

Mis-specified cells die by an active gene-directed process, and inhibition of this death results in cell fate transformation in *Drosophila*

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Summary

Incorrectly specified or mis-specified cells often undergo cell death or are transformed to adopt a different cell fate during development. The underlying cause for this distinction is largely unknown. In many developmental mutants in *Drosophila*, large numbers of mis-specified cells die synchronously, providing a convenient model for analysis of this phenomenon. The maternal mutant *bicoid* is particularly useful model with which to address this issue because its mutant phenotype is a combination of both transformation of tissue (acron to telson) and cell death in the presumptive head and thorax regions. We show that a subset of these mis-specified cells die through an active gene-directed process involving transcriptional upregulation of the cell death inducer *hid*. Upregulation of *hid* also occurs in *oskar* mutants and other segmentation

mutants. In *hid bicoid* double mutants, mis-specified cells in the presumptive head and thorax survive and continue to develop, but they are transformed to adopt a different cell fate. We provide evidence that the terminal *torso* signaling pathway protects the mis-specified telson tissue in *bicoid* mutants from *hid*-induced cell death, whereas mis-specified cells in the head and thorax die, presumably because equivalent survival signals are lacking. These data support a model whereby mis-specification can be tolerated if a survival pathway is provided, resulting in cellular transformation.

Key words: Mis-specification, Cell death, Transformation, *Bicoid*, *Oskar*, *Hid*, *Drosophila*

Introduction

Programmed cell death or apoptosis is an important component of normal development and tissue homeostasis. It is required to remove cells that are no longer needed, cells that have been produced in excess and cells that have sustained genetic damage (Baehrecke, 2002). The molecular mechanisms that activate and execute the cell death program are conserved from worms to flies to mammals (reviewed by Danial and Korsmeyer, 2004). One common feature is the activation of caspases, a highly specialized class of cell death proteases. Produced as inactive zymogens, activation of caspases occurs through proteolytic cleavage, leading to cell death (Salvesen and Abrams, 2004). In *Drosophila*, the cell death-inducing genes *head involution defective* (*hid*; W – FlyBase), *reaper* and *grim* integrate a large number of cell death-inducing stimuli, culminating in activation of caspases (White et al., 1994; White et al., 1996; Grether et al., 1995; Chen et al., 1996). Caspases proteolytically cleave a large number of cellular proteins resulting in death and removal of the affected cell.

During abnormal development, cell death is also a contributing factor to the phenotypes of many mutants in *Drosophila*. This was first noted more than 35 years ago for mutants affecting imaginal development (Fristrom, 1968; Fristrom, 1969) (reviewed by Bonini and Fortini, 1999), and it was later also found in mutants affecting embryonic patterning, including maternal-effect genes, gap genes, pair-rule genes and segment-polarity genes (Lehmann et al., 1986; Tepass et al., 1994; Magrassi and Lawrence, 1988; Martinez-Arias and Ingham, 1985; Perrimon and Mahowald, 1987; Klingensmith et al., 1989; Bejsovec and Wieschaus, 1993; Pazdera et al., 1998; Hughes and Krause, 2001). In these mutants, entire regions of the developing organism are deleted (Nüsslein-Volhard and Wieschaus, 1980). The underlying cause for cell death in these mutants is unknown, but apparently cells are able to monitor their ability to specify and differentiate correctly. If a cell fails to complete its normal developmental program (from now on referred to as mis-specified cell), it undergoes cell death.

However, it is not always the case that mis-specified cells

die. Instead, a transformation to adopt a different cell fate has often been observed. For example, in embryos obtained from *bicoid* mutant females, the acron is transformed to become a telson (see below). Other examples include *sevenless* (*sev*) and *bride of sevenless* (*boss*) mutations, which cause a transformation of the R7 photoreceptor to a cone cell during eye development in the fly (Tomlinson and Ready, 1986; Reinke and Zipursky, 1988). The factors influencing the choice between cell death and cell fate transformation in various mutants are not well understood. To address this issue, we initiated an analysis of the cell death and transformation phenotypes of the maternal effect mutants *bicoid* and *oskar* during embryogenesis.

During *Drosophila* development, the wild-type embryo generates five distinct regions along the anteroposterior axis that are visible in the larval cuticle as acron, head, thorax, abdomen and telson (Fig. 1A,D) (Nüsslein-Volhard et al., 1987). The maternal effect mutants *bicoid* and *oskar* severely disrupt anteroposterior patterning. *bicoid* mutant females produce embryos (from now on referred to as *bicoid* mutants) that lack head and thorax, and a duplicated telson replaces the acron at the anterior tip of the embryo (Fig. 1B,E) (Frohnhofer and Nüsslein-Volhard, 1986; Frohnhofer and Nüsslein-Volhard, 1987). *oskar* mutant females produce embryos (referred to as *oskar* mutants) that lack the entire abdomen, with the telson intact (Fig. 1C,F) (Lehmann and Nüsslein-Volhard, 1986). Development of acron and telson is independent of *bicoid* and *oskar*, and requires the *torso* signaling pathway (Klingler et al., 1988; Schüpbach and Wieschaus, 1986). However, *bicoid* specifies acron versus telson at the anterior tip of the embryo (Fig. 1B) (Frohnhofer and Nüsslein-Volhard, 1986).

In wild-type, *bicoid*⁺ mRNA is maternally localized at the anterior tip of the embryo (Berleth et al., 1988). After fertilization, Bicoid protein forms an exponential concentration gradient along the anteroposterior axis with a maximum at the anterior tip, the source of its translation (Driever and Nüsslein-Volhard, 1988a) (reviewed by Ephrussi and St Johnston, 2004). Bicoid, which is a homeodomain-containing transcription factor, induces target gene expression in a dose-dependent manner, which is required for proper specification of head and thorax (Driever and Nüsslein-Volhard, 1988b; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). Thus, loss of Bicoid results in failure to provide this specification, and the mutant embryos do not develop head and thorax (Fig. 1B,E). Similarly, *oskar* mRNA is localized at the posterior tip of the embryo where it is required to localize the posterior determinant *nanos* (Ephrussi et al., 1991; Kim-Ha et al., 1991). In the absence of *oskar* function, posterior development is disturbed, and the entire abdomen fails to develop (Fig. 1C,F).

