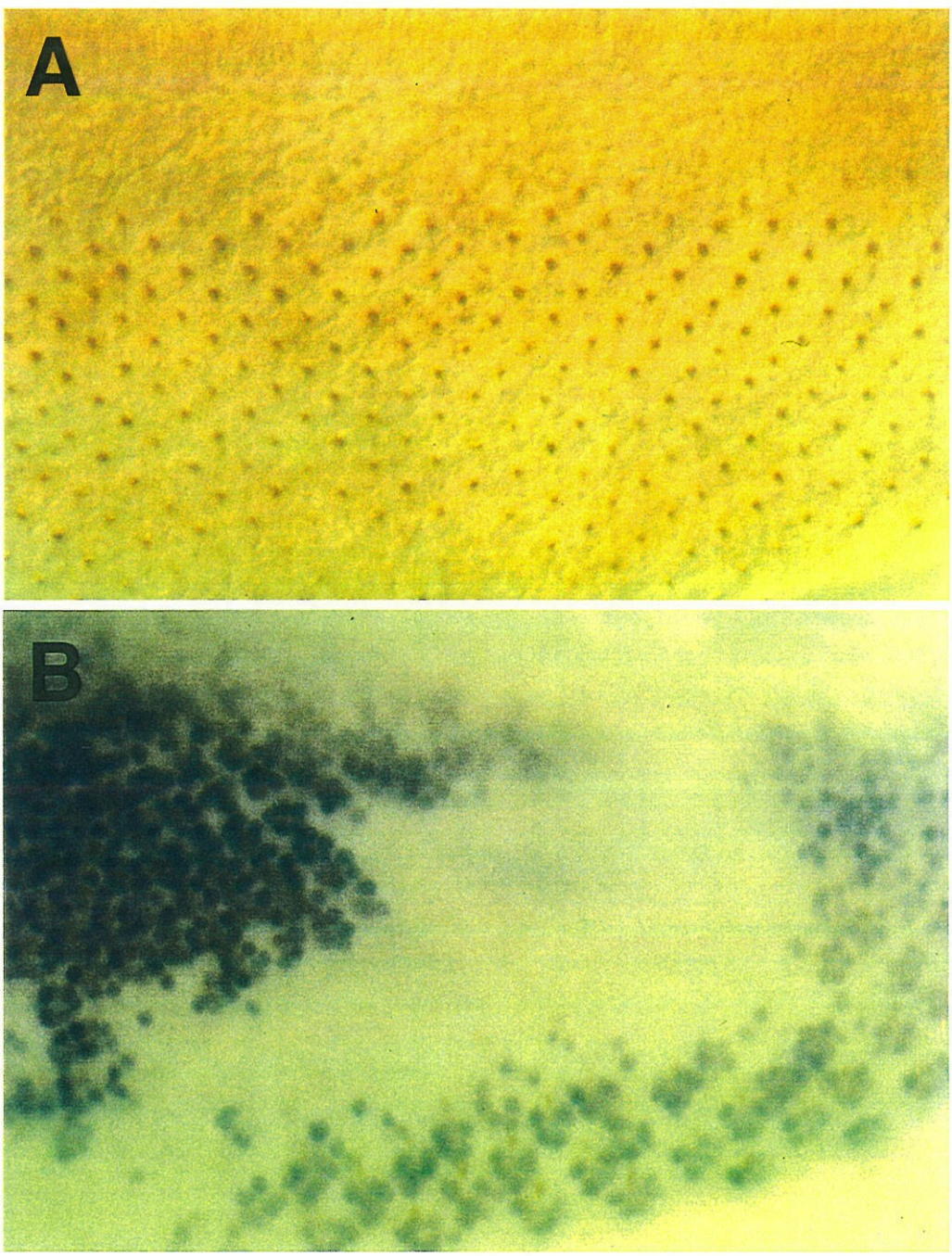
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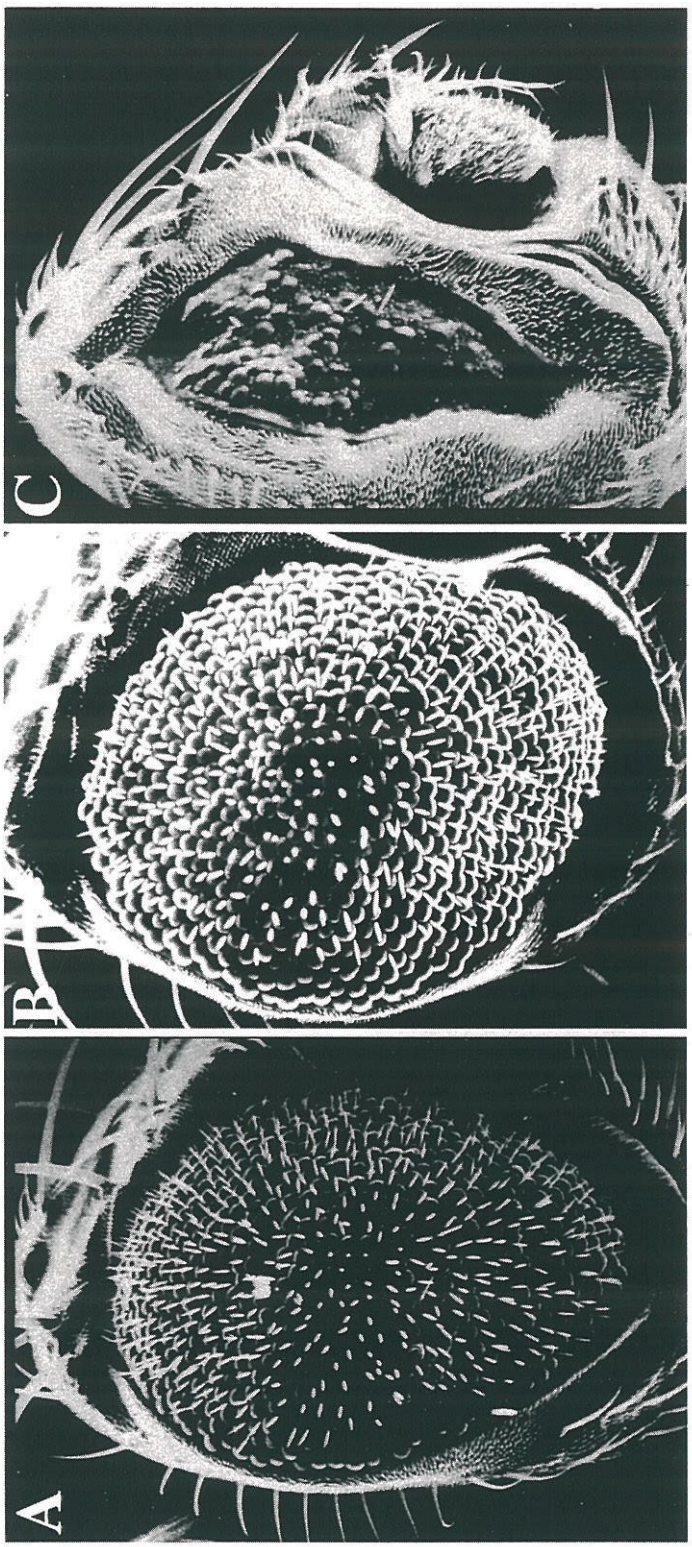
