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Drosophila Neuroblast 7-3 Cell Lineage: A Model System for Studying Programmed Cell Death, Notch/Numb Signaling, and Sequential Specification of Ganglion Mother Cell Identity

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ABSTRACT
Cell lineage studies provide an important foundation for experimental analysis in many systems. *Drosophila* neural precursors (neuroblasts) sequentially generate ganglion mother cells (GMCs), which generate neurons and/or glia, but the birth order, or cell lineage, of each neuroblast is poorly understood. The best-characterized neuroblast is NB7-3, in which GMC-1 makes the EW1 serotonergic interneuron and GW motoneuron; GMC-2 makes the EW2 serotonergic interneuron and a programmed cell death; and GMC-3 gives rise to the EW3 interneuron. However, the end of this lineage has not been determined. Here, we use positively marked genetic clones, bromodeoxyuridine (BrdU) labeling, mutations that affect Notch signaling, and antibody markers to further define the end of the cell lineage of NB7-3. We provide evidence that GMC-3 directly differentiates into EW3 and that the sibling neuroblast undergoes programmed cell death. Our results confirm and extend previous work on the early portion of the NB7-3 lineage (Novotny et al. [2002] Development 129:1027–1036; Lundell et al. [2003] Development 130:4109–4121). J. Comp. Neurol. 481:240–251, 2005.

Indexing terms: cell lineage; neuroblast; programmed cell death; Notch; sanpodo; numb; serotonin; corazonin; NB7-3

Cell lineage analysis provides an important foundation for a molecular, genetic, or experimental investigation of the mechanisms regulating the generation of cell diversity. A variety of methods have been used to do lineage analysis, including direct observation (Taghert and Goodman, 1984; Doe and Technau, 1993; Heid and Hardin, 2000), injection of cytoplasmic or membrane markers (Oster-Granite and Gearhart, 1981; Technau, 1987; Krotoski et al., 1988; Sheard and Jacobson, 1990; Walsh and Cepko, 1990; Birgbauer and Fraser, 1994), or use of heritable genetic markers (Frank and Sanes, 1991; Spena and Salamini, 1995; Cepko et al., 1998; Verberne et al., 1998; Gourdie et al., 2000). Lineage analysis has been done in a wide variety of embryos, including the vertebrates mouse, chick, frog, zebrafish (Luskin et al., 1988; Cepko et al., 1998; Qian et al., 1998, 2000; Moody, 2000); the invertebrates *Drosophila*, *Caenorhabditis elegans*, leech, mollusks, and many others (Sulston et al., 1983; Weisblat et al., 1984; Stuart et al., 1987; Venuti and Jeffery, 1989; Dohmen, 1992; Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999); fungi (Nasmyth, 1983; Klar, 1987); and plants (Irish and Jenik, 2001). In all cases, cell lineage analysis has facilitated subsequent molecular, cellular, or genetic investigation into the mechanisms of generating cell diversity.

The *Drosophila* central nervous system (CNS) is useful for combining lineage analysis with a genetic or experimental analysis of cell fate. There are 30 embryonic neuroblasts (NBs) per hemisegment, and each can be individually identified based on one or more of the following

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features: time of formation, position in the array, green fluorescent protein (GFP) transgene expression, or multiple molecular markers. NBs divide asymmetrically to "bud off" a series of smaller GMCs into the embryo—the number of GMCs produced can vary from as few as 3 (in the NB7-3 lineage; this work) to over 20 (Bossing et al., 1996; Pearson and Doe, 2003) to several hundred (Lee et al., 1999). In all cases, it has been assumed that each GMC produces a pair of postmitotic neuron/neuron or neuron/glial siblings, based on work in other insects and on a few model *Drosophila* GMCs (Doe et al., 1985; Kuwada and Goodman, 1985).

Many lineage studies have been done in the *Drosophila* CNS, but virtually all have been designed to identify the terminal clone of neurons and glia produced by each NB (Bossing et al., 1996; Schmid et al., 1999). While extremely valuable, the next step in lineage analysis is to determine the birth order of all neurons and glia within each NB clone. Information about the neuronal birth order within a NB clone has been determined for the postembryonic mushroom body brain NB lineages, where timed induction of genetic mosaics shows that the γ neurons are born first, the α′/β′ neurons are born second, and the αβ neurons are born last (Lee et al., 1999). In the embryo, a few NB lineages have been partially characterized for neuronal/glial birth order: In NB7-1, it has been shown that the first five GMCs give rise to the U1–U5 motoneurons (Pearson and Doe, 2003). NB4-2 has a first-born GMC that is known to produce the RP2 motoneuron and the RP2 sib (Broadus et al., 1995; Chu-LaGraff et al., 1995). NB1-1 has a first-born GMC that is known to produce the aCC motoneuron and the pCC interneuron (Prokop and Technau, 1994; Bossing et al., 1996). In all aforementioned lineages, it has been shown that a GMC gives rise to two progeny—either two neurons or neuron/glia. In contrast, the first GMC in the NB6-4T lineage is reported to generate two or three glial cells (Higashijima et al., 1996; Akiyama-Oda et al., 1999; Freeman and Doe, 2001).

The NB7-3 lineage has been characterized in both grasshopper and *Drosophila*. In grasshopper, NB7-3 generates two serotonergic neurons from the first GMC in the abdominal segments (Taghert and Goodman, 1984), but the fate of subsequent cells in the lineage is unknown. In *Drosophila*, the abdominal NB7-3 generates just three interneurons (EW1, EW2, EW3) and one motoneuron (GW); additional uncharacterized cells are observed in thoracic segments (Lundell and Hirsh, 1994, 1998; Bossing et al., 1996; Lundell et al., 1996; Schmid et al., 1999). Recently, the early birth order of the NB7-3 lineage has been characterized: GMC-1 makes the EW serotonergic interneuron and GW motoneuron, GMC-2 makes the EW2 serotonergic interneuron and a programmed cell death (PCD), and GMC-3 makes the EW3 corazonin-positive interneuron (Novotny et al., 2002; Lundell et al., 2003). However, the end of this lineage remains unknown. In particular, the EW3 sibling cell is unknown, and the fate of the terminal neuroblast is unknown.

MATERIALS AND METHODS

Fly stocks

Fly stocks used are as follows: *v/v; P[v⁺,k42]/TM3* (*eagle-kinesin-lacZ*; Higashijima, et al., 1996); *Df(3L)H99, kni^{ri-1} p^{p} /TM3, Sb^{1}* (White et al., 1994); *red e spdo^{zz27} / TM3 ftzlacZ Sb^{1}* (White et al., 1994); *numb^{2} pr cn Bc/Cyo ftzlacZ* (Frise et al., 1996), which is the strongest *numb* allele available (Skeath and Doe, 1998); *UAS-Pon-GFP* (Lu et al., 1999); *engrailed-GAL4* (Schmid et al., 1999); *P[ry^{+} act5C-FRT-FRT- tau lacZ] / CyO* (Buenzow and Holmgren, 1995); *pr^{1} pwn^{1} P{ry^{+}t7.2=hsFLP}38 / CyO; Ki^{1} kar^{1} ry^{506}* (Golic and Lindquist, 1989); and *yw* (as the wild-type stock).

Antibody production and immunological staining of embryos

Eagle antibody was made by immunizing mice and rabbits with a full-length Eagle-GST fusion protein for monoclonal and polyclonal production, respectively (University of Oregon Hybridoma center). Immunofluorescent staining was carried out as described in Doe (1992). Primary antibodies diluted were as follows: mouse anti-Zfh-1, 1:1,000 (Lai et al., 1991); rabbit anti-Eyeless, 1:500 (Kammermeier et al., 2001); rat anti–β-gal, 1:5,000 (Spana and Doe, 1996); mouse anti-Islet 1:20 (Thor and Thomas, 1997); mouse anti–Zfh-2, 1:400 (Lai et al., 1991); mouse anti-engrailed 4D9, 1:4 (Patel et al., 1989); rat anti-Hunchback, 1:400 and guinea pig anti-Hunchback, 1:400 (Kosman et al., 1998); rat anti-Huckebein, 1:50 (McDonald and Doe, 1997); rabbit anti–β-gal, 1:3,000 (ICN Pharmaceuticals, Inc.); guinea pig anti-Kruppel, 1:200 (Kosman et al., 1998); rabbit anti-Eagle 1:500 (Dittrich et al., 1997; Freeman and Doe, 2001); rabbit anti-Corazonin, 1:1,000 (Veenstra and Davis, 1993); rabbit anti–phospho-histone H3 1:5,000 (Upstate Biotechnology). Secondary antibodies were species-specific and conjugated to either Alexa Green (Molecular Probes), Red-X, or Cy5 (Jackson ImmunoResearch) and were used at 1:200 dilution. The embryos were dehydrated in a glycerol series and mounted in 70% glycerol: 4% n-propyl gallate. Imaging was done using a Bio-Rad 1024 confocal microscope, and figures were assembled in Adobe Photoshop. In all embryos, only abdominal hemisegments were scored due to the variability in the number of Eg^{+} cells in the thoracic segments.

