Binary cell death decision regulated by unequal partitioning of Numb at mitosis

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SUMMARY

An important issue in Metazoan development is to understand the mechanisms that lead to stereotyped patterns of programmed cell death. In particular, cells programmed to die may arise from asymmetric cell divisions. The mechanisms underlying such binary cell death decisions are unknown. We describe here a *Drosophila* sensory organ lineage that generates a single multidentritic neuron in the embryo. This lineage involves two asymmetric divisions. Following each division, one of the two daughter cells expresses the pro-apoptotic genes *reaper* and *grim* and subsequently dies. The protein Numb appears to be specifically inherited by the daughter cell that does not die. Numb is necessary and sufficient to prevent apoptosis in this lineage. Conversely, activated Notch is sufficient to trigger death in this lineage. These results show that binary cell death decision can be regulated by the unequal segregation of Numb at mitosis. Our study also indicates that regulation of programmed cell death modulates the final pattern of sensory organs in a segment-specific manner.

Key words: Apoptosis, Notch, Asymmetric cell division, Cell lineage, *Drosophila melanogaster*

INTRODUCTION

An important feature of animal development is programmed cell death, or apoptosis. In all apoptotic cells, cell death involves the activation of cysteine proteases known as caspases (Kaufmann and Hengartner, 2001). While the usptream regulators and dowstream targets of caspases are relatively well characterized (Kaufmann and Hengartner, 2001), the mechanisms that control the initial commitment of specific cells to undergo apoptosis during embryonic development are poorly understood.

In *Drosophila*, activation of programmed cell death appears to be regulated mainly at the transcriptional level. Cells fated to die during development express one or several of the four pro-apoptotic genes *reaper* (*rpr*), *hid* (*Wrinkled, W* – FlyBase) *grim* and *sickle* (*skl*) that are organized into a single large complex (Chen et al., 1996; Christich et al., 2002; Grether et al., 1995; Srinivasula et al., 2002; White et al., 1994; Wing et al., 2002a). The proteins encoded by these genes release the inhibition exerted by the inhibitor of apoptosis proteins (IAPs) on caspase activity through three distinct mechanisms. Firstly, all four pro-apoptotic proteins bind to IAPs and these interactions are thought to inhibit the binding of IAPs to caspases (Kaufmann and Hengartner, 2001; Wang et al., 1999). Secondly, Rpr and Hid have been shown to downregulate IAP protein levels by stimulating IAP polyubiquitination, therefore promoting their degradation (Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002b; Yoo et al., 2002). Thirdly, Rpr and Grim seem to inhibit global protein translation, resulting in a differential loss of short-lived proteins such as IAPs (Holley et al., 2002; Yoo et al., 2002). Given the diversity of cells entering apoptosis during *Drosophila* development, this gene complex is thought to integrate various death and survival signals. For instance, programmed cell death of salivary gland cells after pupation is induced by high ecdysone levels. In these cells, the ecdysone-bound EcR/USP nuclear receptor complex directly regulates *rpr* transcription through an essential binding site in its promoter (Jiang et al., 2000).

Cells specifically fated to die have been described in many stereotyped lineages in nematodes (Sulston et al., 1983) and insects (Bossing et al., 1996; Lawrence, 1966; Schmidt et al., 1997). However, the mechanisms underlying binary cell death decisions following asymmetric cell division are poorly understood. For instance, during *C. elegans* development, the NSM mother cell divides asymmetrically and generates a NSM neuron and a cell fated to die (Sulston et al., 1983). Cell death in this lineage requires the activity of the transcription factor CES-2 (Ellis and Horvitz, 1991; Metzstein et al., 1996). CES-2 is thought to activate the expression of the pro-apoptotic gene *egl-1*, a member of the Bcl-2 family, by repressing *ces-1* expression (Metzstein and Horvitz, 1999). However, the mechanisms restricting CES-2 activity to only one daughter cell are not known. Binary cell death decisions have also been studied in the HSN/PHB cell lineage (Guenther and Garriga, 1996). The HSN/PHB neuroblast divides asymmetrically to generate an anterior daughter cell that undergoes apotosis and a posterior HSN/PHB precursor cell. During this division, HAM-1 is localized asymmetrically and is segregated into the

posterior daughter cell. In *ham-1* mutants, the fate of each daughter cell is not properly specified and the anterior daughter cell frequently fails to undergo apoptosis. However, how HAM-1 regulates this cell death decision is not known (Guenther and Garriga, 1996).

We have investigated the mechanisms that regulate binary cell death decisions in the peripheral nervous system of the *Drosophila* embryo. We first describe the lineage of a ventral multidendritic neuron that we named vmd1a. We show that the vmd1a neuron is generated by a sensory organ primary precursor (pI) cell that divides asymmetrically twice. Following each division, one of the two cells expresses the pro-apoptotic genes *rpr* and *grim* and undergoes apoptosis. Numb is unequally inherited at each division. We show that Numb is necessary and sufficient to prevent apoptosis in this lineage whereas activated Notch is sufficient to trigger death in this lineage. This indicates that binary cell death decisions can be regulated by the unequal segregation of Numb at mitosis.

MATERIALS AND METHODS

Drosophila stocks

A *yw* stock was used as a wild-type stock. The Df(1)H99 line carries a deletion in the 75C region that removes the *reaper*, *hid* and *grim* genes (Chen et al., 1996; Grether et al., 1995; White et al., 1994). *numb796*, *insc22*, *Nts* and *N55E11* mutant alleles as well as the arm-GAL4, UAS-p35, hs-Nintra (Lieber et al., 1993) and UAS-Numb-Tmyc (Yaich et al., 1998) transgenes are described in FlyBase (http://fly.ebi.ac.uk:7081/). The CutA3-lacZ line carries a transgene expressing *lacZ* under the control of a *cut* enhancer (Jack and DeLotto, 1995). In the Df(1)H99/TM6 AbdA-lacZ, insc22 B6-2-25/CyO wg-lacZ, numb796/CyO wg-lacZ and hs-Nintra/CyO wg-lacZ stocks, homozygous embryos were identified by the lack of β-galactosidase staining.

