CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

Developmental Biology 336 (2009) 156–168

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

On the roles of *Notch*, *Delta*, *kuzbanian, and inscuteable* during the development of *Drosophila* embryonic neuroblast lineages

Gerald Udolph [a,d,*], Priyadarshini Rath [a], Murni Tio [b,d], Joanne Toh [d], Wanru Fang [d], Rahul Pandey [d], Gerhard M. Technau [c], William Chia [a,b,e]

[a] *Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Singapore*
[b] *MRC for Developmental Neurobiology, King's College London, New Hunt's House, London SE1 1UL, UK*
[c] *University of Mainz, Institute of Genetics, Saarstr. 21, 55122 Mainz, Germany*
[d] *Institute of Medical Biology, 8A Biomedical Grove, Singapore 138648, Singapore*
[e] *Temasek Life Science Laboratories and Department of Biological Science, National University of Singapore, 1 Research Link, Singapore 117604, Singapore*

ARTICLE INFO

Article history:
Received for publication 8 January 2009
Revised 11 September 2009
Accepted 20 September 2009
Available online 25 September 2009

Keywords:
Drosophila
Neurogenesis
Notch
kuzbanian
Asymmetric cell division
Cell fate specification

ABSTRACT

The generation of cellular diversity in the nervous system involves the mechanism of asymmetric cell division. Besides an array of molecules, including the Par protein cassette, a heterotrimeric G protein signalling complex, Inscuteable plays a major role in controlling asymmetric cell division, which ultimately leads to differential activation of the Notch signalling pathway and correct specification of the two daughter cells. In this context, *Notch* is required to be active in one sibling and inactive in the other. Here, we investigated the requirement of genes previously known to play key roles in sibling cell fate specification such as members of the Notch signalling pathway, e.g., *Notch* (*N*), *Delta* (*Dl*), and *kuzbanian* (*kuz*) and a crucial regulator of asymmetric cell division, *inscuteable* (*insc*) throughout lineage progression of 4 neuroblasts (NB1-1, MP2, NB4-2, and NB7-1). *Notch*-mediated cell fate specification defects were cell-autonomous and were observed in all neuroblast lineages even in cells born from late ganglion mother cells (GMC) within the lineages. We also show that *Dl* functions non-autonomously during NB lineage progression and clonal cells do not require *Dl* from within the clone. This suggests that within a NB lineage *Dl* is dispensable for sibling cell fate specification. Furthermore, we provide evidence that *kuz* is involved in sibling cell fate specification in the central nervous system. It is cell-autonomously required in the same postmitotic cells which also depend on *Notch* function. This indicates that KUZ is required to facilitate a functional Notch signal in the *Notch*-dependent cell for correct cell fate specification. Finally, we show that three neuroblast lineages (NB1-1, NB4-2, and NB7-1) require *insc* function for sibling cell fate specification in cells born from early GMCs whereas *insc* is not required in cells born from later GMCs of the same lineages. Thus, there is differential requirement for *insc* for cell fate specification depending on the stage of lineage progression of NBs.

© 2009 Elsevier Inc. All rights reserved.

Introduction

A fundamental question in neurobiology is how complexity and cellular diversity in the central nervous system (CNS) is created by mechanisms such as cell proliferation, determination, and differentiation. *Drosophila* is an ideal model system to study these mechanisms at the level of single identified cells. In the fruitfly, the embryonic CNS derives largely from neural progenitors, called neuroblasts (NBs). During embryonic development, approximately 30 NBs are generated per thoracic/abdominal hemisegment. NBs are neural stem cells that during each division regenerate themselves and give rise to a secondary precursor cell called ganglion mother cell (GMC). Each GMC undergoes a final cell cycle and generates two postmitotic siblings which differentiate into neurons and/or glial cells, or undergo programmed cell death. Cell lineage analysis has demonstrated that each NB generates a stereotypic set of unique and identifiable daughter cells (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997; Udolph et al., 1993) and these identifiable cells can retrospectively serve as indicators for the parent NB lineage from which these cells were generated.

NBs as well as GMCs have been shown to divide in a polarised fashion producing progeny with distinct identities (Jan and Jan, 2000). These asymmetric divisions provide a key mechanism for the generation of cellular diversity within NB lineages. Intrinsic as well as extrinsic cues play crucial roles in the specification of distinct sibling cell fates. Part of the extrinsic mechanisms is the activation of the Notch signalling pathway which depends on the correct asymmetric distribution of Numb for the specification of distinct

* Corresponding author. Institute of Medical Biology, 8A Biomedical Grove, Singapore 138648, Singapore.
E-mail address: gerald.udolph@imb.a-star.edu.sg (G. Udolph).