BrdU pulse labeling

A 1-hour collection of wild-type (*yw*) embryos was aged to the appropriate stage. Embryos were dechorionated in 50% bleach, rinsed in cool tap water, rocked in a 1:1 solution of octane to Schneider's media (Gibco) for 3 minutes, placed in BrdU solution (BrdU 0.4 mg/ml, in Schneider's) for 30 minutes, placed in a small cell culture dish covered with immersion oil (95% heavy 5% light halocarbon oil by Halocarbon Products Corp.), and allowed to develop to stage 17 (~21 hours). Oil was removed from embryos by using heptane, then embryos were fixed in 4% formaldehyde in PEM (Doe, 1992), stored in EtOH.

To stain for BrdU, we followed a protocol adapted from Bruce Edgar (personal communication). BrdU-pulsed embryos were stained with rabbit anti-Eagle and rat anti-Hunchback as described in Doe (1992), exposed to a post-fix of 1:1 heptane and 4% formaldehyde in PEM for 10 minutes, acid treated (2 M HCl and 0.1% Triton-X) for 40 minutes, washed in 0.1 M Borax for 15 minutes, in PBT for 30 seconds, and then stained with mouse anti-BrdU 1:600 (Becton Dickson) following standard staining procedures described above.

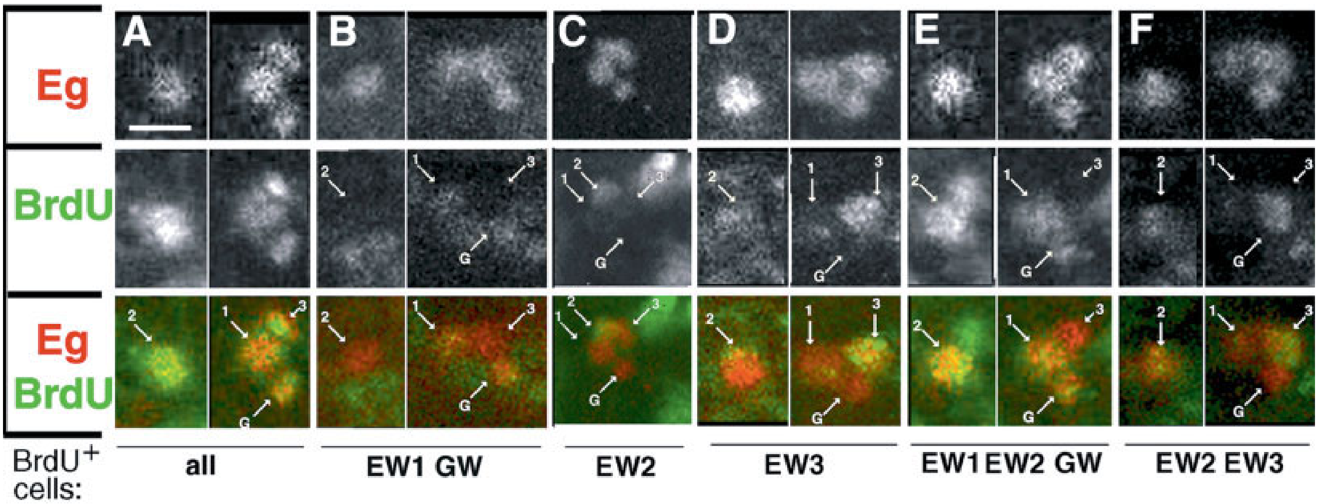

Fig. 1. Sibling relationship between neurons in the neuroblast (NB) 7-3 lineage. Bromodeoxyuridine (BrdU) pulse labeling was done at stages 11–12 and the embryos were aged to stage 17 and stained for Eagle (Eg; to identify all four neurons in the lineage) and BrdU. Top row, Eg expression; middle row, BrdU expression; bottom row, merged image showing Eg (red) and BrdU (green). **A–F:** Each panel shows two focal planes: a ventral focal plane containing the EW2 (2) neuron (left column), and a dorsal focal plane containing the GW (G), EW1 (1), and EW3 (3) neurons (right column). BrdU labeling was detected in the following patterns within the NB7-3 lineage: (A) all cells, n = 209; (B) EW1, GW, n = 14; (C) EW2, n = 32; (D) EW3, n = 89; (E) EW1, GW, EW2, n = 22; (F) EW2, EW3, n = 81. Scale bar = 2.5 μm in A (applies to A–F).

Generation of lacZ-marked cell clones in the CNS

P[ry$^+$ act5C-FRT-stop-FRT- tau lacZ]/CyO and *hs-flp (F-38)* flies (Buenzow and Holmgren, 1995) were mated and placed at 25°C. This method resulted in the appearance of less than five clones per hemisegment. Progeny were allowed to develop until stage 17 then were fixed and stained by standard methods (Doe, 1992) with antibodies to β-gal (to visualize the clone), Eagle (to identify all neurons in the 7-3 lineage), and Hb (to distinguish Hb$^+$ GW/EW1 from Hb$^-$ EW2/EW3 neurons).

RESULTS

Molecular markers uniquely label each neuron in the NB7-3 lineage

Before investigating the cell lineage of NB7-3, we need to identify markers that can be used to distinguish the neuronal progeny of the lineage. Fortunately, the NB7-3 progeny have been well characterized in *Drosophila* and grasshopper embryos, so there are a growing number of markers available. Wild-type stage 17 embryos have four neurons derived from the abdominal NB7-3 lineage: a motoneuron (GW) and three interneurons (EW1–3). Individual neurons can be identified by position, axon projection, and/or expression of a unique combination of molecular markers; in addition, GW can be identified by its small cell body size and unique motoneuron axon projection. Markers that label all neurons include the transcription factor Eagle (Eg) and an *eg-kinesin-lacZ* transgene, which reveals axonal projections (Higashijima et al., 1996), Eyeless (Ey), Engrailed (En), and Islet (Isl). Huckebein (Hkb) is expressed in all progeny of NB7-3 early in development but persists in only three neurons (GW, EW1, EW3) by stage 17. Markers that selectively label subsets of the neurons in this lineage include Kruppel (Kr; Isshiki et al., 2001), Zfh-2, Hunchback (Hb), Zfh-1 and Corazonin (Crz; Isshiki et al., 2001; Novotny et al., 2002; Lundell et al., 2003; Fig. 7).

Sibling relationship between neurons of the NB7-3 lineage

To define the sibling relationships between the four neurons in the NB7-3 lineage, we did BrdU pulse-labeling experiments (see Materials and Methods section for details). Sibling neurons should always be equally labeled, whereas nonsibling neurons should occasionally show unequal labeling (e.g., one positive, one negative). We stained the embryos for Eagle (to identify all four neurons), Hb (to distinguish Hb$^+$ GW/EW1 from Hb$^-$ EW2/EW3 neurons), and BrdU. We find that EW2 and EW3 can label together but are often labeled individually (Fig. 1F, C, and D, respectively), whereas EW1 and GW always label together (Fig. 1B,E). Thus, EW1/GW are siblings derived from a single GMC, whereas EW2 and EW3 are not siblings and must derive from at least two distinct GMCs (the identity of the EW2 and EW3 siblings is addressed below). These data suggest that there are at least three GMCs in the NB7-3 lineage, and they generate four neurons.