Immunostaining, in situ hybridization and microscopy

Staged embryos were fixed and stained as previously described (Orgogozo et al., 2001). For the *Notch* loss-of-function experiment, staged *Notchts1* and *yw* embryos (13-14.5 hours after egg laying at 19°C) were shifted to restrictive temperature (31°C) and were allowed to develop for 45 minutes at 31°C prior to fixation. For the *Notch* gain-of-function experiment, staged hs-Nintra and *yw* embryos (15.5-16.5 or 17.5-18.5 hours after egg laying at 19°C) were heat-shocked 30 minutes at 37°C, then allowed to develop for 45 minutes at 19°C prior to fixation. Primary antibodies were used at the following dilutions: mouse anti-Cut, 1/1000 (DSHB); rabbit anti-Numb, 1/1000 (a gift from Y.-N. Jan); rat anti-Elav, 1/4 (DSHB); rabbit anti-Prospero, 1/1000 (a gift from Y.-N. Jan); guinea-pig anti-Senseless, 1/1000 (a gift from H. Bellen); rabbit anti-β-galactosidase, 1/2000 (Cappel). Secondary antibodies Cy3-, Cy5-, Alexa488- anti-mouse, rat or rabbit, 1/1000 were purchased from Jackson Laboratories or Molecular Probes. The DNA was stained with TOTO-3 (1/3000; Molecular Probes). For in situ detection of *rpr*, *grim* and *hid* transcripts, embryos were pre-hybridized and hybridized as described previously (Wilkie and Davis, 1998). Embryos were then incubated for 2 hours with preabsorbed horseradish peroxidase (HRP)-conjugated sheep anti-digoxigenin Fab fragments (1/1000; Roche) and washed in PBS + 0.1% Tween 20 (PBT). HRP activity was revealed with TSA-FITC (NEN Life Science) for 12 minutes. Embryos were washed four times for 5 minutes in PBT and HRP activity was then inhibited by a 30-minute incubation in 0.3% H_2O_2 followed by five washes in PBT. Embryos were then incubated overnight at 4°C with the anti-Cut, anti-β-galactosidase and/or anti-Pros primary antibodies. After three PBT washes, embryos were incubated with secondary antibodies. The Cut

signal was amplified using biotinylated anti-mouse (1/2000; Jackson Laboratories), avidin DH-biotinylated HRP complexes (Vector Laboratories), TSA-biotine (NEN Life Science) and streptavidine-Alexa 568 (Molecular Probes).

Images were collected on a Leica SP2 confocal microscope and processed using Photoshop software. Figures show the maximal projection of several confocal z-sections.

RESULTS AND DISCUSSION

The vmd1a pI cell divides asymmetrically twice to produce the vmd1a neuron

The vmd1a neuron is located within a cluster of five multidendritic (md) neurons in the ventral region of abdominal segments A1-A7 (Fig. 1A). The vmd1a neuron can be distinguished from the other ventral md neurons (vmd1-4) using the *B6-2-25* enhancer-trap marker (Bier et al., 1989) (this study). The origin of this vmd1a neuron is not known. We have previously established that the vmd1-4 neurons are generated by the four vp1-4 external sensory (es) organ primary precursor (pI) cells (Orgogozo et al., 2001). Each vp1-4 pI cell follows a lineage called the md-es lineage. This lineage is composed of four successive asymmetric cell divisions that generate five distinct cells, the four cells of the es organ at the position where the pI cell has formed and one md neuron that will then migrate to the ventral md cluster (Orgogozo et al., 2001) (Fig. 1B). In the md-es lineage, the membrane-associated protein Numb is segregated into one of the two daughter cells at each cell division (Orgogozo et al., 2001; Rhyu et al., 1994). Numb establishes a difference in cell fate by antagonizing Notch in the Numb-receiving cell (Artavanis-Tsakonas et al., 1999; Guo et al., 1996). Because no es organ is found in the vicinity of the vmd1a neuron, this neuron is probably not generated by a md-es lineage.