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.09.030

fates of sibling cells in several contexts (Buescher et al., 1998; Guo et al., 1996; Spana and Doe, 1996). Numb physically interacts with Notch and negatively regulates Notch signalling in only one of the siblings arising through asymmetric cell division, thus providing a direct link between intrinsic and extrinsic mechanisms during sibling cell fate specification. The current model implies that the fate of one of the two siblings strictly depends on functional Notch signalling (the *Notch*-dependent sibling) whereas the other sibling (the *Notch*-independent sibling) adopts a default fate as it differentiates correctly only if Notch signalling is inhibited. The event of permitting or inhibiting Notch signalling in the respective postmitotic siblings through the asymmetric segregation of the intrinsic cue Numb seems to be crucial for correct cell fate specification, therefore assigning an important role to Notch in the process of cell fate specification. However, a limitation of this model is that most of the sibling cell fate pairs analyzed so far represent early born lineage components normally derived from the first GMC of the respective NBs. Little is known if mechanisms of asymmetric cell division mediated by Insc as well as Notch-mediated cell fate specification are utilized in GMCs derived from later phases of NB lineage progression. Analysis of entire neuroblast lineages would give further insight into the relative importance of intrinsic cues like Inscuteable-mediated asymmetric cell division and extrinsic mechanism of Notch-mediated cell fate specification during both the early as well as the later stages of NB lineage progression.

The Notch signal transduction pathway is highly conserved and its function is tightly regulated. The transmembrane protein Notch undergoes a stepwise process of maturation involving a cascade of proteolytic cleavages. This processing generates the mature and active form of the Notch receptor. After ligand binding, the extracellular part of the Notch protein is cleaved off, a process referred to as ectodomain shedding, resulting in a truncated transmembrane fragment with a very short extracellular domain (Fortini, 2001). This truncation is a prerequisite for a subsequent cleavage in the membrane-anchored intracellular domain of Notch releasing it from the membrane. Its subsequent translocation to the nucleus eventually regulates gene expression. It has been demonstrated that *Drosophila* INSC, a metalloprotease belonging to the ADAM family of proteases, as well as its vertebrate homologues might participate in the cleavage of the extracellular domain of Notch after ligand binding, thus facilitating the subsequent cleavage releasing the intracellular domain (Brou et al., 2000; Mumm et al., 2000). Evidence has also been provided that KUZ processes the Notch ligand Delta (Qi et al., 1999). *kuz* has been found to play a role during heart formation (Albrecht et al., 2006) and it is also required for the localized activation of Notch during oogenesis (Wang et al., 2007). Furthermore, it has been shown that during wing development, *kuz* acts strictly cell-autonomously (Klein, 2002). However, the exact mechanism of *kuz* function has not been studied in the context of nervous system development and NB lineage progression. Similarly, the source of Delta ligand which is utilised to activate Notch in one of two sibling cells appears to differ depending on the system and cellular context (Greenwald, 1989; Kuwada and Goodman, 1985). In the peripheral nervous system (PNS) in *Drosophila*, sibling cell fate resolution seems to require the sibling of the cell activating Notch to provide the appropriate ligand (Zeng et al., 1998). In the CNS, two types of observations (Buescher et al., 1998; Spana and Doe, 1996) suggested that the source of the Notch ligand necessary for activation of Notch signalling can originate from outside of the sibling pair. This conclusion was based on studying two sibling cell fate pairs (vMP2/dMP2 and RP2/RP2sib, respectively) *in vitro* (Spana and Doe, 1996) and of a mutation affecting cell cycle progression (Buescher et al., 1998). It has not been demonstrated whether *Dl* is required or might be redundant within the lineage itself.

Preceding the extrinsic mechanism of sibling cell fate specification are the intrinsic mechanisms involving asymmetric cell divisions that result in distinct sibling cell fate specification. Asymmetric cell division involves the coordinated control of spindle orientation and asymmetric localisation of cellular determinants, such as mRNAs and proteins, into specific compartments of the dividing progenitor prior to cytokinesis. This polarity is mediated by an apically localised protein complex, including Insc (Kraut and Campos-Ortega, 1996; Kraut et al., 1996); the Par proteins: Bazooka (Schober et al., 1999; Wodarz et al., 1999), DaPKC (Wodarz et al., 2000), and DmPar6 (Petronczki and Knoblich, 2001), a protein cassette involved in heterotrimeric G protein signalling: Gαi (Yu et al., 2003), Partner of inscuteable (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), and Locomotion defects (Yu et al., 2005). INSC has been described as a major organizer of the apical complex controlling apical–basal spindle orientation, basal localization of protein determinants as well as asymmetric size of the daughter cells by coupling cell polarity to spindle positioning pathways thereby controlling downstream sibling cell fate specification via the extrinsic mechanisms (Siller and Doe, 2009; Wodarz, 2005).