The mechanisms that cause loss of embryonic tissue in *bicoid* and *oskar* mutants are unclear. In previous studies, these mutants were examined from fertilization to gastrulation, when the wild-type functions of *bicoid* and *oskar* are required for proper specification of cell fates along the anteroposterior axis. Hence, little is known about the events after gastrulation, when the *bicoid* and *oskar* mutant phenotypes, which result in significant tissue loss, are established. In principle, loss of tissue could result from decreased cell proliferation or increased cell death. Because nuclear divisions and cellularization are normal in *bicoid* and *oskar* mutants

(Frohnhofer and Nüsslein-Volhard, 1986; Frohnhofer and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1986), defects in cell proliferation are unlikely to account for tissue loss observed in these mutants. Rather, increased cell death is an attractive mechanism to explain the *bicoid* and *oskar* mutant phenotypes.

Indeed, cell corpses in the abdomen of *oskar* mutants have been observed previously (Lehmann and Nüsslein-Volhard, 1986); however, the underlying cause has never been carefully examined. Here, we show that cell death is responsible for the loss of tissue in *bicoid* and *oskar* mutant embryos. Furthermore, our analysis implies that cellular mis-specification in these mutants triggers cell death through an active gene-directed pathway leading to expression of the cell death-inducing gene *hid*, resulting in caspase-dependent cell death. However, our data also show that if cell death is blocked either by removing *hid* or by providing a survival signaling pathway, mis-specification is tolerated and transformation can occur.

Materials and methods

Fly stocks and genetics

The following fly stocks were used: Canton S (as wild-type reference), *bicoid*^{E1}, *bicoid*^{E2}, *bicoid*^{GB}, *oskar*⁸⁸, *oskar*¹⁶⁶, *torso*¹, *torso*⁴, *hid*^{WR+X1}, *hid*^{H99} and *hid*^{A329}. The *hid*^{H99} *bicoid*^{E2} and *hid*^{A329} *oskar*⁸⁸ double mutants were obtained by meiotic recombination. *hid bicoid* double mutants were maternally mutant for *bicoid* and zygotically mutant for *hid*, and were obtained from females of the genotype *hid*^{H99} *bicoid*^{E2}/*bicoid*^{GB} mated to *hid*^{WR+X1}/+ males. *hid oskar* double mutants are maternally mutant for *oskar* and zygotically mutant for *hid*, and were obtained from *hid*^{A329} *oskar*⁸⁸/*oskar*¹⁶⁶ females mated to *hid*^{H99}/+ males. To inhibit caspases by P35 expression, females of the maternal genotype *UASp-P35*, *bicoid*^{E1}/*bicoid*^{GB} and *UASp-P35*, *oskar*⁸⁸/*oskar*¹⁶⁶ were crossed with *tub-GAL4* males.

For generation of *UASp-P35* transgenic flies, primers GAGCT-TGCGGCCGCAAAATGTGTGTAATTTTTCCGG and TTAGGC-TCTAGATTTTAACATTTATTTAATTGTG were used to amplify the P35-coding region by PCR. The PCR product was treated with *NotI* and *XbaI*, and cloned into *NotI/XbaI*-treated pUASp vector (Rørth, 1998). Transgenic flies were generated by P-element-mediated transformation.

Immunohistochemistry

TUNEL assays, immunohistochemistry, in situ hybridization and Acridine Orange labeling of whole-mount embryos were performed in accordance with standard procedures (Goyal et al., 2000; Patel, 1994; Tautz and Pfeifle, 1989; Abrams et al., 1993). The CM1 antibody as well as monoclonal antibodies against Dlg (4F3), Abd-B (1A2E9) and Antp (8C11) were used at 1:1,000, 1:300, 1:20 and 1:100 dilutions, respectively. For cuticle preparations, differentiated embryos were embedded in Hoyer's medium (van der Meer, 1977).

Results

Cell death and caspase activation in *bicoid* and *oskar* mutants

Reasoning that cell death might be an important contributor to the *bicoid* and *oskar* mutant phenotypes, we employed TUNEL as an assay to visualize cell death (Gavrieli et al., 1992). Compared with wild-type embryos, both *bicoid* and *oskar* mutants showed an increased number of cells undergoing TUNEL-positive cell death (Fig. 1G-I). In *bicoid* mutants, TUNEL labeling is elevated in two distinct stripes in the

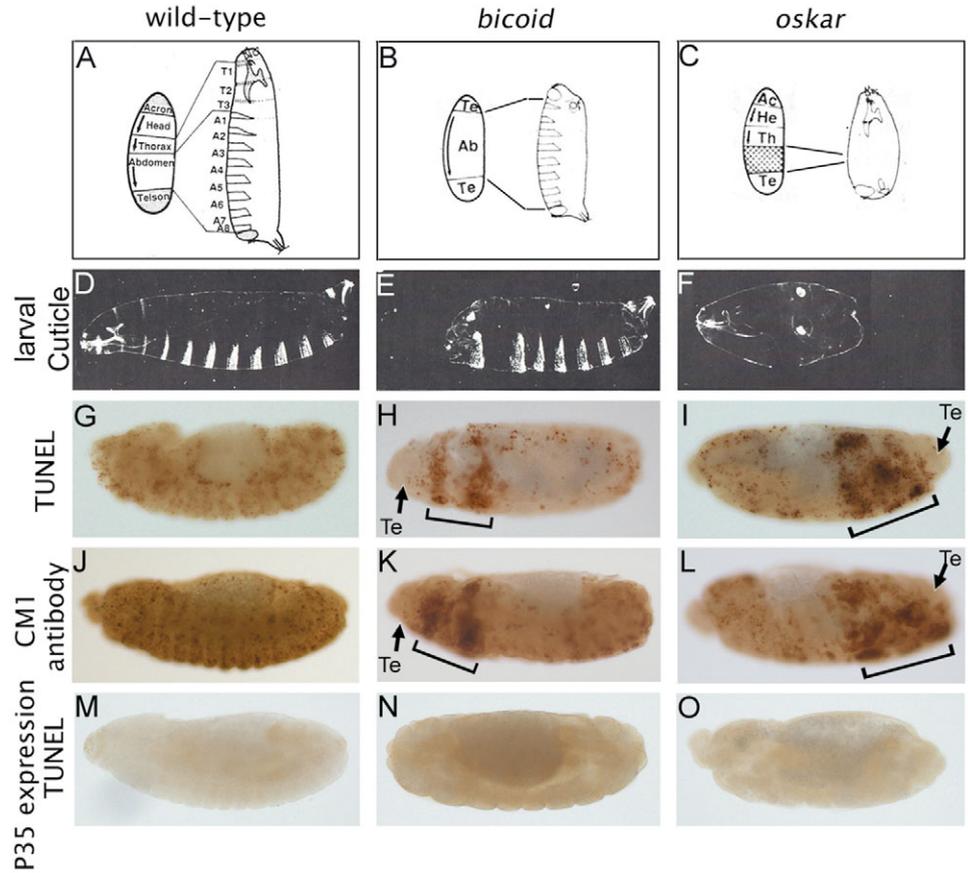
Fig. 1. Caspase-dependent cell death in *bicoid* and *oskar* mutants.