Cut has proved to be a useful lineage marker for establishing the md-es lineage as it is expressed in the pI cell and in all its progeny cells (Blochlinger et al., 1990; Orgogozo et al., 2001). To determine the origin of the vmd1a neuron, we also used Cut as a marker since it accumulates in the vmd1a neuron (Fig. 1A). Our analysis shows that the vmd1a neuron stems from a pI cell that divides asymmetrically twice. This vmd1a pI cell appears as an isolated Cut-positive cell located anteriorly to the pIIa-pIIb cell cluster present at the vp1 position (Fig. 1C; throughout this study, precise staging was determined by counting the number of Cut-positive cells in the vp1 cluster). The vmd1a pI cell divides within the plane of the epithelium with Numb localized asymmetrically and segregating into one daughter cell (Fig. 1D and data not shown). Surprisingly, at a later stage when three cells are present at the vp1 position, only a single Cut-positive cell is detected at the vmd1a position instead of the two seen earlier. We named this cell pIIb. The pIIb cell undergoes an asymmetric division oriented along the dorsal-ventral axis of the embryo, with the cell-fate determinants Numb and Prospero (Pros) (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995) segregating into the dorsal daughter cell (Fig. 1F and data not shown). This second division in the vmd1a lineage produces a dorsal cell with high levels of Pros and a ventral cell with low levels of Pros (Fig. 1G). The ventral cell becomes undetectable around the time when the vp1 cluster is composed of five cells (compare Fig. 1H and 1I). In contrast, the dorsal cell, marked by high level of Pros, accumulates Elav, a neuronal
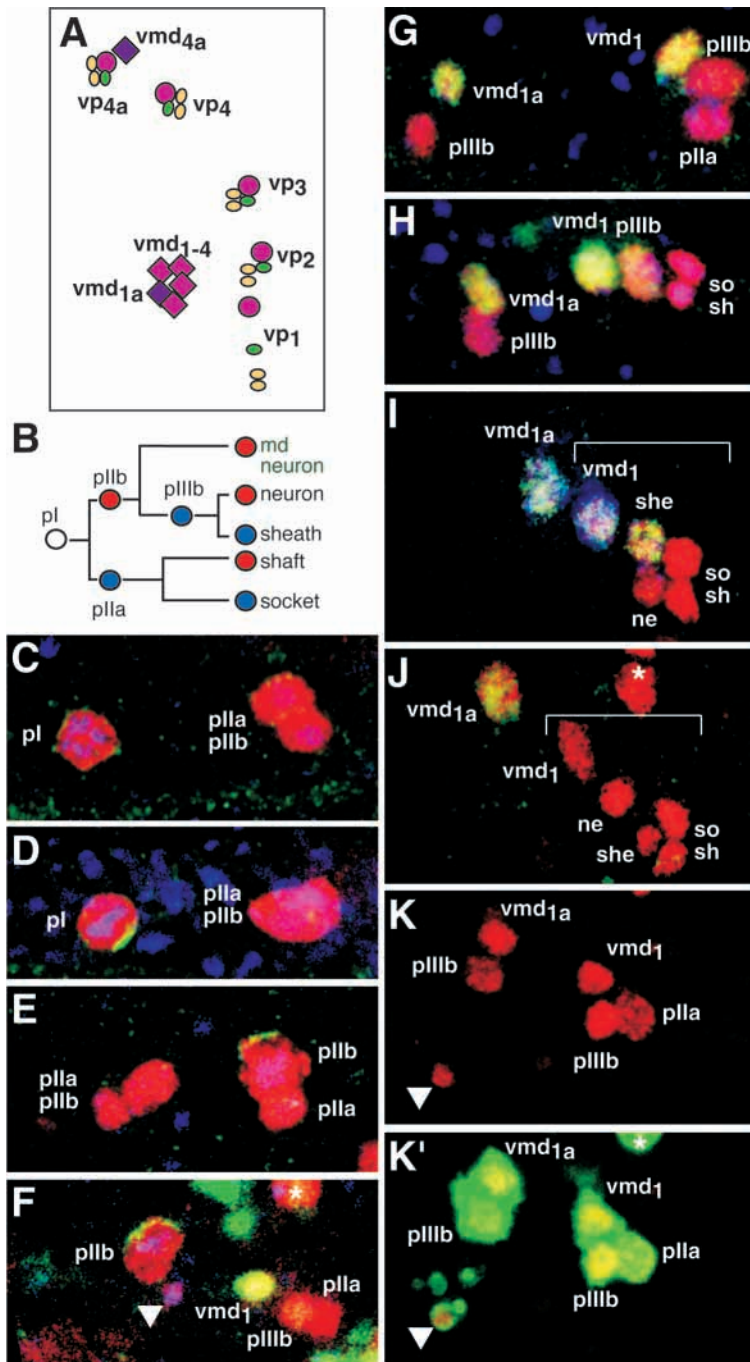
Fig. 1. Analysis of cell divisions in the vmd1a lineage. (A) Diagram of the ventral Cut-positive md neurons and es organs in segments A1-A7. Each vp1-4a organ is composed of an es neuron (large pink circular cell), a sheath cell (green) and a socket/shaft cell pair (yellow). The vmd1a and vmd4a neurons (dark purple diamond-shaped cells) can be distinguished from the vmd1-4 neurons (pink diamond-shaped cells) as they express the *B6-2-25* enhancer-trap marker. (B) Md-es lineage (Orgogozo et al., 2001). Numb-inheriting cells are in red. (C-K) Confocal images showing the vmd1a-vp1 region. (C-D) In stage 12 *CutA3-lacZ* embryos (Jack and DeLotto, 1995) stained for β-galactosidase in red, Numb in green and DNA in blue, the vmd1a pI cell divides asymmetrically soon after the vp1 pI cell has divided. (E) Stage 12 wild-type embryo stained for Cut in red, Numb in green and DNA in blue. (F-H) Stage 13 wild-type embryo stained for Cut in red, Pros in green and DNA in blue. A small Cut-positive dot is detectable at the pIIa position (arrowhead in F). (I) Late stage 13 wild-type embryo stained for Cut in red, Pros in green and Elav in blue. (J) Late stage 13 *B6-2-25* embryo stained for Cut in red and β-galactosidase in green. (I-J) Cells of the vp1 lineage are indicated by a bracket. (K-K′) Same confocal section of a stage 13 *CutA3-lacZ* embryo stained for Cut (K,K′) in red and ß-galactosidase (K′) in green. A small Cut-positive and some β-galactosidase-positive dots are detectable at the position of the pIIa cell (arrowhead in K, K′). In all figures, anterior is left and dorsal is up

embryos homozygous for a deficiency (*H99*) that removes the *rpr*, *hid* and *grim* genes (Chen et al., 1996; Grether et al., 1995; White et al., 1994). In these mutant embryos, one additional es organ comprising four Cut-positive cells is found near the vmd1a neuron in all segments analyzed (100%, *n*=47 segments, Fig. 2A,D). We next examined embryos expressing the caspase inhibitor p35 ubiquitously (arm-Gal4 UAS-p35 embryos). Again, an ectopic es organ was observed close to the vmd1a neuron in many segments (40%, *n*=52, Fig. 2C,D). Since the vmd1a pI cell is the only Cut-positive pI cell identified in this region, we conclude that the ectopic Cut-positive es cells originate from the vmd1a lineage. Hence, we infer that the pIIa and pIIIb cells in the vmd1a lineage die via apoptosis (Fig. 2E). Consistently, TUNEL-positive nuclear fragments as well as β-galactosidase-positive cytoplasmic fragments of *CutA3-lacZ*-expressing cells were observed at the position of the pIIa and pIIIb cells (arrowheads in Fig. 1F, K-K′; data not shown). We interpret these fragments as the nuclear and cytoplasmic remnants of the two dying cells.