Here, we investigate the involvement of *N*, *Dl*, *kuz*, and *insc* during the process of asymmetric cell division and sibling cell fate specification in the embryonic ventral nerve cord by performing clonal analysis of complete embryonic CNS lineages derived from single *N*, *Dl*, *kuz*, or *insc* mutant cells. Our data show that *Notch* as well as *kuz* exhibited identical cell fate specification phenotypes in NB clones in a cell-autonomous manner. *Dl* mutant lineages were completely wild type suggesting that Delta is not required for activation of Notch in cells within the lineages. However, Delta is required for sibling cell fate specification per se as in *Dl* mutant embryos sibling cell fate specification defects could be observed. This suggests a non-autonomous function of *Dl* which might originate from outside of the NB lineages such as from the underlying ectoderm and/or the surrounding mesoderm. We also extend the current understanding on the roles of *insc* in differential cell fate specification of sibling cells. We demonstrate that although Insc is essential for cell fate specification in the first-born cells of the studied NB lineages, it was not required for cell fate specification in later born cells in the same lineage where Notch is still essential. We demonstrate that *Notch* is required more generally and beyond the requirement of *insc* for cell fate specification in NB lineages. Our findings therefore uncouple intrinsic mechanisms of asymmetric cell division in early born cells within NB lineages mediated by INSC and extrinsic mechanism of cell fate specification in both early and late born cells via the Notch signalling pathway.

Materials and methods

Fly stocks

The following fly stocks were used: $Canton^{S}$, N^{55e11}, Dl^{9P}, *sca-Gal4*, $kuz^{1405(FRT40A)}$ (all from Bloomington Stock Center); *UAS-*kuz^{DN} (from G. Rubin); $insc^{22}$ (from R. Kraut).

Transplantation technique

The transplantation procedure was performed according to previously published techniques (Prokop and Technau, 1993). Generally, cells were taken from ventral positions of the neuroectoderm (0%–20% VD) of donor embryos and were isotopically implanted into *wt* host embryos of same age. After transplantation, embryos were raised until stage 16, fixed, stained, and resulting cell lineages in the nervous system were compared to their wild type counterparts. The heterogenetic transplantations were generally performed as follows: neuroectodermal cells (stage 7) were removed from HRP-labelled mutant donors at 0%–20% ventral–dorsal diameter. Single cells were subsequently implanted into an equivalent position of *wt* host embryos at the same stage. Homozygous mutant donor embryos were identified by staining each donor separately for the presence of a

blue-balancer chromosome (see also Udolph et al., 1998). Subsequently, embryos were subjected to the standard post-transplantation protocol (Prokop and Technau, 1993). Neuroblast lineages were identified based on the analysis of indicator cell types generated within lineages. Criteria such as lineage context of cells, their location with respect to landmarks and lineage siblings and axonal projection patterns were used. For description of *wt* lineages and their identified cell types, see Bossing et al. (1996), Schmid et al. (1999), Schmidt et al. (1997), and http://flybrain.neurobio.arizona.edu/Flybrain/html/contrib/1998/technau/index.html. Description of individual neurons was based on the nomenclature developed by Bossing et al. (1996), except for the EVE-positive neurons in the NB7-1 which were termed U-neurons according to Pearson and Doe (2003). kuz^{DN}-expressing cells were taken from *sca-Gal4:UAS-*kuz^{DN} donor embryos and were implanted into *wt* hosts.

Generation of kuz germ line clones

kuz germ line clone embryos were produced using the FLP/DFS technique that is routinely used to generate germ line mosaics in *Drosophila* (Chou and Perrimon, 1992). The kuz^{1405}*-FRT/CyO*; *hsFlp*, *MKRS*/+ strain was crossed to $ovo^{D1\text{-}18}$, *FRT/CyO* males. Offspring of this cross were heat shocked for 1 h at the third instar larval stage on two consecutive days. Females of the genotype kuz^{1405}/kuz^{1405}; *hsFLP*, *MKRS* were collected and backcrossed to the parental males of kuz^{1405}/ *Cyo, ftz-lacZ.*

Immunohistochemistry

Antibody staining was done according to published information (Schmidt-Ott and Technau, 1992). The following primary antibodies were used: α-Even-skipped (Frasch et al., 1986); α -Zfh-1 (Lai et al., 1991); α-22C10, α-Delta (DSHB, Iowa); α-β-GAL (Promega); α-ODD (Ward and Coulter, 2000); α-INSC (Tio et al., 2001); and α-MEF2 (Bour et al., 1995). Secondary antibodies used throughout this study were coupled either to FITC, Cy3, or HRP (Jackson). Topro-3 was used to label DNA. Images were taken using either an upright confocal microscope (Olympus) or an Axioimager upright system (Zeiss).