Timing of neuronal birth dates in the NB7-3 lineage

We used the same BrdU pulse-labeling experiment to determine the birth order of the four neurons. We pulsed from 4.5 hours of development (which typically labels the entire clone; data not shown) to 8.5 hours of development (which typically labels no cells in the clone; data not shown). These time points define the window of time in which cell divisions occur in the short NB7-3 lineage. By focusing on 1-hour intervals within this time window, we were able to determine the birth order of the neurons. Early pulses (4.5 hours) preferentially label the GW/EW1

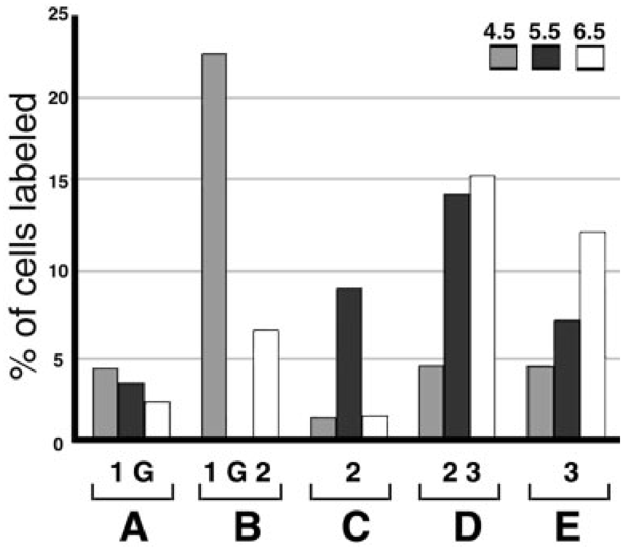

Fig. 2. Timing of neuronal birth dates in the neuroblast (NB) 7-3 lineage. Bromodeoxyuridine (BrdU) pulse labeling was done for 1 hour beginning at the indicated hour of embryonic development (shading key, top right). Embryos were then aged to stage 17 and quantitated for the identity of BrdU$^+$ neurons in the lineage (see Materials and Methods section for details). **A–E:** The different subsets of neuronal labeling observed; each bar represent the percentage that particular pattern was observed for each time point, excluding lineages with all or no Eg$^+$ cells labeled: (A) EW1, GW; (B) EW1, GW, EW2; (C) EW2; (D) EW3; (E) EW2, EW3. Neuron names abbreviated EW1, 1; GW, G; EW2, 2; EW3, 3.

sibling neurons (Fig. 2A) or the GW/EW1 sibling neurons plus the EW2 neuron (Fig. 2B). This finding shows that the GW/EW1 sibling neurons are born first and suggests that the EW2 neuron is born next. Indeed, a slightly later pulse (5.5 hours) preferentially labels the EW2 neuron (Fig. 2C) or EW2 and EW3 together (Fig. 2D). An even later pulse (6.5 hours) preferentially labels the EW3 neuron (Fig. 2E). Taken together, our data suggests that EW1/GW are born first, EW2 is born second, and EW3 is born last.

Lineage relationship between GMCs in the NB7-3 lineage

The BrdU-labeling data are consistent with a lineage in which NB7-3 sequentially produces three GMCs, with the first generating the GW/EW1 sibling neurons, the second generating EW2, and the third producing EW3 (Fig. 3A top), supporting the results of Novotny et al. (2002). However, alternative models were not addressed. Does the first-born GMC generate three cells (GW/EW1 siblings and EW2) as has been proposed for the first-born GMC from the Eg$^+$ NB6-4T lineage (Akiyama-Oda et al., 1999, 2000a,b)? To distinguish between GW/EW1 siblings and EW2 all being produced from the first GMC (Fig. 3A, middle panel) versus GW/EW1 and EW2 being produced from two different GMCs (Fig. 3A, top panel), we used genetic mosaic analysis to determine the sibling relationship between GMCs within the NB7-3 lineage and the Pros antibody marker to determine the number of GMCs produced by NB7-3. By using the hs-flp/FRT system (Buenzow and Holmgren, 1995), we induced heritably expressed β-galactosidase$^+$ cell clones within the CNS (see Materials and Methods section). We used hs-flp to induce clones at a low frequency of two to three clones per segment, thus ensuring that each cluster of β-galactosidase$^+$ cells represents a single clone. We find clones in which two EW neurons (presumably EW2 and EW3 by their position) are labeled together, whereas GW/EW1 are unlabeled (Fig. 3A bottom panel); this type of two-cell EW clone can only occur if the clone is induced in the NB after it produces GMC-1, and it allows us to make two conclusions: (1) GW/EW1 are produced before EW2 and EW3, confirming our BrdU pulse label data; and (2) the first GMC cannot generate the three GW/EW1 and EW2 neurons (ruling out a lineage tree similar to that proposed for NB6-4 (Akiyama-Oda et al., 1999, 2000a,b). We conclude that GMC-1 produces the GW/EW1 sibling neurons and that the subsequent GMC-2 and GMC-3 produce the EW2 and EW3 neurons, respectively (we have already determined that they are not siblings of a single GMC and that EW2 is born before EW3; see previous section).

To confirm that at least three GMCs are made by NB7-3, we stained *eg-kinesin-lacZ* embryos for β-galactosidase (to identify all cells in the NB7-3 lineage), Prospero (Pros; to identify both the NB, which has cortical Pros protein, and the GMCs/young neurons, which have nuclear Pros protein), and phospho-histone H3 (PH3; to identify mitotic cells). We observed lineages where there was a PH3$^+$, cortical Pros$^+$ mitotic NB7-3 (Fig. 3B, top panel), and in a more internal focal plane, we saw three nuclear Pros$^+$ smaller cells (Fig. 3B, bottom panel). This finding indicates that NB7-3 has divided at least twice (to produce GW/EW1 and GMC-2) and is in the process of budding off GMC-3. These data, taken together with data from the previous sections, allow us to conclude that NB7-3 makes 3 GMCs, which produce four neurons: the first GMC makes the GW/EW1 sibling neurons, the second GMC makes EW2, and the third GMC generates EW3.

EW2 sibling undergoes PCD

It is typically assumed that all GMCs produce a pair of neurons (Bauer, 1904; Goodman and Doe, 1993). We cannot detect a sibling for EW2 (or EW3), however, by BrdU-labeling experiments. In theory, the EW2 sibling could migrate away from the clone, die, or down-regulate Eg expression; alternatively, GMC-2 may differentiate directly into EW2 and have no sibling. We can rule out the possibility that the EW2 sibling migrates away or down-regulates Eg expression, because virtually all clones derived from DiI-labeled NB7-3 have only four cells in the clone (and in the entire segment containing the clone; Schmid et al., 1999). Furthermore, we can detect NB7-3 clones containing a mitotic NB and four nuclear Pros$^+$ cells more internally (Fig. 3C), which suggests that the first two GMCs have produced four neurons, and the mitotic NB is in the process of generating GMC-3. This finding shows that EW2 has a sibling neuron, subsequently called EW2sib, and that GMC-2 does not directly differentiate into EW2.

PCD had been observed in the 7-3 lineage using the deficiency *H99* (see Materials and Methods section for full genotype; Novotny et al., 2002). *H99* removes the *reaper* and *hid* cell death genes, and embryos homozygous for the *H99* deficiency (subsequently called "H99 embryos") have no detectable apoptosis in the embryonic CNS (White et al., 1994). In wild-type embryos, the NB7-3 lineage gen-