In the md-es lineage, the pIIa and pIIIb cells generate the shaft/socket and the neuron/sheath cell pairs, respectively. In the absence of apoptosis, we observed that the pIIa and pIIIb cells in the vmd1a lineage generate ectopic shaft/socket and neuron/sheath cell pairs. We conclude that in the absence of apoptosis, the vmd1a lineage is completely transformed into an md-es lineage.

marker. This cell is identified as the vmd1a neuron as it also expresses the enhancer-trap markers *E7-2-36* (an md marker) and *B6-2-25* (a vmd1a marker) (Bier et al., 1989) (Fig. 1I-J and data not shown). We conclude from this lineage study that the vmd1a neuron is born from a pI cell that divides asymmetrically twice to produce the vmd1a neuron and two daughter cells of unknown fate. The latter cells were named pIIa and pIIIb (Fig. 1E-G).

The pIIa and pIIIb cells die by apoptosis

A possible fate for the pIIa and pIIIb cells is death. To test this hypothesis, we analyzed the progeny of the vmd1a pI cell in embryos in which cell death is inhibited. We first studied

All abdominal Cut-positive md neurons that are not associated with any es organs are generated by a similar apoptotic lineage

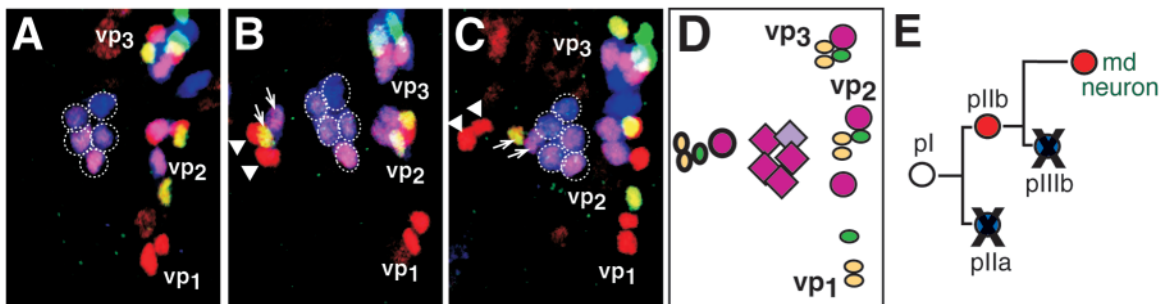Interestingly, the apoptotic lineage described above and the

Fig. 2. Genetic evidence for cell death in the vmd1a lineage. (A-C) Ventral Cut-positive md neurons and es organs in segments A1-A7 of wild-type (A), *Df(1)H99* (B) and *arm-Gal4 UAS-p35* (C) embryos stained for Cut (red), Elav (blue) and Pros (green). (D) Diagram of the ventral-most Cut-positive md neurons and es organs in segments A1-A7 of *Df(1)H99* or *arm-Gal4 UAS-p35* embryos. The Cut-positive md neurons (diamond-shaped cells) are either highly Cut-positive (pink) or weakly Cut-positive (light purple). Es neurons, sheath cells and socket/shaft cell pairs are as in Fig. 1A. One ectopic es organ (bold outline in D) is observed near the vmd1a neuron in *Df(1)H99* (B) and *arm-Gal4 UAS-p35* (C) embryos. This es organ is composed of one Elav-positive es neuron (arrow), one Pros-positive sheath cell (arrow) and two Cut-positive cells identified as the socket and shaft cells (arrowheads; one of these two cells accumulates Suppressor of Hairless [Su(H)], a socket cell marker; data not shown (Gho et al., 1996)). This phenotype is observed in 40% of the segments of *arm-Gal4 UAS-p35* embryos (*n*=52). In other segments, we observed three Cut-positive cells (12%), two Cut-positive cells (12%) or no additional Cut-positive cell (36%). Therefore, in these cases, the level of p35 accumulation may not be high enough to fully inhibit apoptosis in the vmd1a lineage. (E) Model proposed for the vmd1a lineage. The pIIa and pIIIb cells die.