Construction of elav-mCD8-GFP transgene

mCD8-GFP was amplified by PCR using primers RP9 (AATT GCGGCCGC CAA AAT GGC CTC ACC GTT GAC C) and RP10 (AATT GAGCTC TTA TTT GTA TAG TTC ATC CAT GCC AT) from *pUAST-mCD8-GFP* (Lee and Luo, 1999) and cloned into Bluescript (pBS, Strategene) with *Not*I and *Sac*I as the cloning sites. This construct was then cut with *Kpn*I and *Not*I and ligated to a 3.5-kb *Kpn*I–*Not*I-digested fragment containing the *elav* promoter (Yao and White, 1994). Finally, *elav-mCD8-GFP* was cut with *Sal*I and cloned into *Xho*I cleaved transformation vector pCaSpeR4. Germ line transformants were generated by Bestgene Inc, CA.

Results

Our previous study on the role of *insc* in the context of lineage development and asymmetric cell fate choice has been focused on a particular atypical NB cell lineage, MP2 (Rath et al., 2002). Here, we extend our investigations on the roles of *N*, *Dl*, *kuz*, and *insc* in sibling cell fate specification in 4 neuroblast lineages (NB1-1, MP2, NB4-2, and NB7-1). These lineages include well-described sibling cell pairs such as the aCC/pCC neurons and the RP2/RP2sib neurons which are easily identifiable by cell specific markers and morphology. Furthermore, as a group, they contain a simple lineage consisting of only 2 cells (MP2), two neuroblasts giving rise to neurons only (NB4-2, NB7-1) and a neuroglioblast lineage producing both neurons and glia (NB1-1).

Notch is cell-autonomously required for cell fate specification during early and late neuroblast lineage development

The transplantation technique allows for analysis of complete cell lineages of individual NBs thereby giving access to early as well as late lineage components at the resolution of individual cells. Specific neuroblast lineages were identified by the stereotypic location of cells with regards to typical landmarks of the VNC, the presence of typical indicator cells and typical projection patterns of neurons. Donor cells derived from N^{55e11} embryos were transplanted into *wt* hosts and our analysis was focused on 4 stereotypic neuroblast lineages that contain well-described sibling pairs of neurons with distinct identities, which could be scored on the basis of morphological criteria and differential marker gene expression. In the following, we compare neuroblast lineages derived from wild type cells to those produced by *Notch* mutant cells.

NB1-1 (Figs. 1A and B; Suppl. Tab. 1): The *wt* abdominal NB1-1A consists of typical indicator cells, among them the aCC/pCC neurons, 2–3 glial cells which belong to the group of subperineural glia (SPG) (Ito et al., 1995) and 4–5 clustered interneurons with typical projections along the ipsilateral connective towards the posterior (Bossing et al., 1996; Udolph et al., 1993). The aCC and its sibling pCC are derived from the first GMC of NB1-1. Two of the SPG cells are located in close proximity to the aCC/pCC neurons and are termed the A and B glia (Klämbt and Goodman, 1991). A third SPG (LV–SPG) is found ventral to the cluster of interneurons.

We found major changes in the cellular composition of the NB1-1 lineage derived from *Notch* mutant cells (Fig. 1B; Suppl. Tab. 1; see also Udolph et al., 2001). Like in *wt* clones, we detected two neurons in dorsal position of the nerve cord where normally the aCC/pCC neurons are located. However, the axonal projections of both cells were typically aCC-like and innervated the dorsal muscle 1 (DO1). In some of the specimens where we could not directly observe the innervation of muscle DO1, the aCC motoaxon nevertheless projected to the dorsal-most territory and in the vicinity of DO1. Cells with pCC-like projections could not be detected in *Notch* clones. Thus, we found the expected duplication of the aCC neuron at the expense of the pCC neuron. Our experiments revealed that differentiation of other cell types of the NB 1-1A lineage were also affected in *Notch*. In *Notch* mutant clones, the typical SPG glial components were lost and this loss was always accompanied by a gain of neurons within the interneuronal cluster, indicating a sibling relationship between neurons and glia (Udolph et al., 2001). Since the neurons of this cluster normally tightly fasciculate, it was difficult to analyze any change in the projection pattern of individual neurons. In some cases, however, the fascicle of the posterior projections seemed to contain more axons as judged from an increased size of the fascicle.