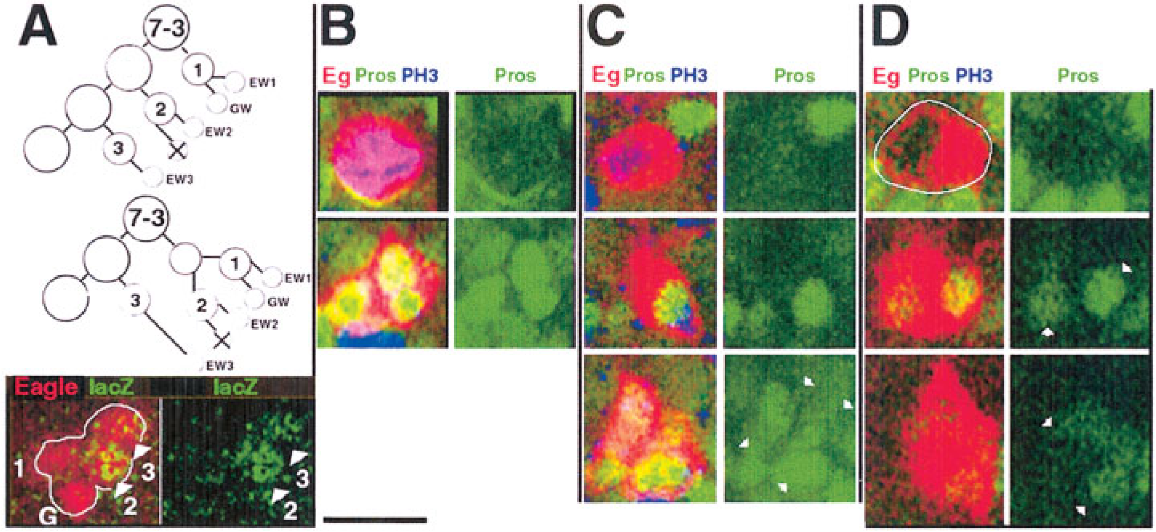

Fig. 3. Lineage relationship between ganglion mother cells (GMCs) in the neuroblast (NB) 7-3 lineage. **A:** FLP-FRT clonal analysis reveals GMC-1 produces only two neurons: GW and EW1. The top panels show two candidate NB7-3 lineages: the canonical NB lineage (top), and a noncanonical lineage similar to that proposed for NB6-4T (middle; Akiyama-Oda et al., 1999, 2000a,b). Bottom panel: A stage 17 embryo stained for Eagle (Eg; red) to detect the four neurons in the NB7-3 lineage in which we have induced a βgal^{+} cell clone (green) that specifically labels two EW neurons (presumably EW2 and EW3 by position; GW can be uniquely identified by size and posterior position and is βgal-negative). This clone could be obtained in a canonical lineage (gray shading, top lineage) but not in the noncanonical lineage (see text for details). B–D: Timeline of GMC and neuronal production in the NB7-3 lineage. The left column of each panel shows triple labeling of *eg-kinesin-lacZ* embryos for cytoplasmic β-gal (Eg; red; to label the NB7-3 lineage), Prospero (Pros; green; to distinguish NBs from GMC/neurons), and phospho-histone H3 (PH3; blue; to identify mitotic cells); the same image showing just Pros (green) is shown to the right. Each row shows a different focal plane of the same NB7-3 lineage, from the ventral NB layer (top) to more dorsal GMC/neuronal layers (middle and bottom). Midline, to left; anterior, up. **B:** NB7-3 produces three GMCs. At stage 13, NB7-3 has generated three nuclear Pros^{+} progeny (lower panel; the GW/EW1 neurons and GMC-2) and is dividing to produce GMC-3 as shown by the PH3^{+} cortical Pros^{+} mitotic NB (top panel). **C:** GMC-1 and GMC-2 each produce a pair of neurons. At stage 13–14, the NB7-3 has residual phospho-histone H3 (top panel), suggesting it has just budded of a GMC (GMC-3; middle panel); the lineage also contains four nuclear Pros^{+} neurons (bottom panel; white arrows), which develop from the earlier-born GMC-1 and GMC-2. **D:** NB7-3 can be transiently detected after all progeny are produced. At stage 14–15, NB7-3 has a triangular morphology with the nucleus pushed to one side (top panel). The final arrangement of the four EW1, EW2, EW3, and GW (labeled 1, 2, 3, G, respectively) neuronal progeny can be detected (middle and bottom panels). The last-born EW3 neuron has the strongest residual nuclear Pros staining. Scale bar = 4.9 μm B (applies to A–D).

erates four neurons at stage 17 (Fig. 4A,G,M,S,Y). Like Novotny et al. (2002), we find that *H99* embryos have an increase in the number of Eg+ cells in the 7-3 cluster. We find that *H99* embryos usually show five neurons at stage 17 (57%; Fig. 4B,H,N,N′,T,T′,Z), with the remainder of the lineages showing either four neurons like wild-type (20%; data not shown) or six neurons (16%; data not shown).

To determine the identity of the extra neurons in *H99* embryos, we used molecular markers that can uniquely identify all four mature neurons in the lineage. In wild-type embryos, GW and EW1 neurons express Zfh-1 and/or Hb (Figs. 4A,G, 5), whereas EW3 specifically expresses the neurotransmitter Crz (Fig. 4Y, 5). In *H99* embryos, there is little or no increase in the number of neurons expressing these markers (Fig. 4B,H,Z, 5), indicating that the extra neurons are not the GMC-1–derived GW/EW1 or the GMC-3–derived EW3. In wild-type embryos, the EW2 neuron expresses Kr and Zfh-2 (Fig. 4M,S, 5); *H99* embryos show an extra Kr^{+} neuron (30%; Figs. 4N′, 5) and an extra Zfh-2^{+} neuron (32%; Figs. 4T′, 5), consistent with the survival of a neuron with EW2-like cell fate. We conclude that GMC-2 divides by the canonical mode to generate two neurons, EW2/EW2sib, but the EW2sib normally undergoes PCD. When EW2sib is prevented from executing PCD, it differentiates as an interneuron expressing similar genes as EW2.

EW3 sibling appears to directly differentiate into EW3

We cannot detect a sibling of EW3 by BrdU-labeling experiments. As with EW2, it could have a sibling neuron that dies, migrates away, or down-regulates Eg; it also might directly differentiate from GMC-3. We can rule out the possibility that the EW3 sibling migrates away or down-regulates Eg expression, because virtually all clones derived from DiI-labeled NB7-3 have only four cells in the clone (and in the entire segment containing the clone; Schmid et al., 1999). Thus, GMC-3 either generates a pair of siblings with one undergoing PCD (similar to the GMC-2 lineage) or GMC-3 directly differentiates into EW3. We can distinguish between these models by examining *sanpodo* and *numb* mutations, which equalize sibling cell fate of all sibling neurons tested (Skeath and Doe, 1998; Novotny et al., 2002; Lundell et al., 2003). If GMC-3 divides to make EW3 and a sibling that undergoes cell death, then we should expect a pair of EW3 neurons in either *sanpodo* or *numb* mutants. We observe zero EW3 neurons in *numb* mutants; therefore, if this was a canonical GMC lineage, we should see two EW3 neurons in *sanpodo* mutants. In fact, we see this only 20% of the time (and it was only observed 9% of the time in Lundell et al., 2003). We propose that this unusual phenotype is due to EW3 and the terminal neuroblast being sibling cells