(A-C) Schematic illustration of the wild-type (A), *bicoid* (B) and *oskar* (C) phenotypes. In each panel, the embryonic fate maps are shown on the left, the differentiated larvae on the right. During development, wild-type embryos specify five distinct regions along the anteroposterior axis that are visible in the larval cuticle as Acron (Ac), Head (He), Thorax (Th), Abdomen (Ab) and Telson (Te).

Arrows indicate the polarity of the tissues. T1-3 and A1-8 denote thoracic and abdominal segments, respectively. In *bicoid* and *oskar* mutants, this pattern is severely affected and some of the regions are missing. In addition, in *bicoid* mutants, the anterior acron is transformed into a telson (B).

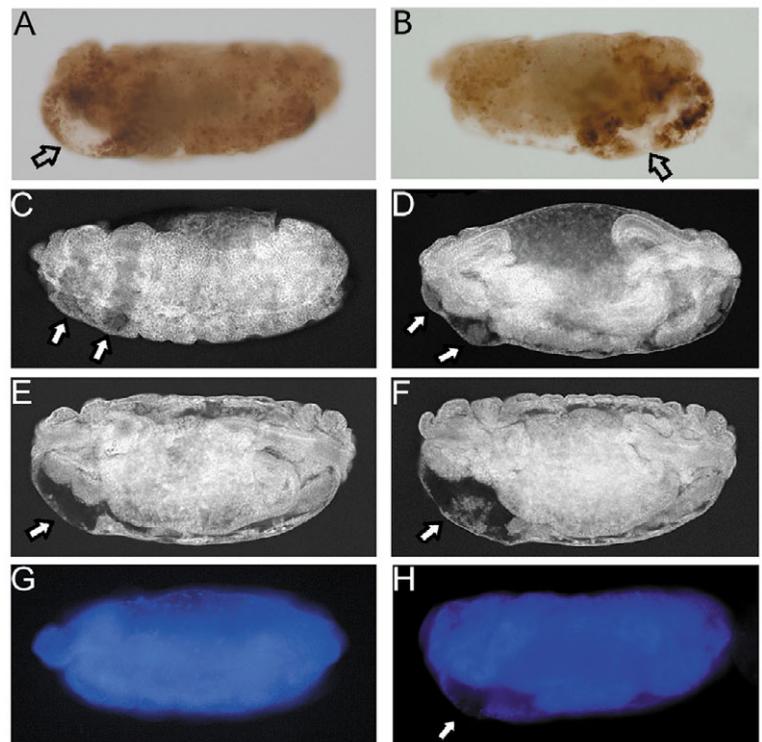
Modified, with permission, from Nüsslein-Volhard et al. (Nüsslein-Volhard et al., 1987). (D-F) Lateral views of larval cuticle preparations of wild-type (D), *bicoid* (E) and *oskar* (F) mutants. (G-I) Lateral views of TUNEL-labeled embryos of wild-type (G), *bicoid* (H) and *oskar* (I) mutants. (H,I) Brackets indicate areas of increased cell death; arrows indicate the presumptive telson (Te) areas, which are TUNEL negative.

(J-L) CM1 labeling to detect active DrICE in wild-type (J), *bicoid* (K) and *oskar* (L) mutants. Lateral views. (K,L) Brackets highlight areas of increased caspase activation; arrows indicate the presumptive telson (Te) areas, which lack caspase activation. (M-O) Expression of the caspase inhibitor P35 blocks TUNEL-positive cell death in wild-type (M), *bicoid* (N) and *oskar* (O) mutants.



presumptive head and thorax region of the embryos, whereas almost the entire posterior half of *oskar* mutants is strongly TUNEL positive. These TUNEL-positive regions correspond to the missing tissues of the larvae (Fig. 1E,F). It is not clear why TUNEL-labeling in *bicoid* embryos starts out in two distinct stripes. However, later in development these stripes become wider and eventually fuse (see Fig. 2). Similar results were obtained by labeling embryos with Acridine Orange, an alternative method to labeling dying cells (see Fig. 4A). The presumptive telson regions in *bicoid* and *oskar* embryos are TUNEL negative (arrows in Fig. 1H,I). However, in *torso*

Fig. 2. Development of the areas of clearance. (A,B) CM1 labeling of stage 15 *bicoid* (A) and *oskar* (B) mutants. Open arrows indicate areas of clearance. (C-F) Confocal images of lateral views of stage 14 (C), stage 15 (D), stage 16 (E) and stage 17 (F) *bicoid* mutants stained with anti-Discs large (Dlg) antibody to visualize cell outline. Clearance of tissue is initially detectable in two distinct zones (see arrows in C and D), but becomes broader over time, fuses (E) and enlarges (F). Similar results were obtained for *oskar* (not shown). (G,H) DAPI staining of wild-type (G) and *bicoid* (H) mutants to visualize chromosomal DNA. The areas of clearance (arrow in H) do not contain DNA.



mutants, acron and telson tissues also undergo TUNEL-positive cell death (data not shown).

In wild-type embryos, normal developmental cell death starts at embryonic stage 11 (~7 hours) (Abrams et al., 1993). However, TUNEL-positive cell death in *bicoid* and *oskar* mutants is already detectable at stage 10, about 1 hour before the onset of normal cell death in wild-type embryos, and remains detectable in the affected regions throughout development until shortening of the mutant embryos is visible (data not shown). These results suggest that cell death contributes to the loss of tissue in *bicoid* and *oskar* mutants.