MP2 (Figs. 1C and D; Suppl. Tab. 2): The wild type MP2 precursor normally divides once producing two sibling neurons, dMP2 and vMP2. vMP2 extends a single axon projecting along the ipsilateral connective in an anterior direction whereas the dMP2 has a bipolar axon projecting anteriorly as well as posteriorly (Fig. 1C). Thirteen MP2 clones were obtained from N^{55e11} transplantations. With the exception of one clone which showed *wt* projection patterns, the remaining 12 clones showed dMP2-like axonal projections by both daughter cells that is also accompanied by the absence of vMP2 axonal projections indicating a vMP2 to dMP2 cell fate transformation (Fig. 1D) (Broadus et al., 1995).

NB4-2 (Figs. 1E and F; Suppl. Tab. 3): The first-born neurons from GMC4-2a of the *wt* NB4-2 lineage are the RP2/RP2sib pair of sibling neurons. RP2 is a motor neuron with a unique position within the nerve cord. It extends a motor axon into the periphery innervating the dorsal muscle DO2. The potential RP2sib neuron also occupies a characteristic position ventral to the neuropile. The lineage further consists of a cluster of neurons of which some are motor neurons (4-2MN) that fasciculate and leave the CNS through the intersegmental

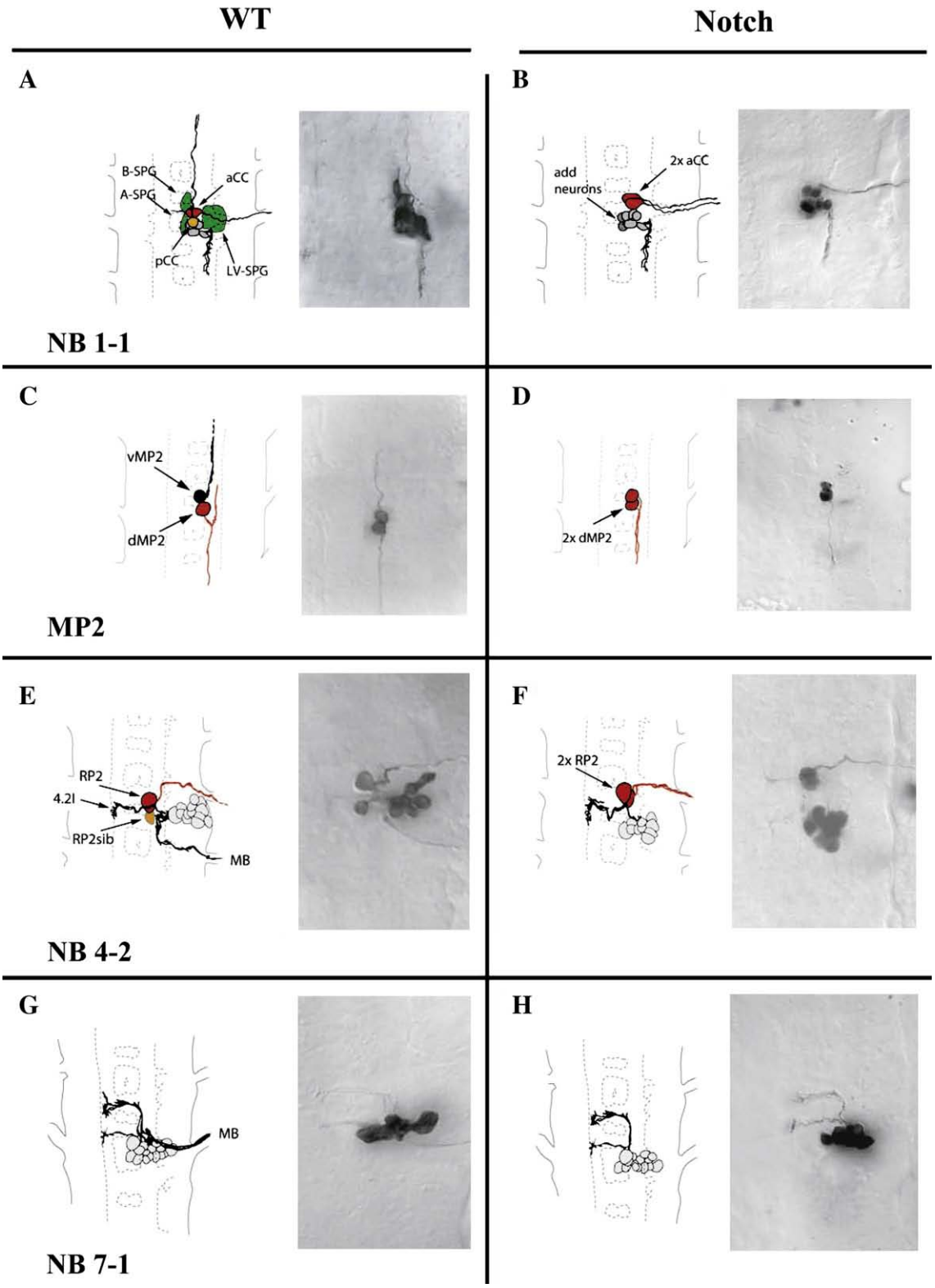


Fig. 1. Comparison of *wt* neuroblast lineages of NB1-1, MP2, NB4-2, and NB7-1 to lineages derived from transplantation of *Notch* mutant cells into *wt* background. Schematic drawings as well as photographs of specimen are presented in a horizontal view with anterior to the top. (A, C, E, G) Wild type lineages of neuroblasts as indicated. (B, D, F, H) Neuroblast lineages derived from *Notch* mutant cells. (A) The wild type lineage of NB1-1 consists of the aCC motor neuron (red) and the pCC interneuron (orange), a set of 2–3 glial cells (green), and a cluster of 5–6 interneurons (grey). (B) *Notch* mutant NB1-1 shows two aCCs (red) at the expense of the pCC neuron, absence of glial cells and additional neurons in the cluster of interneurons (dark grey). (C) The wild type MP2 lineage consists of the vMP2 (blue) and the dMP2 (red) neurons. (D) The mutant MP2 lineages duplicate the dMP2 neuron at the expense of its sibling vMP2. (E) The wild type lineage of NB4-2 consists of the RP2 motoneuron (red) and RP2sib neuron (orange), and a lateral cluster of cells (grey) which includes motoneurons with a motoneuronal bundle (MB) projecting out of the ventral nerve cord, and interneurons (4.2I) projecting toward and through the posterior commissure. (F) The mutant NB4-2 lineage consists of two RP2 neurons (red) and does not produce any RP2sib cell. While the MB from the lateral cluster is absent, the 4.2I interneurons are still visible. (G) The wt NB7-1 lineage consists of up to 5 pairs of U-sibs and U-neurons which are all located in a group of neurons (grey). The U-neurons are motor neurons forming a motoneuronal bundle (MB) projecting to the periphery. Note the interneuronal projections through both commissures to the contralateral neuropile. (H) The mutant NB7-1 lineage shows a cell number comparable to the wt clone; however, the MB bundle is undetectable.

nerve. In addition, a single interneuron projects contralaterally through the anterior commissure (NB4-2I).

We obtained three NB4-2 clones derived from *Notch* mutant cells. In all of these clones, we found duplication of the RP2 neurons at the expense of its siblings RP2sibs (Fig. 1F; Suppl. Tab. 3). We could detect two cells in the dorsal position of the nerve cord typical of the RP2 neuron, with motoneuronal projections leaving the CNS through the segmental nerve (SN). The duplication of these dorsal cells was accompanied by the loss of the ventral RP2 sib neurons. In addition, the 4-2MN projections were entirely missing indicating a function of *Notch* in their specification or axonal pathfinding.

NB7-1 (Figs. 1G and H; Suppl. Tab. 4): The *wt* lineage of NB 7-1 consists of interneurons as well as motor neurons. NB7-1 gives rise to about 16-22 neurons from potentially 8 to 11 GMCs. The first 5 GMCs from NB7-1 give rise to the 5 U-neurons which are motoneurons and 5 U-sib interneurons (Pearson and Doe, 2003). U-neurons can also be identified by the expression of Eve. Their axons leave the ventral nervous system through the intersegmental nerve (ISN) (Bossing et al., 1996; Pearson and Doe, 2003). The Eve-negative U-sib interneurons project through the anterior and posterior commissures. The remainder of the cells in the lineage (6–12) do not have any obvious axonal phenotype in the embryo.

In total, we analyzed 10 *Notch* mutant NB 7-1 clones. In 8 of the clones, we found complete absence of the motoneuronal fascicle which indicates a loss of U-neurons (Fig. 1H). In the other 2 clones, we found at least 1 U-neuron motoaxon and the fascicles leaving the CNS appeared thinner as a consequence. We also observed thickened axonal fascicles running through the anterior and posterior commissures in some clones, potentially indicating the presence of more interneurons, possibly U-sib neurons. These observations are consistent with the notion that U-neurons are transformed into U-sib neurons.