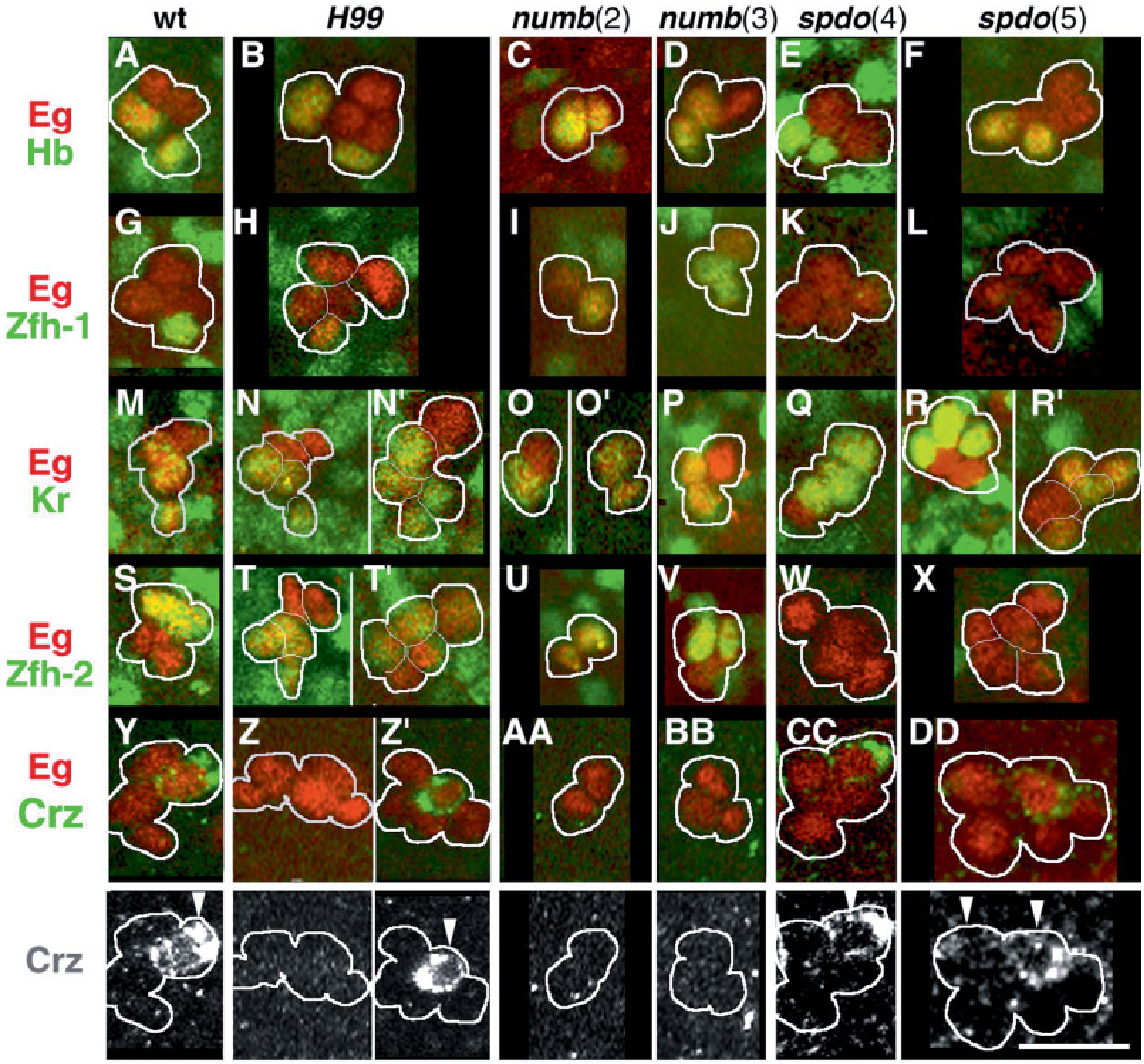

Fig. 4. The role of cell death and Notch/Numb signaling on the neuroblast (NB) 7-3 lineage. Stage 17 embryos stained for Eagle (Eg, red) and the indicated neuron-specific markers (green). Eg^+ cells are outlined in thick white lines; in some cases, individual Eg^+ cells are outlined in thin white lines. Kr-stained embryos are shown at stage 15. The bottom row shows Crz as a single label for clarity (cells identical to the double label row above). **A,G,M,S,Y:** Wild-type (wt) embryos: (A) Hb stains EW1, GW; (G) Zfh-1 stains GW; (M) Kr stains EW1, GW, EW2; (S) Zfh2 stains EW2, EW3; (Y) Crz stains EW3. **B,H,N,N′,T,T′,Z,Z′:** *H99* embryos containing five neurons in the NB7-3 lineage: (B) Hb labels EW1, GW; (H) Zfh-1 labels GW; (N,N′) Kr labels EW1, GW, EW2 61% of the time (N) and EW1, GW, EW2, EW2sib 36% of the time (N′); (T,T′) Zfh-2 labels EW2, EW3 61% of the time (T), but EW2, EW2sib, EW3 32% of the time (T′); (Z,Z′) Crz labels EW3 50% of the time. **C,I,O,O′,U,AA:** $numb^2$ embryos containing two neurons in the NB7-3 lineage: (C) Hb labels EW1, GW; (I) Zfh-1 labels EW1, GW; (O,O′) Kr labels EW1, GW 63% of the time (O) and either EW1 or GW 31% of the time (O′); (U) Zfh-2 labels two cells (see Discussion section); (AA) Crz does not label EW3. **D,J,P,V,BB:** $numb^2$ embryos containing three neurons in the NB7-3 lineage: (D) Hb labels EW1, GW; (J) Zfh-1 labels EW1, GW; (P) Kr labels EW1, GW; (V) Zfh2 labels three cells, but one is always much lighter (see Discussion section); (BB) Crz does not label EW3. **E,K,Q,W,CC:** $sanpodo^{zz27}$ embryos containing four neurons in the NB7-3 lineage: (E) Hb labels EW1, GW; (K) Zfh-1 does not label EW1, GW; (Q) Kr labels EW1, GW, EW2; (W) Zfh-2 labels no cells (see Discussion section); (CC) Crz labels EW3. **F,L,R,R′,X,DD:** $sanpodo^{zz27}$ embryos containing five neurons in the NB7-3 lineage: (F) Hb labels EW1, GW; (L) Zfh-1 does not label EW1, GW; (R,R′) Kr labels EW1, GW, EW2 63% of the time (R), but labels EW1, GW, EW2, EW2sib 36% of the time (R′); (X) Zfh-2 labels no cells (see Discussion section); (DD) Crz labels EW3 72% of the time and EW3. Arrowheads indicate Crz^+ cells. Scale bar = 4.5 μm.

(see Discussion section). It remains possible that GMC-3 may sometimes divide to make EW3/EW3sib (20% of the time) and sometimes directly differentiate into EW3 (80% of the time).

What is the fate of the terminal neuroblast in the NB7-3 lineage? After GMC-3 is born, at stage 13, we can transiently detect NB7-3 as a superficially positioned triangular-shaped cell with an accumulation of β-gal at

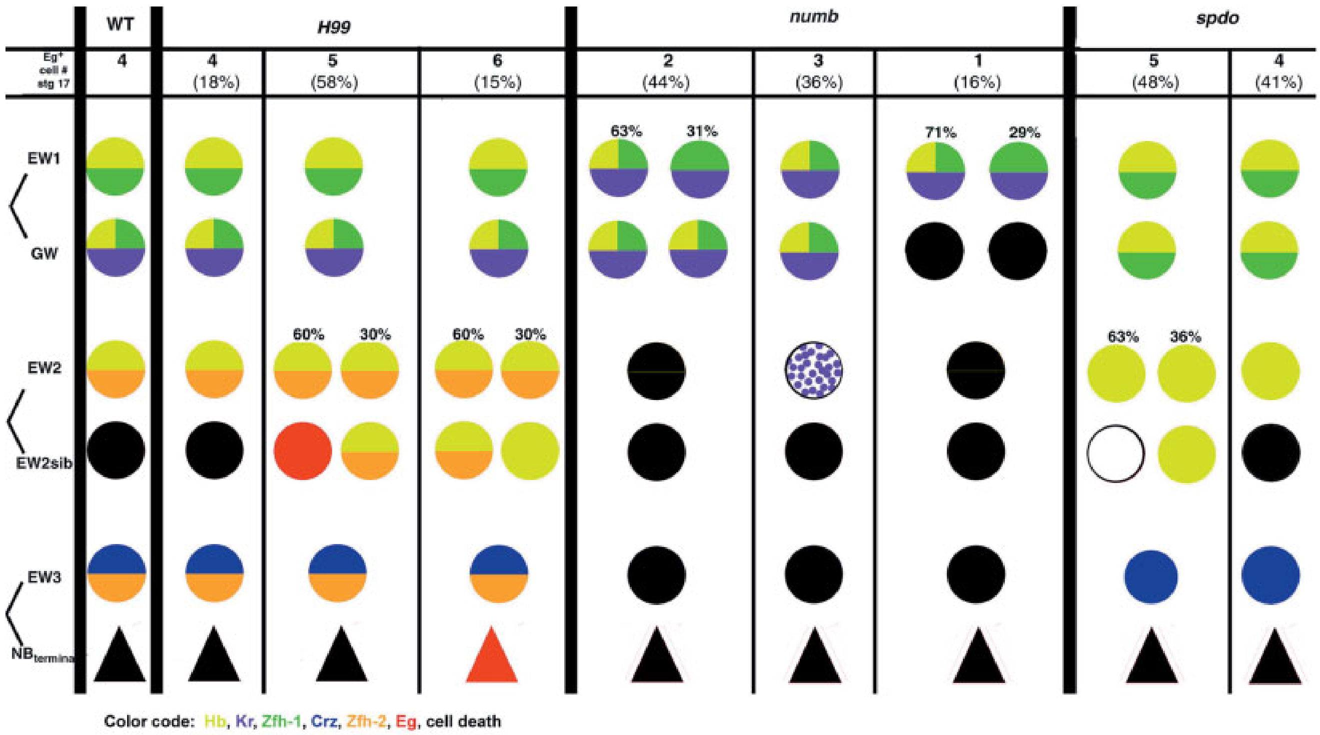

Fig. 5. Summary of the cell death and Notch/Numb signaling phenotypes in the neuroblast (NB) 7-3 lineage. Schematics showing the type of cell fates observed in the NB7-3 lineage in wild-type, *H99*, *numb*^{2}, and *sanpodo*^{zz27} mutant embryos. Circles represent postmitotic neurons; triangle is the terminal NB7-3. The normal EW1 is yellow/green; the normal EW2 is yellow/green/purple; the normal EW2sib is black (cell death); the normal EW3 is blue/orange; the normal EW3sib is black (cell death); and the normal terminal NB is black (cell death). In the mutant backgrounds, often we observe molecular marker combinations that do not precisely match a wild-type cell and presumably reflect a mixed or abnormal cell fate. The molecular marker color code used is Hb, yellow; Zfh-1, green; Crz, blue; Zfh-2, orange; Eg, red; cell death, black; Kr, purple; light Kr staining, purple dots. See Figure 4 and text for details.

one side (from *eg-kinesin-lacZ* expression; Fig. 3D). Although β-gal levels appear to remain constant, at later stages we cannot detect NB7-3. We speculate that NB7-3 undergoes PCD. Although we never or rarely detect it as a differentiated neuron in *H99* embryos, this finding may be because its cell type (NB) leads it to become mitotically quiescent and remain undifferentiated, similar to other NB's that survive until larval stages, rather than differentiate as a neuron.