We analyzed whether the increased cell death observed in *bicoid* and *oskar* mutants is caspase dependent. Cleavage and activation of caspases was determined immunohistochemically using the CM1 antibody (Srinivasan et al., 1998), which recognizes the active *Drosophila* caspase DrICE (Yu et al., 2002). Compared with wild-type embryos, increased CM1 labeling is detectable in *bicoid* and *oskar* mutants (Fig. 1J-L) in the regions that were identified by increased TUNEL labeling (Fig. 1H,I). The telson areas are spared from caspase activation. Similar to the onset of TUNEL-positive cell death, CM1 labeling is first detectable at stage 10, suggesting a direct correlation between caspase activation and cell death in these domains. Next, we analyzed whether caspase activation is required for increased cell death in *bicoid* and *oskar* mutants. The caspase inhibitor P35 has been shown to inhibit developmental cell death in *Drosophila* embryos (Fig. 1M) (Hay et al., 1993). Thus, we expressed P35 in *bicoid* and *oskar* mutants. TUNEL-positive cell death is strongly reduced in these embryos (Fig. 1N,O), suggesting that cell death in *bicoid* and *oskar* mutants is dependent on caspase activation.

Cell death causes clearance of tissue in *bicoid* and *oskar* mutants

CM1 labeling is present in the affected regions of the embryos until they are shortened. We detected an interesting intermediate phenotype in *bicoid* and *oskar* mutants, beginning at stage 14. Labeling with the CM1 antibody revealed large areas with no staining signal surrounded by immunopositive tissue (Fig. 2A,B, arrows). These areas also fail to give signals with antibodies recognizing ubiquitously expressed proteins such as Tubulin and Dronc, another *Drosophila* caspase (data not shown). We refer to these areas as 'areas of clearance'.

Areas of clearance are not observed in wild-type embryos and in P35-expressing embryos (Fig. 1N,O), suggesting that they develop as a direct consequence of cell death in *bicoid* and *oskar* embryos. Hence, we characterized them in a time course using an antibody against the ubiquitously expressed membrane-associated Discs large (Dlg) protein (Parnas et al., 2001). Initially, in stage 14 *bicoid* embryos, beginning of clearance is visible in two distinct zones similar to TUNEL labeling and caspase activation (Fig. 2C). However, the two zones fuse over the next few stages and the areas of clearance enlarge significantly (Fig. 2D-F). Later these areas collapse, and the final phenotype of *bicoid* mutants is established. Similar data were obtained for *oskar* mutants (data not shown). These areas also do not contain DNA (Fig. 2G,H). Thus, DNA and proteins are cleared as a result of the apoptotic events in these tissues. Moreover, because Dlg is membrane associated, these data indicate that de-cellularization, the end result of the apoptotic process, has occurred. In Fig. 5 and Fig. S2 (see

supplementary material), we are using the areas of clearance as markers of cell death in response to developmental mis-specification.

Expression of *hid* in *bicoid* and *oskar* embryos

During *Drosophila* embryogenesis, the genes *reaper*, *hid* and *grim* are essential for cell death through activation of caspases (White et al., 1994; White et al., 1996; Grether et al., 1995; Chen et al., 1996). We determined whether they are involved in the cell death response of *bicoid* and *oskar* mutants. Compared with wild-type embryos, *hid* expression is significantly elevated in those parts of *bicoid* and *oskar* embryos that showed high levels of TUNEL-positive cell death and activated DrICE (Fig. 3A-C). *hid* expression is not detectable in the presumptive telson regions. Expression of *reaper* and *grim* was not upregulated (data not shown). The increased expression of *hid* is first visible in stage 9 embryos, preceding cell death by ~1-2 hours, suggesting that transcriptional induction of *hid* triggers cell death in *bicoid* and *oskar* mutants. Expression of the caspase inhibitor P35 did not affect expression of *hid* (data not shown), demonstrating that caspase activation occurs downstream of *hid*. This is consistent with the proposed role of *hid* as caspase activator (Grether et al., 1995). Interestingly, *hid* expression in the affected parts of *bicoid* and *oskar* mutants is maintained for less than 2 hours, and then downregulated.

To determine whether cell death in *bicoid* mutants requires *hid*, embryos double mutant for *hid* and *bicoid* (see Materials and methods) were analyzed by TUNEL and Acridine Orange labeling. Cell death was not detectable in stage 10 *hid bicoid* double mutant embryos (Fig. 4B,D), suggesting that *hid* is genetically required for increased cell death in *bicoid* embryos. In summary, this analysis highlights the fact that cells in the affected regions of *bicoid* and *oskar* mutants do not simply die by a passive mechanism because of a lack of appropriate developmental information. Instead, these cells induce a

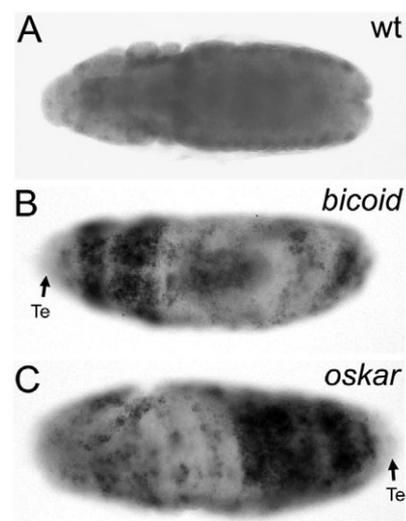


Fig. 3. Expression of *hid* in *bicoid* and *oskar* mutants. Ventral views of stage 9 wild-type (A), *bicoid* (B) and *oskar* (C) mutant embryos, labeled for expression of *hid* by in situ hybridization. The arrows in B,C indicate the presumptive telsons (Te), which lack *hid* expression.

transcriptional response leading to expression of *hid*. Thus, they die by an active gene-directed process.