md-es lineage probably account for the production of all embryonic Cut-positive md neurons. Indeed, most Cut-positive md neurons can be associated to one es organ, suggesting that they originate from a md-es lineage. However, two or three single Cut-positive md neurons in the dorsal region of segments A1-A7 (mean value = 2.6, *n*=33 segments, Fig. 3A-A′) cannot be associated with any es organ, similarly to the vmd1a neuron. Remarkably, in embryos homozygous for the H99 deficiency, we observed two or three ectopic es organs in the dorsal region of segments A1-A7 (mean value = 2.9, *n*=20, Fig. 3B-
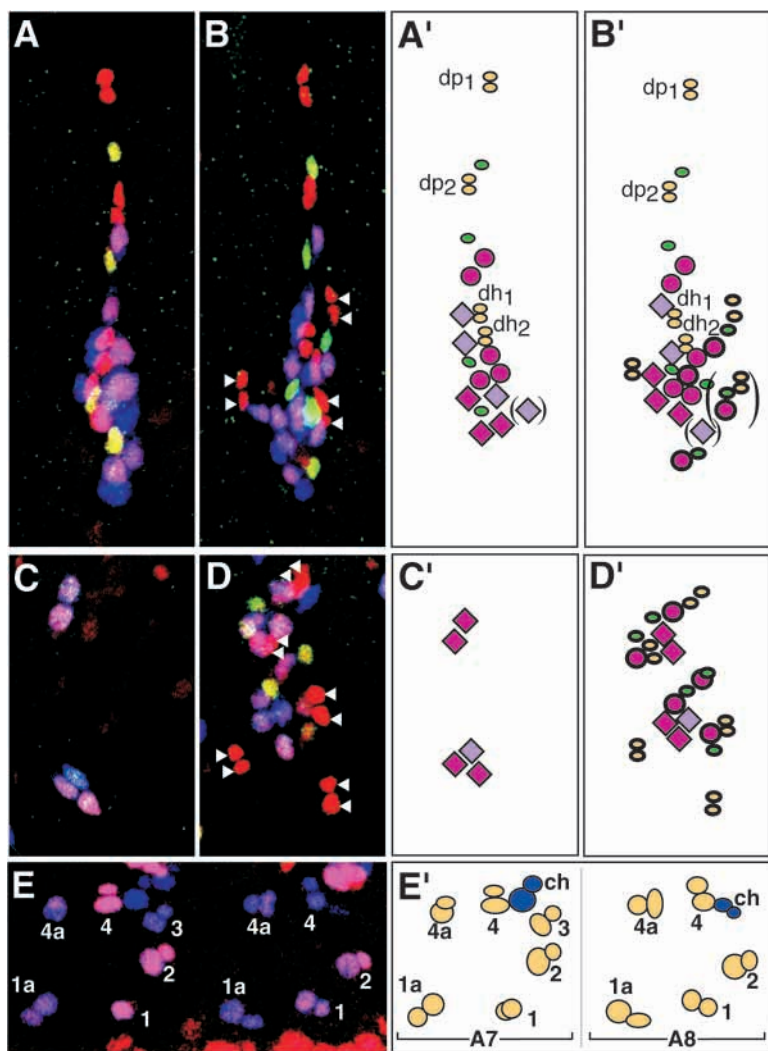
Fig. 3. Additional apoptotic lineages similar to the vmd1a lineage in other embryonic regions. (A-D′) Cut-positive md neurons and es organs in the dorsal region of segments A1-A7 (A,B) and in the ventral region of segment A8 (C,D) of wild-type (A,C) and *Df(1)H99* (B,D) embryos stained for Cut (red), Elav (blue) and Pros (green). The schematic representations of the Cut-positive md neurons and es organs (A′,B′,C′,D′) are as in Fig. 2. Two or three ectopic es organs are observed in the dorsal region of segments A1-A7, as well as five ectopic es organs in the ventral region of segment A8. Arrowheads indicate the ectopic socket and shaft cells. Cells shown into brackets in A′,B′ are present in 58% of the segments of wild-type embryos (A′; *n*=33) and in 90% of the segments of *Df(1)H99* embryos (B′; *n*=20). Ectopic cells in B′ and D′ have a bold outline. (E) Ventral region of segments A7-A8 of a wild-type stage 12 embryo stained for Cut (red) and the sensory organ marker Senseless (Sens; blue (Nolo et al., 2000)). (E′) Schematic representation of the Cut-positive and Sens-positive sensory organ precursor cells (yellow) and of the chordotonal (ch) organ precursor cells (blue) which are Cut-negative and Sens-positive. At this stage, six pIIa-pIIb cell clusters are seen in the ventral region of segment A7. In the ventral region of segment A8, five pIIa-pIIb cell clusters are observed at positions corresponding to the vmd1a, vp1, vp2, vp4 and vp4a positions in segment A7.
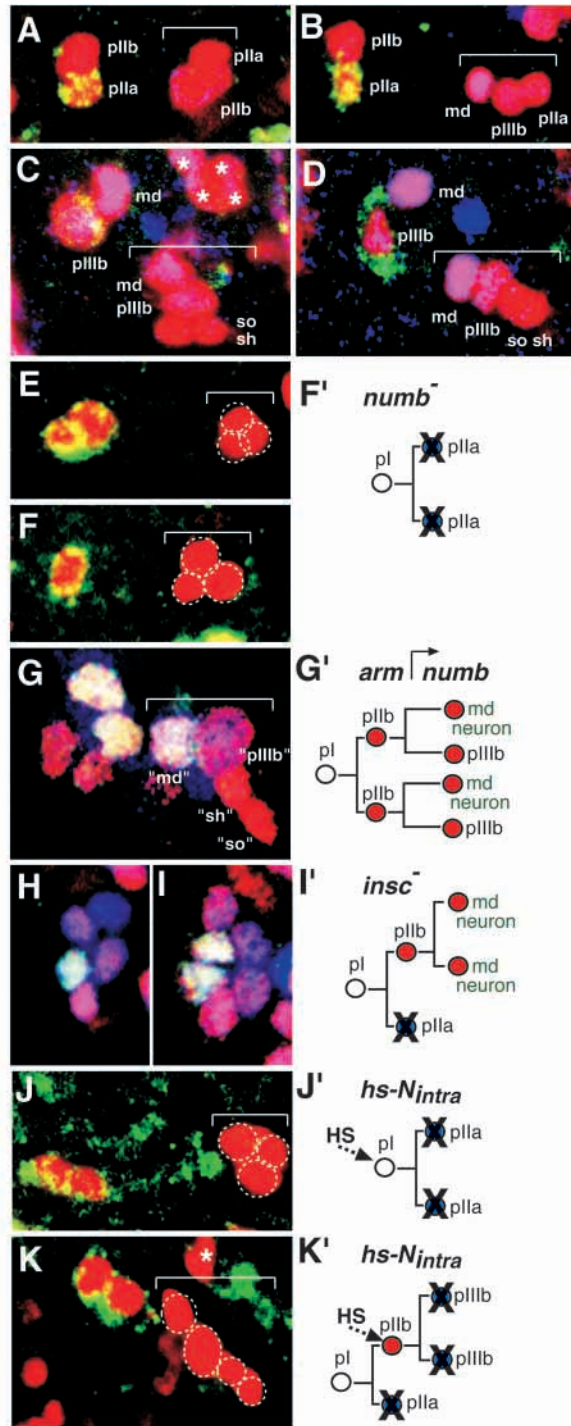
Fig. 4. *Numb* inhibits the *Notch*-mediated expression of *rpr* and *grim* in the vmd1a lineage. (A-D) Wild-type embryos stained for Cut in red, Pros in blue and *rpr* transcripts (A,C) or *grim* transcripts (B,D) in green. The *rpr* and *grim* genes are specifically expressed in the pIIa and pIIIb cells of the vmd1a lineage. No expression is seen in the vp1 lineage cells. (E-F) In *numb796* mutant embryos stained for Cut in red and *rpr* in green, *rpr* transcripts accumulate in both vmd1a pI daughter cells (E) or in the only pI daughter cell remaining (F). No expression is seen in the vp1 lineage cells. This indicates that both vmd1a pI daughter cells undergo cell death in the absence of *numb* (F′). (G) In *arm-Gal4 UAS-numb* embryo stained for Cut in red, Pros in green and Elav in blue, four Cut-positive cells accumulating various levels of Pros and Elav can be observed at the vmd1a position, suggesting that the pIIa cell did not die and was transformed into a pIIb cell (G′). Note that in G, the pIIa cell of the vp1 lineage was not completely transformed into a pIIb cell since two Pros-negative cells accumulating high levels of Cut, corresponding to socket-like and shaft-like cells, are observed. (H-I′) *B6-2-25* (H) and *B6-2-25 insc22* mutant embryos (I) stained for Cut in red, β-galactosidase in green and Elav in blue. In the ventral md cluster of *insc22* mutant embryos (I), six md neurons instead of the five seen in wild-type (H) are found. Two of them express the vmd1a-specific enhancer-trap marker *B6-2-25*. This indicates that the pIIIb cell did not die and was transformed into a vmd1a neuron (I′). (J,K) Heat-shocked *hs-Nintra* embryos stained for Cut in red and *rpr* (J) or *grim* (K) in green. *rpr* (J) or *grim* (K) transcripts accumulate in both pI (J) and pIIb (K) daughter cells. This indicates that in these conditions, both pI (J′) or pIIb (K′) daughter cells undergo cell death. Staged hs-Nintra and control *yw* embryos (15.5-16.5 or 17.5-18.5 hours after egg laying at 19°C) were heat-shocked 30 minutes at 37°C, to induce the expression of Nintra, and allowed to develop for another 45 minutes at 19°C prior to fixation. We only analyzed embryos in which at least one segment shows a dividing vp1 pIIb cell or a dividing vp1 pIIIb cell. In each panel, the vp1 lineage cells are indicated by a bracket.