Notch/Numb signaling directs sibling cell fate in the 7-3 lineage

It has been shown in several neural and myogenic lineages that Notch and Numb are involved in distinguishing sibling cell fate (Uemura et al., 1989; Guo et al., 1996; Spana and Doe, 1996; Carmena et al., 1998; Skeath and Doe, 1998). Numb inhibits Notch signaling (Frise et al., 1996); asymmetric localization of Numb protein into one sibling cell results in one sibling receiving Notch signaling (the Numb$^-$ cell) and the other sibling lacking Notch signaling (the Numb$^+$ cell; Uemura et al., 1989; Rhyu et al., 1994; Hirata et al., 1995; Spana and Doe, 1995, 1996; Guo et al., 1996; Kraut et al., 1996; Carmena et al., 1998; Skeath and Doe, 1998). To explore the role of Notch/Numb regulation of sibling fate in the NB7-3 lineage, we tested for asymmetric localization of a Partner of Numb (PON) -GFP fusion protein, which is known to be a reliable indicator of Numb protein localization (Lu et al., 1999). We find asymmetric basal localization of PON-GFP in the dividing NB7-3 (data not shown), similar to its reported pattern of localization in neuroblasts (Lu et al., 1999). In addition, we find asymmetric localization of PON-GFP in GMC-1 of the NB7-3 lineage (Figs. 6, 7).} This finding suggests that Numb is unequally distributed in the newborn GW/EW1 sibling neurons, so we decided to use a genetic approach to test the role of Notch/Numb signaling in specifying sibling cell fates within the NB7-3 lineage.

To determine how Numb affects the NB7-3 lineage, we stained homozygous *numb*^{2} *pr cn Bc* embryos (subsequently called *numb*^{2} embryos) for neuron-specific markers in the 7-3 lineage (Fig. 4; Table 1). This is the strongest *numb* allele available (Skeath and Doe, 1998) and is stronger than the *numb*^{1} allele used in Novotny et al. (2002) to study the NB7-3 lineage. In wild-type embryos, there are always four Eg$^+$ neurons. In *numb*^{2} embryos, there are most commonly just two neurons (46%; Fig. 4C,I,O, O′,U,AA); less common are clones with three neurons (38%; Figs. 4D,J,P,V,BB, 5) or one neuron (15%; data not shown). In clones with two neurons, both neurons express markers characteristic of the GW motoneuron (Hb$^+$ Fig. 4C, Zfh-1$^+$ Fig. 4I, Kr$^+$ Fig. 4O,O′ and Crz$^-$ Fig. 4AA), showing that Numb is required for specifying the EW1 sibling fate at the expense of the GW fate. That both EW2 and EW2sib fail to survive suggests that Numb is required

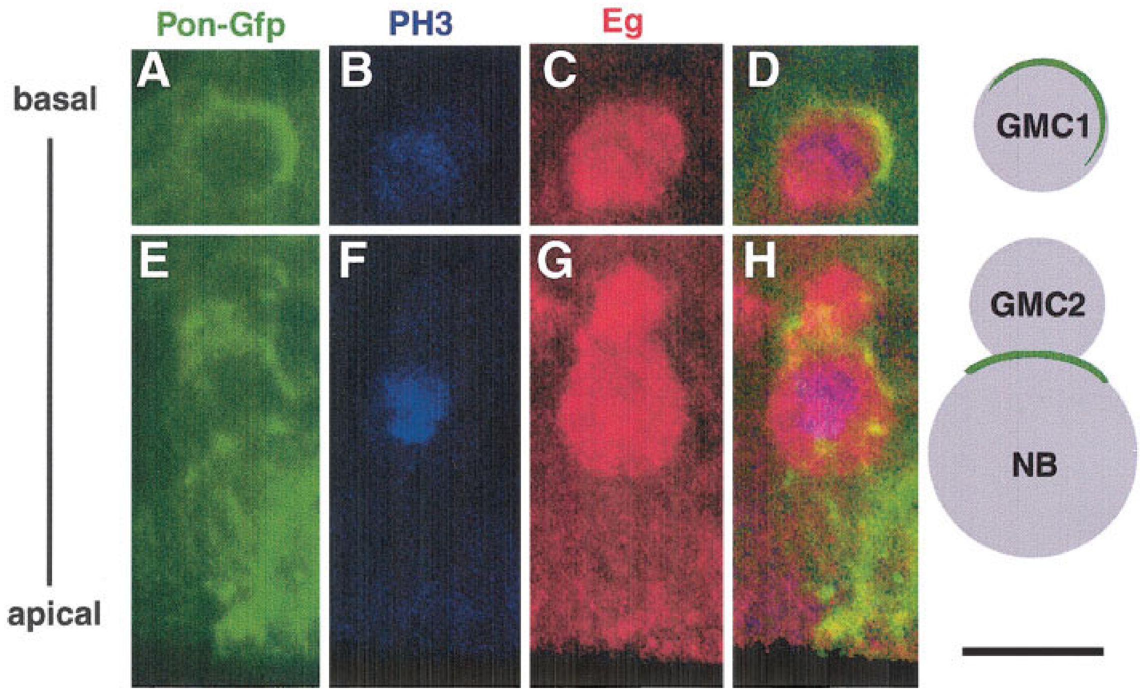

Fig. 6. Partner of Numb–green fluorescent protein (PON-GFP) fusion protein is asymmetrically localized in ganglion mother cell-1 (GMC1) in the neuroblast (NB) 7-3 lineage. Lateral view of a stage 12 embryo expressing a PON-GFP fusion protein (see Materials and Methods section) stained for Eagle (Eg; red, to mark the NB7-3 lineage), PON-GFP (green), and phospho-histone H3 (PH3, to mark mitotic cells). The top panel shows a focal plane containing the more internal GMC-1, which has an asymmetric cortical crescent of PON-GFP. The lower panel shows an adjacent focal plane containing the dividing NB7-3 (large cell) and the nondividing GMC-2. A merged image of all three single images is shown at right; schematic summarizing the expression pattern seen in this lineage shown at far right. Scale bar = 4.5 μm.

for the EW2 cell fate at the expense of the EW2sib (PCD) fate. Finally, the observation that EW3 fails to survive in all clones (clones of 1, 2, or 3 cells never express the EW3-specific Crz neurotransmitter; Fig. 4AA,BB) suggests that Numb is required for the EW3 cell fate at the expense of the EW3sib (PCD) fate. This phenotype for Numb function in a GMC is similar to that seen by Lundell et al. (2003; see Discussion section). Thus, we conclude that Numb acts to distinguish all sibling fates in the NB7-3 lineage, with Numb promoting EW1 over GW1, EW2 over EW2sib, and the EW3 over EW3sib.