The ability to block cell death in *hid bicoid* double mutants allowed us to address the fates of cells that normally undergo cell death in *bicoid* single mutants. Cuticle preparations of *hid bicoid* double mutant larvae revealed that the patterning defect (loss of head and thorax, and transformation of acron into telson, see Fig. 1B) is not rescued, as expected, because the wild-type function of *bicoid* is still missing (Fig. 4F). However, the anterior-most abdominal segment is considerably expanded in size, whereas the remaining abdominal segments are of normal size (Fig. 4F, see enlargement in Fig. 4H). This expansion is not present in either *bicoid* (Fig. 4E,G) or *hid* single mutants (data not shown). Hence, we conclude that this expansion is derived from cells which would normally die in *bicoid* mutants. Significantly, the rescued cells secrete cuticle elements, suggesting that they are terminally differentiated. Similarly, it has also been observed in *C. elegans* that cells that are programmed to die are able to differentiate if cell death is blocked (Avery and Horvitz, 1988).

Rescued mis-specified cells in *hid bicoid* double mutants have posterior identity

It has previously been proposed that mis-specified cells have at least two options: they either die or they survive and are transformed to adopt a different fate (reviewed by Bonini and Fortini, 1999). The underlying cause for this distinction is unclear. The *bicoid* mutant phenotype combines both transformation of tissue (acron to telson, Fig. 1B) and cell death in the presumptive head and thorax region (Fig. 1H). Thus, we asked why some mis-specified cells in *bicoid* mutants die, while others survive. To address this, we closely examined

the identity of the expanded first abdominal segment in *hid bicoid* double mutants. Interestingly, the polarity of the denticles and the width of the segment resemble segment A8, the posterior-most segment (Fig. 1A), suggesting that anterior tissue may have been transformed toward a posterior identity in *bicoid* mutants. As mentioned above, transformation of acron into telson in *bicoid* mutants has previously been reported (Frohnhofer and Nüsslein-Volhard, 1986) (Fig. 1B). However, the *hid bicoid* double mutant analysis revealed that the transformation of anterior tissue into posterior identity expands beyond the telson, and that this expansion undergoes *hid*-induced cell death in *bicoid* single mutants.

To verify this interpretation, we analyzed the posterior identity of anterior tissue in more detail using an antibody raised against the Abdominal-B (Abd-B) protein as posterior marker. In wild-type embryos, Abd-B protein is present in the posterior part of the embryo (Fig. 5A,C) (Celniker et al., 1989). However, in *bicoid* mutants it is found in both posterior and anterior poles of the embryo (Fig. 5B,D), consistent with our morphological observation. Thus, in *bicoid* mutants, anterior cells are incorrectly specified because they receive a developmental signal that in wild-type embryos is present only posteriorly.

To determine whether mis-specified cells induce *hid* expression and cell death in *bicoid* mutants, we analyzed Abd-B protein distribution in wild-type, *bicoid* single and *hid bicoid* double mutant embryos. In stage 15 wild-type embryos, Abd-B protein is detectable in two populations of cells at the posterior pole, a dorsally located component that gives rise to the telson (Fig. 5C, bracket), and a ventrally located component which specifies segment A8 (Fig. 5C, red arrow). In *bicoid* mutants, Abd-B protein distribution is mirror-imaged at the anterior pole (Fig. 5D). Significantly, however, part of the *Abd-B*-expressing ventral tissue in *bicoid* mutants is cleared as a consequence of cell death (Fig. 5D, open arrow; compare with Fig. 2). Consistently, in *hid bicoid* double mutants, tissue clearance does not occur and Abd-B protein persists (Fig. 5E, red arrow). Thus, the mis-specified ventral tissue expressing *Abd-B* survives in the double mutant. Similar data were obtained using a different abdominal marker, *abd-A* (see Fig. S1 in the supplementary material). Taken together, these findings suggest that mis-specified cells in *bicoid* mutants can induce *hid* expression and undergo cell death. Furthermore, the red arrows in Fig. 5C-E indicate the highest expression levels of *Abd-B* specifying abdominal segment A8 in wild-type, *bicoid* mutants and *hid bicoid* double mutants. Thus, the rescued cells in *hid bicoid* double mutants experience highest levels of

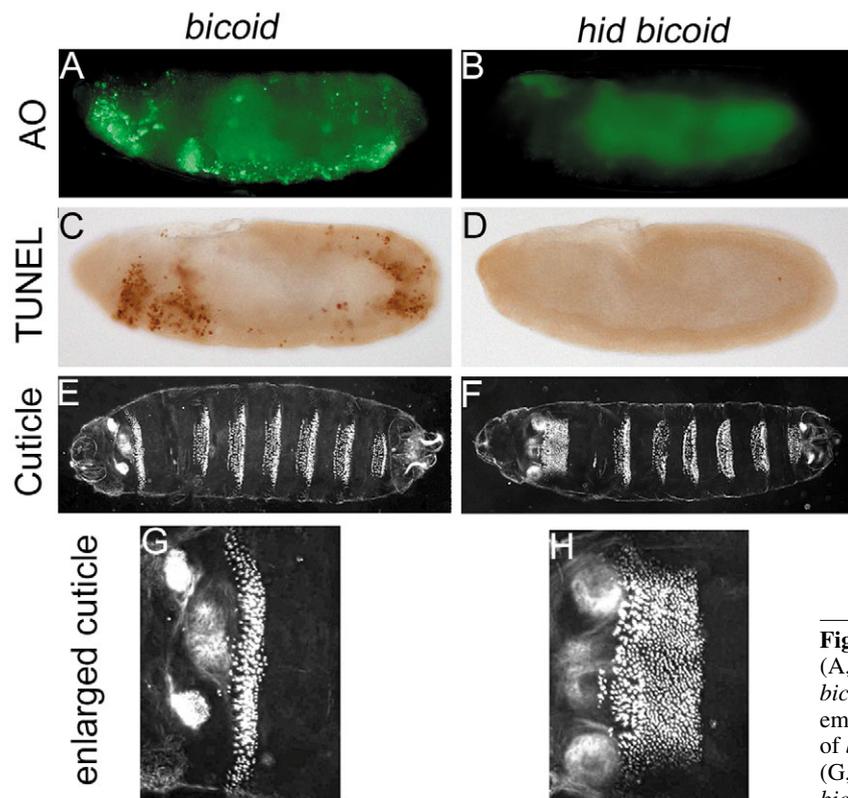


Fig. 4. *hid bicoid* double mutant analysis. Acridine Orange (A,B) and TUNEL (C,D) labeling of dying cells in stage 10 *bicoid* mutant (A,C) and *hid bicoid* double mutant (B,D) embryos. (E,F) Ventral views of larval cuticle preparations of *bicoid* (E) and *hid bicoid* (F) mutants. (G,H) Enlargements of the first abdominal segment of *bicoid* (E) and *hid bicoid* (F) mutants, respectively.