neurons are found (100%, *n*=14, Fig. 3C). By contrast to segments A1-A7, no external sensory organs are observed in this ventral region. To test whether each of the five ventral A8 md neurons is generated via an apoptotic lineage similar to the one described for vmd1a, we analyzed the ventral region of segment A8 in embryos homozygous for the H99 deficiency. Remarkably, five ectopic es organs (100%, *n*=11, Fig. 3D-D′) were observed in this region. These data indicate that five pI cells follow an apoptotic lineage similar to the vmd1a lineage in the ventral region of segment A8.

Furthermore, analysis of wild-type stage 12 embryos (Fig. 3E-E′) showed that five Cut-positive pI cells form in the ventral region of segment A8 at positions corresponding to the vmd1a, vp1, vp2, vp4 and vp4a pI cells in segments A1-A7. We therefore assume that these A8 pI cells are homologous to the vmd1a, vp1, vp2, vp4 and vp4a pI cells of segments A1-A7. Together, these observations indicate that the main difference in sensory organ patterns in the ventral region between segment A8 and segments A1-A7 is that the pI cells at positions 1, 2, 4 and 4a follow an apoptotic lineage in segment A8 and an md-es lineage in segments A1-A7. A recent study has revealed that the homeotic *Ultrabithorax* (*Ubx*) gene acts at different steps in sensory organ development to regulate the bristle pattern in the thoracic legs (Rozowski and Akam, 2002). Indeed, *Ubx* was shown to control the absence of two particular bristles in the third thoracic segment relative to the second thoracic segment by two distinct mechanisms. For the posterior

B′). This strongly suggests that these Cut-positive md neurons originate from an apoptotic lineage as the one described for vmd1a. Thus, all abdominal Cut-positive md neurons likely originate either from an apoptotic lineage as the one described for vmd1a or from an md-es lineage. We conclude that regulation of cell death specifies the relative number of Cut-positive md neurons and es organs within a segment.

Programmed cell death modulates the final pattern of sensory organs in a segment-specific manner

In the ventral region of segment A8, five Cut-positive md

Table 1. Expression of the *rpr* and *grim* genes in the vmd1a lineage cells

	Genotype			○○	○●	●●	○	●
				% segments showing the following outcome in the vmd1a lineage				
A	*yw*	*rpr*	(n=134)	25	71	0	4	0
		grim	(n=53)	8	77	0	15	0
	numb796	*rpr*	(n=55)	13	16	54	4	13
		grim	(n=54)	13	26	52	0	9
	yw +HS	*rpr*	(n=98)	32	51	0	17	0
		grim	(n=60)	40	40	0	20	0
	hs-Nintra +HS	*rpr*	(n=61)	21	43	6	20	10
		grim	(n=67)	21	46	6	25	2
B	*yw*	*rpr*	(n=55)	6	78	0	16	0
		grim	(n=78)	6	91	0	3	0
	yw +HS	*grim*	(n=86)	4	90	0	6	0
	hs-Nintra +HS	*grim*	(n=89)	13	62	7	13	5