To determine whether Notch signaling is required to specify Numb-independent sibling fates, as has been shown in many other lineages, we used the mutation *sanpodo*^{zz27} to block Notch signaling in the CNS. This mutation is a molecularly defined null sanpodo allele (O'Connor-Giles and Skeath, 2003). Embryos homozygous for *sanpodo* (subsequently called *sanpodo* ^{zz27} embryos) have been shown to mimic loss of Notch signaling in all sibling neurons tested (Buescher et al., 1998; Skeath and Doe, 1998), and this mutation has none of the massive neuroblast hypertrophy that makes classic Notch pathway mutations difficult to interpret for GMC and neuronal phenotypes. Wild-type embryos always have four Eg$^+$ neurons in the NB7-3 lineage, but *sanpodo* ^{zz27} embryos most commonly have five Eg$^+$ cells (48%; Fig. 4F,L,R,R′,X,DD), although clones are observed with four neurons (35%; Fig. 4E,K,Q,W,CC) and six neurons (15%; data not shown). What is the fate of the sibling neurons in *sanpodo* ^{zz27} embryos? All clones duplicate the EW1 interneuron (Hb$^+$ Fig. 4E,F, Zfh-1$^-$ Fig. 4K,L, Kr$^+$ Fig. 4Q,R,R′) at the expense of the GW motoneuron, a reciprocal phenotype to that seen in *numb*^{2} embryos. The five- and six-cell clones contain an extra neuron that lacks markers for GW (Fig. 4K,L) but expresses the EW2 marker Kr only 30% of the time (Figs. 4R′, 5), suggesting a partial transformation of EW2sib to the EW2 fate (not full survival, nor full expression of EW2 markers; see Discussion section). Again, this finding is reciprocal to the *numb*^{2} phenotype. Finally, the five-cell clones contain a Crz-positive neuron (72%; Fig. 4CC), but occasionally contain an extra Crz$^+$ neuron (20%; Fig. 4DD), suggesting a low frequency transformation of the terminal NB to the GMC-3/EW3 fate. Thus, we conclude that Notch signaling acts to distinguish all sibling fates in the NB7-3 lineage, promoting GW over EW1, EW2sib over EW2, and EW3sib over EW3.

DISCUSSION

Sibling relationships and the end of the NB7-3 lineage

We use BrdU labeling, positively marked clonal analysis, and antibody markers to define the complete cell lineage of NB7-3. The possibility of EW1 GW and EW2 all arising from the first GMC stem from several experiments from a different lineage NB6-4T and the cyclin A mutants in the NB7-3 lineage (Akiyama-Oda et al., 1999, 2000a,b; Novotny et al., 2002). Our experiments using positively marked clones conclusively rule out EW1, GW, and EW3 being siblings.

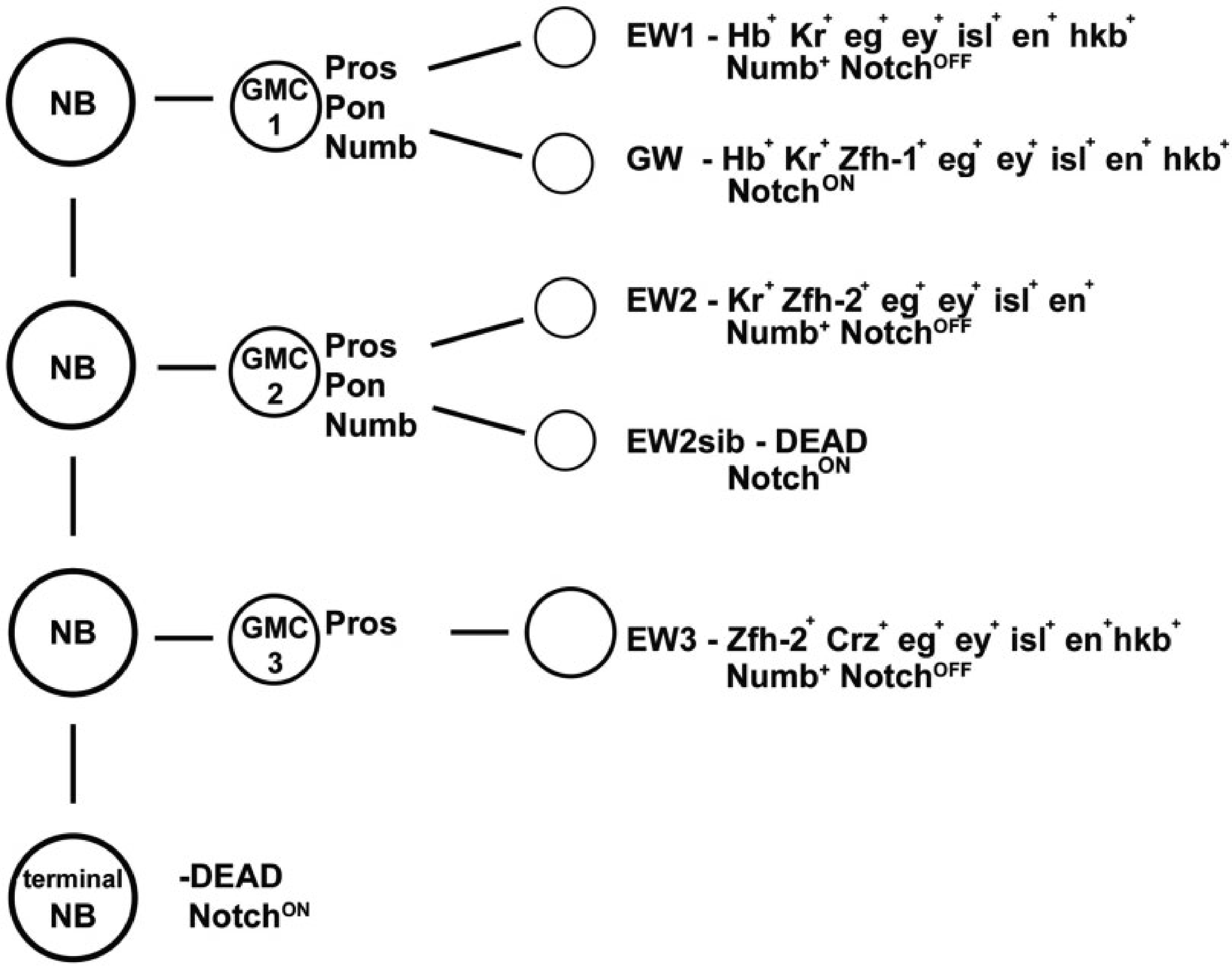

Fig. 7. Summary of the neuroblast (NB) 7-3 lineage. The NB7-3 divides to give rise to ganglion mother cell-1 (GMC-1) and a regenerated NB. This regenerated NB divides to give rise to GMC-2 and another regenerated NB. The final NB divides to give rise to GMC-3 and the terminal NB, which later undergoes PCD. GMC-1 divides to form EW1 and EW2. GMC-1 expresses Pros, which is divided equally between the sibs, and PON and Numb, which are asymmetrically segregated into EW1. GMC-2 divides to give rise to EW2 and EW2 sib with the latter undergoing PCD. GMC-2 also expresses Pros and PON in a manner similar to GMC-1, with pros being equally divided between the sibs and PON and Numb being localized into EW2. GMC-3 expresses Pros and directly differentiates into EW3. The genes expressed in the differentiated progeny are also shown.

TABLE 1. Quantitation of the Cell Death and Notch/Numb Signaling Phenotypes in the NB7-3 Lineage[1]

	Wild-type (y w)	numb[2]	sanpodo[zz27]	H99
Eagle	4.0 ± .29 (222)	2.3 ± .72 (343)	4.8 ± .73 (175)	5.0 ± .82 (282)
Hunchback	1.9 ± .24 (222)	1.8 ± .45 (121)	2.0 ± .13 (62)	2.0 ± 0 (199)
Zfh-1	1.0 ± .22 (123)	2.2 ± .72 (73)	0.1 ± .36 (14)	1.2 ± .55 (66)
Kruppel	3.1 ± .26 (215)	1.7 ± .58 (60)	3.4 ± .60 (48)	3.4 ± .60 (63)
Zfh-2	2.0 ± .23 (113)	2.1 ± .69 (144)	0.0 ± .18 (62)	2.3 ± .69 (57)
Corazonin	1.0 ± .22 (78)	2.8 ± .85 (65)	1.0 ± .42 (51)	0.9 ± .61 (83)

[1]The data represent the number of cells expressing neuron-specific markers for the NB7-3 lineage in the indicated genotypes. Rows indicate molecular markers; columns indicate homozygous genotype (see the Materials and Methods section for full genotypes).

We show that GMC-1 and GMC-2 divide by the classic mode to produce a pair of neurons (Bauer, 1904): GMC-1 produces the EW1 interneuron and GW motoneuron, and GMC-2 produces the EW2 interneuron and EW2sib cell (which undergoes PCD). We think the *sanpodo* and *numb* data argues against the model of GMC-3 development in which it undergoes a typical division to make two sibling neurons (EW3 and PCD) as suggested by Lundell et al. (2003). *numb* and *sanpodo* mutants give the opposite sibling cell fate transformations in all GMCs tested to date.