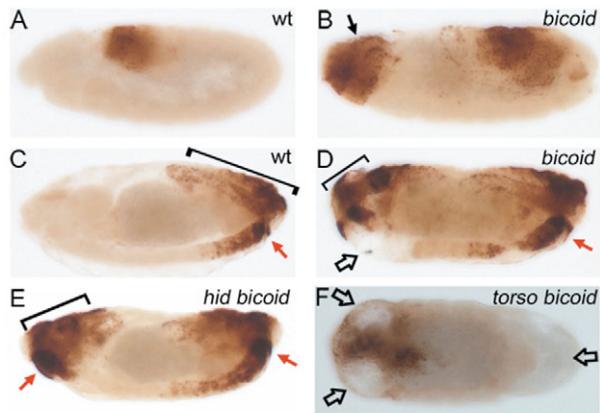


Fig. 5. Abd-B distribution in wild-type, *bicoid*, *hid bicoid* and *torso;bicoid* embryos. Lateral views of stage 8 (A,B) and stage 15 (C-F) wild-type (A,C), *bicoid* (B,D), *hid bicoid* (E) and *torso;bicoid* (F) mutants labeled with anti-Abd-B antibodies. Wild-type embryos (A,C) contain Abd-B protein in the posterior region only. In *bicoid* mutant embryos (B,D), Abd-B protein is present at the posterior and at the anterior pole (see arrow in B). The brackets in C-E indicate the dorsally located component of Abd-B expression that develop into telson structures. Cell death clears part of the mis-specified area at the anterior (open arrow in D). Tissue clearance does not occur in *hid bicoid* double mutants (E). The red arrows in C-E indicate the relative position that specifies A8 segments in wild-type (C), *bicoid* (D) and *hid bicoid* (E) mutants. In *torso;bicoid* double mutants (F), the dorsally located component of anterior Abd B expression as well as the ventral and the posterior component are cleared (open arrows in F).

Abd-B and develop into a segment with A8 identity (Fig. 4F). We also determined that cell death in *oskar* mutants largely affects mis-specified tissue, and that *hid oskar* double mutants rescue this tissue (see Fig. S2 in the supplementary material). Thus, similar to *bicoid*, mis-specified cells in *oskar* mutants induce *hid* to undergo cell death.

In *torso;bicoid* double mutants all Abd-B-expressing tissue is removed

From the analysis presented above, it is also clear that not all mis-specified cells at the anterior of *bicoid* mutants undergo cell death. The tissue containing the dorsally located component of Abd-B (bracket in Fig. 5D) is spared from tissue clearance, and thus cell death. This tissue undergoes transformation from acron to telson in *bicoid* mutants (Fig. 1B), for which it requires *torso* signaling (Nüsslein-Volhard et al., 1987; Frohnhöfer and Nüsslein-Volhard, 1986; Klingler et al., 1988; Schüpbach and Wieschaus, 1986). To determine whether *torso* signaling protects this mis-specified tissue from cell death, we performed a double mutant analysis. In stage 15 *torso;bicoid* double mutants, areas of clearance are detectable in both the ventral and dorsal components of Abd-B-expressing tissue at the mis-specified anterior tip, as well as at the posterior tip, which is also affected in *torso* mutants (Fig. 5F) (Klingler et al., 1988; Schüpbach and Wieschaus, 1986). Consistently, expression of *hid* is also upregulated in the presumptive acron and telson regions of *torso* mutants (data not shown). In summary, this analysis suggests that *torso*⁺ protects the mis-specified dorsal component at the anterior tip

of *bicoid* embryos from *hid*-induced cell death, providing an explanation of why these cells survive. This analysis suggests that if an alternative survival signaling pathway is provided, such as *torso*⁺ in this example, mis-specification is tolerated and transformation can occur (see Discussion). Thus, the posterior to anterior transformation in *bicoid* embryos affects cells differently with respect to survival depending on their relative location. The telson survives because the *torso* signaling pathway is intact in *bicoid* embryos. However, the anterior tissue with posterior identity next to the telson undergoes cell death in *bicoid* embryos suggesting that it lacks an ‘anterior’ survival signal or is otherwise unable to inhibit expression of *hid*.

Expression of *hid* in segmentation mutants

Because *hid* is transcriptionally upregulated in dying mis-specified cells of *bicoid* and *oskar* mutants, we determined whether induction of *hid* expression represents a broader mechanism that also applies to mis-specified cells in other developmental mutants. We analyzed mutants of gap genes [*knirps* (*kni*)], pair-rule genes [*odd-skipped* (*odd*)] and segment-polarity genes [*wingless* (*wg*)]. These mutants are characterized by loss of tissue (Nüsslein-Volhard and Wieschaus, 1980), and ectopic cell death has been reported for these mutants (Tepass et al., 1994; Perrimon and Mahowald, 1987; Klingensmith et al., 1989; Bejsovec and Wieschaus, 1993; Pazdera et al., 1998; Hughes and Krause, 2001). As in the case of *bicoid* and *oskar* mutants, *hid* expression is upregulated during stage 9 of embryogenesis in the regions of the mutant embryos that are later deleted in the larvae (Fig. 6). This is most evident for *odd*, which lacks every other segment in the larvae. Correspondingly, we detected upregulation of *hid* in every other segment (Fig. 6C, arrowheads). In *kni* mutants, upregulation of *hid* is detectable in the posterior part of the embryo (Fig. 6B), where *kni*⁺ function is required (Nauber et al., 1988; Pankratz et al., 1992). In *wg* mutants, *hid* expression is detectable in every segment (Fig. 6D), consistent with the notion that the segment polarity phenotype is the result of regional mis-specification and subsequently cell death (Klingensmith et al., 1989; Perrimon and Mahowald, 1987). Similar data were also obtained for additional mutants, including *hunchback*, *Krüppel*, *fushi-tarazu*, *hedgehog* and *engrailed* (data not shown). Thus, these data support the notion that upregulation of *hid* in mis-specified cells is a common feature in many developmental mutants.