The expression of the *rpr* and *grim* genes was analyzed following the division of the pI (upper part of the table, A) and pIIb (lower part of the table, B) cells in the vmd1a lineage. +HS means that embryos were heat shocked. *n* is the number of segments analyzed for each genotype. ○○, two cells, neither of which expresses *rpr* or *grim*; ○●, two cells, one of which expresses *rpr* or *grim*; ●●, two cells, both expressing *rpr* or *grim*; ○,one cell that does not express *rpr* or *grim*; ○⁞ one cell expressing *rpr* or *grim*.

sternopleural bristle, *Ubx* blocks the selection of the pI cell from the proneural cluster whereas for the apical bristle, it inhibits the differentiation of the pIIa and pIIb cells. Our analysis suggests that homeotic genes may also regulate the final pattern of sensory organ by a third mechanism, i.e. by regulating the programmed cell death of the pIIa and pIIIb cells. Since *Abdominal-B* (*Abd-B*) regulates the homeotic identity of segment A8 (Kuhn et al., 1992), we propose that *Abd-B* regulates cell death in sensory organ lineages in segment A8. It remains to be determined whether *Abd-B* acts in the proneural cluster or in the pI cell to specify its lineage or whether it more directly regulates the expression of pro-apoptotic genes in the pIIa and pIIIb cells.

The vmd1a pIIa and pIIIb cells specifically express the *reaper* and *grim* genes

Since the *rpr*, *hid* and *grim* genes are included in the H99 region required for cell death in the vmd1a lineage, we analyzed their expression in this lineage. We found that *rpr* and *grim*, but not *hid*, are expressed specifically in the pIIa and pIIIb cells of the vmd1a lineage. By contrast, these genes are not expressed in cells of the vp1-4 lineages. In embryos in which a pIIb cell divides at the vp1 position in at least one abdominal segment, most segments contain a vmd1a pIIa-pIIb pair with one cell expressing *rpr* (71%, Fig. 4A; Table 1) or *grim* (77%, Fig. 4B; Table 1). This cell is the pIIa cell fated to die. In some other segments, neither of these two cells accumulates *rpr* (25%) or *grim* (8%). Since the development of segments is not perfectly synchronous, we assume that this represents a situation preceding the onset of *rpr* and *grim* expression in the pIIa cell. In the remaining segments, a single Cut-positive cell is detected indicating that the pIIa cell has died. In those segments, expression of *rpr* and *grim* is never detected in the remaining pIIb cell (Table 1).

We next analyzed the expression of *rpr* and *grim* in the pIIb daughter cells. In embryos in which at least one abdominal segment shows a dividing vp1 pIIIb cell, most segments contain a pIIIb-md pair with the ventral pIIIb cell (identified by a low level of Pros accumulation) expressing *rpr* (78%, Fig. 4C; Table 1) or *grim* (91%, Fig. 4D, Table 1). In some segments (6%), the expression of *rpr* and *grim* is not detected in the pIIIb and md cells. These segments are probably at a stage preceding

the onset of *rpr* and *grim* expression. Finally, we also observed segments with no pIIIb cell and only one highly Pros-positive cell that does not express *rpr* or *grim*, corresponding to the vmd1a neuron (Table 1). Thus, induction of apoptosis in the pIIa and pIIIb cells involves the transcriptional activation of the *rpr* and *grim* genes specifically in these cells.

Numb prevents apoptosis in the vmd1a lineage

During the pIIb division, Numb was shown to segregate into the dorsal pIIb daughter cell. This cell is not fated to die and differentiates as a vmd1a neuron. By contrast, we could not directly determine which one of the two pI daughter cells inherits Numb. Indeed, since the orientation of the vmd1a pI cell division is random, we could not identify the pIIa and pIIb cells from their relative positions. Nevertheless the vmd1a pIIa and pIIIb cells appear to generate ectopic shaft/socket and neuron/sheath cell pairs when cell death is prevented. In the md-es lineage, these cell pairs are the progeny of the cells that do not inherit Numb (Fig. 1B). This suggests that both the vmd1a pIIIb cell and the pIIa cell do not inherit Numb. Thus, Numb appears to segregate in the cells that do not die in the vmd1a lineage.

We therefore tested the role of Numb in regulating *rpr* and *grim* expression as well as cell death in the vmd1a lineage. In *numb* mutant embryos in which a secondary precursor cell divides at the vp1 position in at least one abdominal segment, we observed that the two Cut-positive vmd1a pI daughter cells accumulate *rpr* or *grim* transcripts (54% of the segments for *rpr*, 52% for *grim*; Fig. 4E, Table 1). In other segments we observed a single Cut-positive pI daughter cell accumulating *rpr* (13%) or *grim* (9%, Fig. 4F, Table 1). In these segments one pI daughter cell has already died and the other one is undergoing apoptosis. These two phenotypes are not seen in wild-type embryos. Thus, in the absence of *numb*, both pI daughter cells undergo programmed cell death. Consistently, no Cut-positive cell is observed at the vmd1a position in *numb* mutant embryos in most segments (19/23). We conclude that *numb* is required to inhibit the expression of *rpr* and *grim* and to prevent cell death in the pIIb cell.

To test whether *numb* is sufficient to prevent cell death, we analyzed the progeny of the vmd1a pI cell in arm-Gal4 UAS-numb embryos that express high levels of Numb. In wild-type
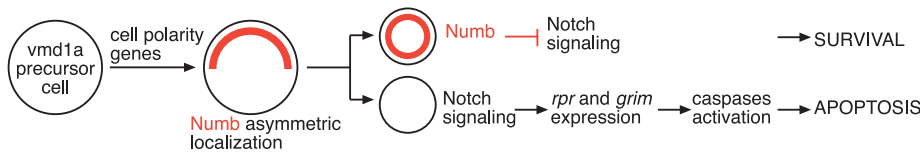
Fig. 5. Regulation of cell death in the vmd1a lineage. Alternative cell death decision is regulated by the unequal partitioning of Numb. Numb inhibits the pro-apoptotic function of Notch in one of the two daughter cells, while activation of Notch induces the expression of *rpr* and *grim* in the other cell.

embryos in which a vp1 pIIIb cell is dividing in at least one segment, one or two Cut-positive cells are observed at the vmd1a position (Table 1). In contrast, four Cut-positive cells are observed in 50% of the segments (*n*=18) in arm-Gal4 UAS-numb embryos at the same stage. In 8 out of the 9 segments with four cells, two cells accumulating high levels of Pros and two cells accumulating low levels of Pros are seen (Fig. 4G), suggesting that these cells are two vmd1a neurons and two pIIIb cells. These data indicate that the pIIa cell death was inhibited and that the pIIa cell was transformed into a pIIb-like cell (Fig. 4G′).