We find that *numb* mutants give zero EW3 neurons (similar to Lundell et al., 2003) but that *sanpodo* mutations give two EW3 neurons only 20% of the time. Thus, GMC-3 is atypical in its response to loss of *sanpodo*. We propose that this unusual phenotype is due to EW3 and the terminal neuroblast being sibling cells. It is also possible that GMC-3 may have a variable division pattern (perhaps based on its size): in some cases, it divides to make EW3/EW3sib (20% in this study), and in the remaining cases, it directly differentiate into EW3. In the latter situation, the difference in sibling cell types (neuroblast/GMC) may make it difficult or impossible for *sanpodo* mutations to equalize the cell fates. Both GMC-3 and the terminal neuroblast are exceptionally small, and this finding may contribute to both cells exiting the cell cycle. GMC-3 would differentiate into EW3, whereas the terminal neuroblast would undergo PCD. This model is consistent with all available data, including that of Lundell et al. (2003).

How does the NB7-3 lineage end? We suggest that NB7-3 undergoes PCD after generating GMC-3. We can transiently detect the NB after GMC-3 is born, but during this time window, it acquires a novel triangular morphology, the nucleus moves to a peripheral position, and then the cell rapidly disappears. We also do not observe the presence of the NB in DiI lineage studies (Schmid et al., 1999). In addition, death of the terminal NB is supported by TUNEL assays on wild-type embryos that find a fifth Eg^{+} cell present in the NB7-3 cluster during early stages but later undergoes cell death (Lundell et al., 2003). When we block the cell death of the terminal NB in the 7-3 lineage, we cannot say whether it has a normal NB identity or a novel identity, because we have no markers to identify the cell in late stage embryos. Permanently marking the NB using genetic mosaic techniques (Buenzow and Holmgren, 1995) or DiI labeling (Bossing et al., 1996; Schmid et al., 1999) in *H99* embryos would help resolve the fate of the terminal NB.

Lineage-specific PCD of an identified neuron

It is well known that cell death occurs in the embryonic CNS (Abrams et al., 1993; White et al., 1994, 1996), but whether this death is a stochastic occurrence or a reproducible fate of identified neurons has never been addressed. The idea of PCD in the NB7-3 lineage was first presented by Novotny et al. (2002). Although our results support the role of PCD in shaping the lineage, we add to this discovery by providing the first analysis of the identity of the extra cells found in *H99* mutants. In H99 mutants, we find 57% show an increase in Eg+ cells from four to five. We show that the fifth cell is the surviving EW2 sib as evidence by staining for EW2-specific markers, Kr and Zfh-2. Is cell death within the NB7-3 lineage evolutionarily conserved? In grasshopper embryos, NB7-3 has been proposed to generate two serotonergic interneurons from GMC-1 in most abdominal segments, and three serotonergic neurons from the first two GMCs in the first thoracic segments, and there is even a suggestion that a fourth GMC in this lineage may undergo PCD (Taghert and Goodman, 1984). These serotonergic neurons have similar axon projection profiles as the two serotonergic neurons in the *Drosophila* NB7-3 lineage (EW1 and EW2; Lundell et al., 1996). These data are consistent with cell death of a neuron in the GMC-2 and GMC-3 lineages, but they differ in finding two serotonergic interneurons derived from GMC-1, instead of the interneuron/motoneuron pair observed in *Drosophila*. Perhaps the grasshopper lineage does not include a GW motoneuron, or it is possible that this motoneuron was missed due to lack of heritable lineage markers. If so, it is possible that the two serotonergic interneurons do in fact derive from GMC-1 and GMC-2 as seen for *Drosophila*. Of interest, there is even an observation of PCD in this lineage, although the identity of the cell was though to be GMC-4, it may have been EW2sib (Taghert and Goodman, 1984).

Role of Notch/Numb signaling in the NB7-3 lineage

This is not the first time Notch/Numb signaling has been shown to play a role in sibling specification. Notch/Numb signaling regulates sibling cell fate of the MP2 CNS precursor (Spana et al., 1995), several identified embryonic GMCs (Buescher et al., 1998; Skeath and Doe, 1998; Wai et al., 1999), embryonic muscle founder cells (Ruiz Gomez and Bate, 1997; Carmena et al., 1998), and embryonic and adult external sense organ precursor lineages (Guo et al., 1996; Reddy and Rodrigues, 1999). In all cases assayed, loss of *numb* or *Notch* results in a reciprocal sibling cell fate duplication. We believe that Notch signaling is generically used to split one parental cell fate into two distinct sibling cell fates: one sibling has active Notch signaling and acquires the "A" fate, whereas the other sibling has delayed or no Notch signaling and acquires the "B" fate (Skeath and Doe, 1998). It is the identity of the parental cell that is critical for determining the final identity of A and B fates: if the parental cell is GMC-1 in the NB7-3 lineage, then the siblings are the EW1 interneuron and GW motoneuron; if the parental cell is the MP2, then the siblings are the dMP2 and vMP2 interneurons (Spana et al., 1995); and so on. It will be interesting to determine how Notch signaling interacts with parental cell-specific factors to confer distinct sibling cell fates.

Our work supports that of Lundell et al. (2003) in which they used different mutants (*numb*^{1} and *spdo* ^{G104}) and TUNEL to show that Notch/Numb directed cell death is an active player in shaping the fate of EW2 and EW3 in the NB 7-3 lineage. However, they do not address Numb localization, cell death in the fate of the terminal NB, nor the role of Notch/Numb in specification of the progeny of GMC-1, EW1, and GW. We have made two observations, relating to Notch/Numb signaling: (1) based on the asymmetric localization of Partner of Numb, Numb is present in GMC-3. (2) Notch/Numb are acting in GMC-1 to direct GW versus EW1 cell fate, such that Numb is required for EW1 cell fate.

One drawback to our lineage study is the limited number of molecular markers available for assaying neuronal identity. Although we offer some new markers for studying 7-3 lineage, including Kr, Isl, Hkb, and Eyeless, clearly more markers would be better. Any single marker may respond to a particular genetic background in a way inconsistent with its use as a cell fate marker (a trivial example is that Numb would not be a good marker for assaying *numb*^{2} mutations). In our case, we find that the marker Zfh-2 is expressed in a manner inconsistent with the cell fates it is supposed to mark in *numb*^{2} and *sanpod*^{zz27} mutants. In *numb*^{2} embryos, all markers except Zfh-2 indicated that the one or two remaining neurons have the GW fate, yet these "GW" neurons are abnormally Zfh-2^{+} (Fig. 4U,V). Conversely, in *sanpodo*^{zz27} embryos, none of the four to five neurons are Zfh-2^+, despite all other markers showing the presence of EW2 and EW3 cell fates, which normally are Zfh-2^+ (Fig. 4W,X). We conclude that Zfh-2 is expressed in direct response to Notch signaling in the NB7-3 lineage, which renders it useless as a marker for EW2/EW3 cell fates.

Knowledge of the NB7-3 cell lineage is a valuable resource for investigating the mechanisms controlling birth-order specification of GMC fates. In addition to the mutant studies done here, it has already been used by our lab and others to help understand the role of Hb and Kr, in specifying sequential GMC identity in the NB7-3 lineage and several other NB lineages (Isshiki et al., 2001). We have applied this information to study other GMC- or neuron-specific transcription factors. Information gleaned from studying these types of mutants may provide additional insight into GMC birth-order specification, motoneuron versus interneuron specification, induction of PCD, or axon pathfinding mechanisms. Finally, we have started by analyzing the simplest NB lineage in the embryo, but it should be possible to extend this type of study to more complex NB lineages. We have already performed preliminary studies showing that none of the five Eve^+ neurons (U1–U5) derived from the NB7-1 lineage are siblings (Pearson and Doe, 2003), consistent with previous work showed that each of these neurons has an Eve^- sibling (Skeath and Doe, 1998). Work of our lab and others has revealed the complete clone of neurons and glia produced by each embryonic neuroblasts (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). This study is a step toward our ultimate goal of extending our understanding to include the complete birth-order and sibling relationship (i.e., cell lineage) for all embryonic neuroblasts.

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