Discussion

The events leading to cell death and tissue loss in response to mis-specification in *bicoid* and *oskar* mutants are summarized in Fig. 7. These mutants do not contain the correct developmental information for proper specification of the anterior and posterior parts of the embryos, respectively. Subsequently, the affected cells induce a transcriptional response leading to *hid* expression, which triggers caspase activation and cell death. Finally, clearance of the dying tissue occurs, and the embryos become visibly shortened.

Interestingly, it takes approximately 6 hours from caspase activation (stage 10; 7 hours of age) to the onset of cellular clearance (stage 14; 13 hours of age). During this time, the contents of the affected cells, including protein and DNA, are

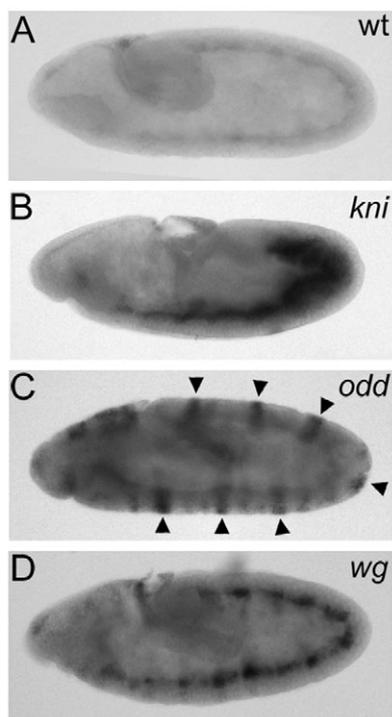


Fig. 6. *hid* expression in segmentation mutants. Lateral views of stage 9 wild-type (A), *kni* (B), *odd* (C) and *wg* (D) embryos. Arrowheads in C indicate the pair-rule expression of *hid* in *odd* mutants.

completely degraded and the cells are removed. This analysis highlights that developmental cell death occurs very rapidly, and that the *bicoid* and *oskar* mutants provide a convenient model with which to analyze the individual events of cell death with high temporal and spatial resolution because many cells die almost synchronously in these mutants.

Mis-specified cells in patterning mutants induce *hid* expression

Although we largely focus our analysis on the maternal effect mutants *bicoid* and *oskar*, it is likely that the principles we have uncovered are of broader significance. Segmentation mutants acting downstream of *bicoid* and *oskar*, including mutants of gap genes (*Krüppel*, *knirps*), pair-rule genes (*odd*, *fushi-tarazu*) and segment polarity genes (*wg*, *hedgehog*, *engrailed*) induce expression of *hid* (Fig. 6). These mutants are characterized by loss of larval tissue (Nüsslein-Volhard and Wieschaus, 1980), and for some of them cell death has been documented (Tepass et al., 1994; Perrimon and Mahowald, 1987; Magrassi and Lawrence, 1988; Klingensmith et al., 1989; Bejsovec and Wieschaus, 1993; Pazdera et al., 1998; Hughes and Krause, 2001). As in the case

of *bicoid* and *oskar*, *hid* expression is upregulated during stage 9 of embryogenesis in the regions of the mutant embryos that are later deleted in the larvae. In addition, *hid* mutants rescue the cuticle phenotype of *armadillo* mutants (Cox et al., 2000). Finally, we also found *hid* expression accompanied by TUNEL-positive cell death in *dorsal* and *Toll^{10b}* mutants which cause dorsalizing and ventralizing phenotypes, respectively, along the dorsoventral axis of *Drosophila* embryos (data not shown). Thus, these data support the notion that upregulation of *hid* appears to be a common trigger for a caspase-dependent cell death program in mis-specified cells of patterning mutants.

Furthermore, mutations affecting imaginal disc development result in loss of the adult appendage due to inappropriate cell death (Fristrom, 1968; Fristrom, 1969) (reviewed by Bonini and Fortini, 1999). We are currently determining whether these mutants also require *hid* expression to develop the final phenotypes. Moreover, many gene disruptions in mice result in inappropriate cell death in the tissue that requires the function of the disrupted gene (Rossel and Capecchi, 1999; McKay et al., 1994; Swiatek and Gridley, 1993), suggesting that similar mechanisms might exist in mammalian development. Finally, cell death may be an important contributing factor to human congenital birth defects. Thus, an understanding of the underlying mechanisms is of general interest.

Interestingly, not all segment polarity mutants analyzed induce *hid* expression and cell death. Embryos mutant for *patched*, which encodes the *hedgehog* receptor, were not found to express *hid* and do not contain increased cell death (data not shown), although *hedgehog* mutants both upregulate *hid* and contain increased amounts of cell death. The reasons for these differences are not known, but partial redundancy might account for lack of *hid* expression in *patched* mutants. The *Drosophila* genome encodes another *patched* homolog, *patched-related*, which might provide the survival requirement for mis-specified cells in *patched* mutants.

Mis-specified cells in *bicoid* and *oskar* mutants induce expression of *hid*. We did not observe increased *reaper* or *grim* expression in these mutants. However, expression of *reaper* has been reported in *crumbs* mutants, which affect epithelial integrity (Nordstrom et al., 1996). X-ray-treated embryos also preferentially respond by upregulation of *reaper* (Nordstrom et al., 1996; Brodsky et al., 2000; Ollmann et al., 2000), rather than *hid* (M. E. Grether, PhD thesis, MIT, 1994). Although we have not analyzed *crumbs* mutants for *hid* expression, it appears that cells contain several developmental checkpoints, which activate different cell death-inducing regulators depending on the type of abnormal cellular development.