To test whether physiological levels of Numb are sufficient to inhibit apoptosis, we used the *inscuteable* (*insc*) mutation that disrupts the polarity of the pIIb cell in the md-es lineage (Orgogozo et al., 2001). In the vmd1a lineage, Insc specifically accumulates in the pIIb cell where it localizes asymmetrically (data not shown). In *insc* mutant embryos, a duplication of the vmd1a neuron was seen in 13% of the segments (*n*=77, Fig. 4H,I). This indicates that the pIIIb cell has survived and was transformed into a second vmd1a neuron. We interpret this cell-fate change as resulting from a mispartitioning of Numb which then inhibits the death of the pIIIb cell (Fig. 4I′). Together, these results show that *numb* is both necessary and sufficient to inhibit cell death in the vmd1a lineage.

Activated Notch triggers apoptosis in the vmd1a lineage

Numb is known to function by antagonizing Notch activity (Artavanis-Tsakonas et al., 1999; Guo et al., 1996). This therefore suggests that Notch promotes cell death in the vmd1a lineage and that Numb blocks this activity of Notch. Unfortunately, the strong effect of *Notch* loss-of-function alleles on the selection of the vmd1a pI cell meant that it was not possible to test directly whether *Notch* is required for cell death in the vmd1a lineage. We therefore used the conditional *Notch^{ts1}* allele. However, when *Notch^{ts1}* embryos are shifted to a restrictive temperature (31°C) soon after the specification of the vmd1a pI cell (i.e., at 13-14.5 hours after egg laying at 19°C), we observed no significant reduction in the number of *rpr*- or *grim*-expressing pIIa cells. A stronger *Notch^{ts1}*/ *Notch^{55e11}* combination causes the appearance of additional vmd1a pI cells even at the permissive temperature (19°C). It is therefore not possible to determine whether an increase in the number of *rpr*- or *grim*-negative cells results from a lack of Notch-dependent apoptosis or from an excess of vmd1a pI cells due to reduced Notch signaling during lateral inhibition.

We therefore tested whether an activated form of Notch, Nintra (Lieber et al., 1993), can promote the death of the pIIb cell when expressed around the time of the vmd1a pI cell division. In 6% of the segments (*n*=128) from embryos in which at least one segment shows a dividing vp1 pIIb cell, *rpr* or *grim* transcripts accumulate in both vmd1a pI daughter cells

(Fig. 4J; Table 1). In other segments, a single Cut-positive cell remains at the vmd1a position and accumulates *rpr* (10%, *n*=61 segments) or *grim* (2%, *n*=67 segments, Table 1). These expression patterns are not seen in heat-shocked control embryos (Table 1). Importantly, these observations are similar to those made in *numb* mutant embryos. Thus, both loss of *numb* activity and ectopic Notch signaling lead to transcriptional activation of pro-apoptotic genes in the pIIb cell (Fig. 4J′). Finally, a similar effect of Nintra on *rpr* and *grim* expression was seen in the vmd1a pIIb daughter cells when Nintra expression was induced at a later stage, i.e. when the vmd1a pIIb cell is dividing (Fig. 4K-K′). Together, these results indicate that Notch signaling is sufficient to promote cell death in the vmd1a lineage.

Studies in vertebrates have shown that Notch activation can either protect cells from death or trigger apoptosis depending on the cellular context (Artavanis-Tsakonas et al., 1999; Deftos et al., 1998; Ohishi et al., 2000; von Boehmer, 1999). The mechanisms by which Notch regulates apoptosis in vertebrates are poorly understood. We show here that Notch activation triggers the transcriptional activation of the pro-apoptotic genes *reaper* and *grim*. Activated Notch acts as a transcriptional co-activator for Supressor of Hairless (Su(H)), a sequence-specific DNA-binding protein (Artavanis-Tsakonas et al., 1999). In the vmd1a lineage, activated Notch possibly regulates the *rpr* and *grim* gene expression directly via the putative Su(H)-binding sites present in the 5′ and 3′ flanking regions of the *rpr* and *grim* genes. Identification of the functional Su(H) binding sites located within this 300 kb gene complex is a difficult task since none of the available *rpr*- and *grim*-containing cosmids (Chen et al., 1996; White et al., 1994) include all the regulation sequences required for expression of *rpr* and *grim* in the vmd1a lineage (data not shown).

Importantly, Nintra does not induce the expression of *rpr* and *grim* in the vp1-4 lineages. Thus, depending on the cell lineage, Notch activity can either have no influence on cell death (vp1-4 lineage) or induce cell death (vmd1a lineage). A better understanding of the mechanisms underlying binary cell death decisions will require the identification of the factors influencing Notch decision in a lineage-specific manner.

In summary, we have described the lineage generating the vmd1a neuron. This lineage is composed of two asymmetric divisions following which one daughter cell undergoes apoptosis. These two binary cell death decisions are regulated by the unequal segregation of Numb at mitosis. Therefore, our data provide the first experimental evidence that alternative cell death decision can be regulated by the unequal segregation of a cell fate determinant (Fig. 5). The conserved role of Numb and Notch in neuronal specification in flies and vertebrates suggests that Numb-mediated inhibition of Notch may play a similar role in regulating cell death decisions in vertebrates.

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