In summary, we observed *Notch*-related cell fate transformations in all four NB lineages studied here. The observation of changes with regards to axonal projection patterns of indicator cells born later in these lineages are novel. Our data indicate that *Notch* function is also required for cells which are born from later GMCs during NB lineage progression. The clonal analysis also demonstrates that *Notch* acts in a cell-autonomous manner during sibling cell fate specification in NB lineages.

Dl functions non-autonomously in sibling cell fate specification

To study whether the Notch ligand Delta is required autonomously or non-autonomously for sibling cell fate specification, we harvested neuroectodermal cells from Dl^{9P} donors and transplanted them individually into *wild type* hosts. The resulting NB lineages were analyzed on the cellular level. We examined 12 clones representing *Dl* mutant lineages of NB1-1 ($n=4$; Suppl. Tab.1), MP2 ($n=7$; Suppl. Tab. 2), and NB7-1 ($n=1$; Suppl. Tab. 4). We did not obtain any NB4-2 lineage. It was observed that the MP2 clones correctly resolved into dMP2 and vMP2 neurons as judged by cellular morphology, positioning of cells, and axonal projection patterns. The NB1-1 lineages correctly resolved into the sibling pair of aCC and pCC neurons. In addition, the appropriate numbers of *Notch*-dependent glial cells at correct positions within the nerve cord were produced. We also did not detect any cell fate changes in the NB7-1 lineage as the motoneuronal fascicle was present in all clones, suggesting correct specification of the U-neurons and their siblings, the U-sibs. Thus, cell fate changes could not be detected in these three lineages. Therefore, *Dl* is not required for cell fate specification in the mutant lineages.

In order to study the requirement for *Dl* during cell fate specification we analyzed the phenotype of Dl^{9P} mutant embryos in more detail. A scoreable indicator pair of sibling neurons is represented by the progeny of the first GMC of NB4-2, GMC4-2a, which gives rise to the sibling neurons, RP2/RP2sib. A distinctive feature of RP2sib is that it initially expresses EVE but extinguishes EVE later in development (Buescher et al., 1998). In wild type embryos at about stage 13, RP2sibs with fading EVE expression can be detected. In Dl^{9P} mutants, we observed RP2 multiplication which is at least partly caused by the strong neural hyperplasia of *Dl*. However, we did not observe cells with fading EVE expression in the position of RP2/RP2sib. Thus, RP2/RP2sib cell fates are not correctly resolved and presumably only RP2 neurons are made in Dl^{9P}. In addition, we quantified the number of Odd-skipped (ODD) positive cells within the MP2 lineage (Fig. 2). Odd is a specific molecular marker for differential cell fate in the MP2 lineage. In young stages of wild type embryos, ODD is initially expressed in the MP2 progenitor and in the two postmitotic daughter cells, vMP2 and dMP2 as well as in the two MP1 neurons. However, in later stages, ODD expression is lost in the vMP2 cell whereas expression is maintained in the dMP2 cell (Spana et al., 1995) (Fig. 2A). At an early stage when three cells (vMP2, dMP2 and MP1) per hemisegment normally still express ODD in the wild type, in Dl^{9P}, we observed 31.8 ± 8.7 ODD-positive cells per segment ($n=13$; Fig. 2B). However, in later stages when *wt* embryos normally extinguished ODD expression in the vMP2s, we still found an average of 29.9 ± 8.9 ODD-positive cells per segment ($n=25$; Fig. 2C). This suggests a transformation of vMP2 into the dMP2 cell fate. Our findings are consistent with the notion that *Delta* is required to resolve asymmetric cell fates among asymmetric siblings such as RP2/RP2sib and vMP2/dMP2 (Suppl. Tab. 5).