Cell death versus cell fate transformation

Mis-specified cells can survive if an alternative survival pathway is provided. The example presented here is the acron into telson transformation in *bicoid* mutants, which is mediated

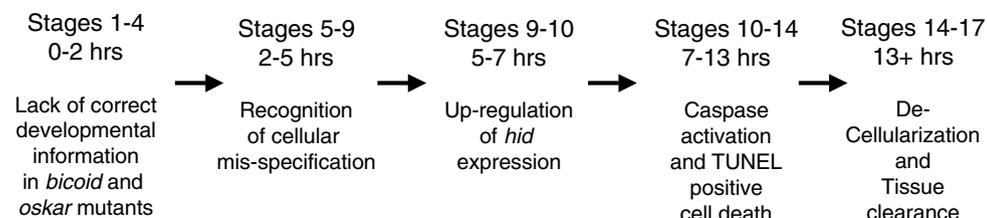


Fig. 7. Time course of the establishment of the *bicoid* and *oskar* mutant phenotypes. The developmental stages and approximate age of the embryos are indicated. The events that occur as development proceeds are summarized. See text for details.

by the *torso* signaling pathway. Although the cells giving rise to telson structures at the anterior tip are mis-specified based on Abd-B-labeling experiments, they survive because they receive a survival signal from the *torso* signaling system. In this case, transformation rather than cell death is favored. It has previously been shown that activation of the Ras/Mapk pathway protects cells from *hid*-induced apoptosis, both by transcriptional repression of *hid* (Kurada and White, 1998) and by phosphorylation of Hid protein by Mapk (Bergmann et al., 1998). Because Torso, which encodes a receptor tyrosine kinase (RTK) (Sprenger et al., 1989), is known to activate Ras and Mapk (Ghiglione et al., 1999; Gabay et al., 1997), we tested whether manipulation of active Mapk levels using a gain-of-function allele, *Mapk^{Sem}*, can suppress *hid* expression and cell death in *bicoid* mutants. However, this was found not to be the case (data not shown). Thus, *torso* appears to protect mis-specified cells independently of Mapk activation.

The *hid bicoid* double mutant analysis reveals that the transformation of anterior into posterior identity expands beyond the telson, and that this expansion undergoes *hid*-induced cell death in *bicoid* single mutants. The rescued cells secrete larval cuticle elements, suggesting that mis-specified cells have the developmental capacity to terminally differentiate. However, in *hid⁺* background, they instead die, presumably because equivalent survival signals are lacking. We propose that mis-specified cells undergo cell death if no alternative survival pathway is provided to protect them.

An alternative survival mechanism might also operate in other developmental mutants where transformation rather than cell death occurs. Mutations in the *sev* RTK and its ligand *boss* result in transformation of the R7 photoreceptor cell into a non-neuronal cone cell (Tomlinson and Ready, 1986; Reinke and Zipursky, 1988). Survival of this cell could be mediated by the *Drosophila* Egf receptor (Egfr), another RTK, which is required to maintain cell survival in the developing eye disk (Baker and Yu, 2001). Accordingly, activation of the Ras/Mapk pathway by Egfr would inhibit *hid* expression and support survival of the presumptive R7 photoreceptor cell. This interpretation is also consistent with observations that *egfr⁻* clones are small and undergo cell death (Diaz-Benjumea and Garcia-Bellido, 1990; Xu and Rubin, 1993), and that this death can be suppressed in *hid* mutants (Yu et al., 2002). Thus, transformation of the R7 photoreceptor to a cone cell rather than R7 cell death in *sev* and *boss* mutants could occur because of survival signaling by the Egfr.

Why do mis-specified cells die?

The *hid bicoid* double mutant analysis suggests that mis-specified cells can continue to develop and differentiate. Yet, they die. Presumably, this cell death protects the organism from potentially dangerous cells. For example, it is conceivable that in mammals, surviving mis-specified cells might lie dormant in the host organism for years. During this time, they might acquire additional genetic alterations that could drive the progressive transformation of these cells into malignant cancer. In wild-type embryos, mis-specification probably occurs in cells in isolation, and elimination of these cells does not interfere with development and survival of the organism. Only in extreme situations, such as the patterning mutants analyzed here, is the mis-specification caused by aberrant development so severe that the affected organism dies.

What is the nature of the mechanism that recognizes mis-specified cells?

The cause of mis-specification in each segmentation mutant is different. Usually, the expression of other segmentation genes is shifted and expanded, resulting in flattened gradients (e.g. Rivera-Pomar and Jäckle, 1996; Mullen and DiNardo, 1995). Yet, irrespective of the cause of mis-specification, most of these mutants have in common that they induce *hid* expression. It is currently unknown how the mis-specified fate of cells is recognized, and how *hid* expression is induced. One possibility might be that the protein gradients established by *bicoid⁺* and *oskar⁺*, as well as other segmentation genes (Driever and Nüsslein-Volhard, 1988a; Ephrussi et al., 1991; Dahanukar and Wharton, 1996; Rivera-Pomar and Jäckle, 1996; Mullen and DiNardo, 1995) are used as readout for proper cellular specification. The steepness of protein gradients as a means to determine life or death decisions has recently been proposed (Moreno and Basler, 2004; de la Cova et al., 2004). Such a model would imply that cells are able to determine their position in a graded field and compare this readout with their neighbors. Because in *bicoid* and *oskar* mutants these gradients do not form, the concentration difference between neighboring cells would be zero. If the concentration difference between two neighboring cells is below a crucial threshold, they induce the expression of *hid* and undergo cell death. This model could also explain embryonic pattern repair, which was described in embryos that express six copies of the *bicoid* gene (Namba et al., 1997). In these embryos, the head and thorax primordia are expanded because of the presence of six copies of *bicoid*. However, this expansion is corrected for by induction of cell death, and relatively normal larvae develop (Namba et al., 1997). In this case, the Bicoid protein gradient does form, but would be flatter compared with wild type. Thus, the concentration difference between neighboring cells would be below a critical threshold, sufficient to induce *hid*-dependent cell death. However, it is largely unknown how cells compare their position in a graded field with those of their neighbors. It has been proposed that short-range cell interactions mediated via the cell-surface proteins Capricious and Tartan provide cues that support cell survival during wing development (Milan et al., 2002). Cells unable to participate in these interactions are eliminated by cell death. It is unclear, however, whether short-range interactions are sufficient to explain the cell death phenotype in *bicoid* and *oskar* mutants.

Irrespective of the underlying mechanism for sensing mis-specification, our results highlight the role of an active gene-directed process that removes mis-specified cells during development. However, if a survival mechanism is provided, mis-specified cells can survive and adopt a different fate. In wild-type embryos, mis-specification probably occurs in cells in isolation, and hence is difficult to study. However, in *bicoid* and *oskar* mutants, large regions of neighboring cells are mis-specified and undergo cell death simultaneously, providing a unique opportunity to clarify the signals that initiate cell death in situations where cells are developmentally mis-specified.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/24/5343/DC1>

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