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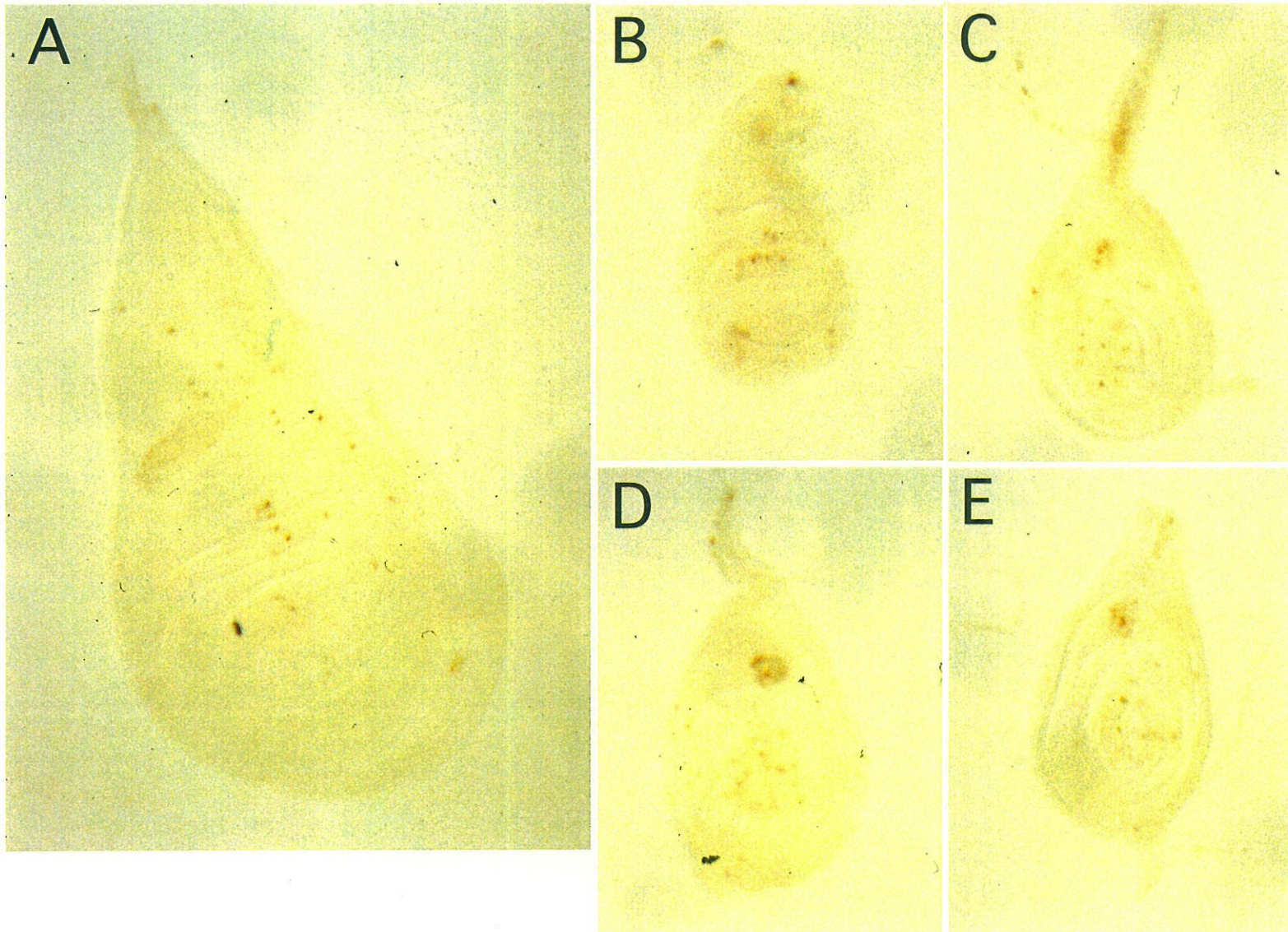
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