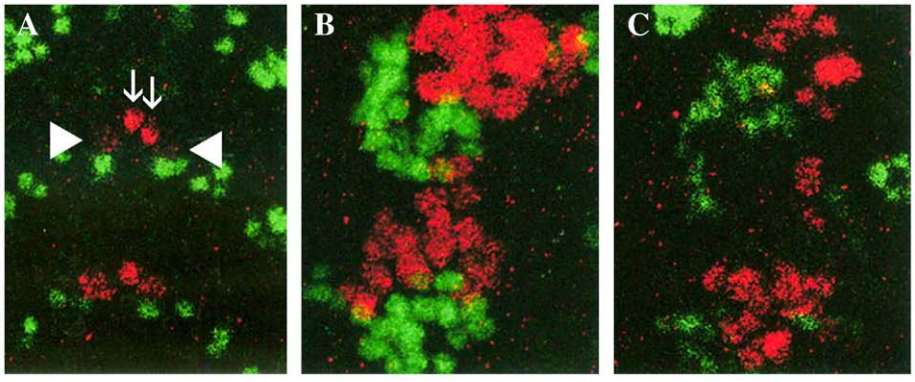


Fig. 2. *Dl* functions in sibling cell fate specification in the MP2 lineage. Anterior is to the top. (A) Stage 13–14 *wt* and (B, C) stage 13–14 and stage 16 Dl^{9P} embryos, respectively, double-labelled with anti-EVE (green) and anti-ODD (red). (A) ODD expression can be seen in the dMP2s (arrows) and being extinguished from the vMP2s (arrowheads) in the *wt* embryo. The ODD-positive MP1 cells are in a different focal plane (not shown). In the Dl^{9P} embryos (B and C), comparable numbers of ODD-positive cells can be observed in stage 13–14 (B) and stage 16 (C) embryos suggesting a requirement for *Dl* for vMP2 specification; note that in (C), not all positive cells are in the focal plane shown.

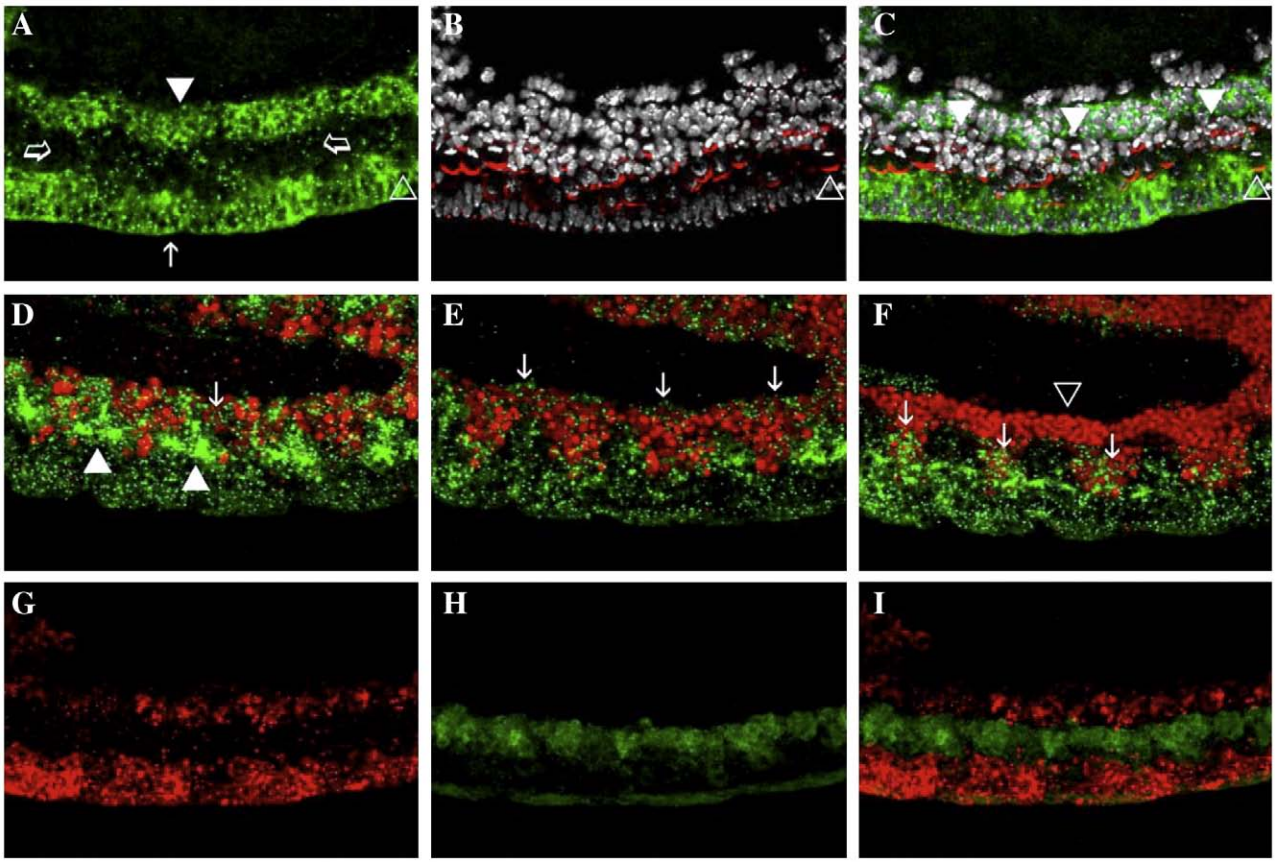
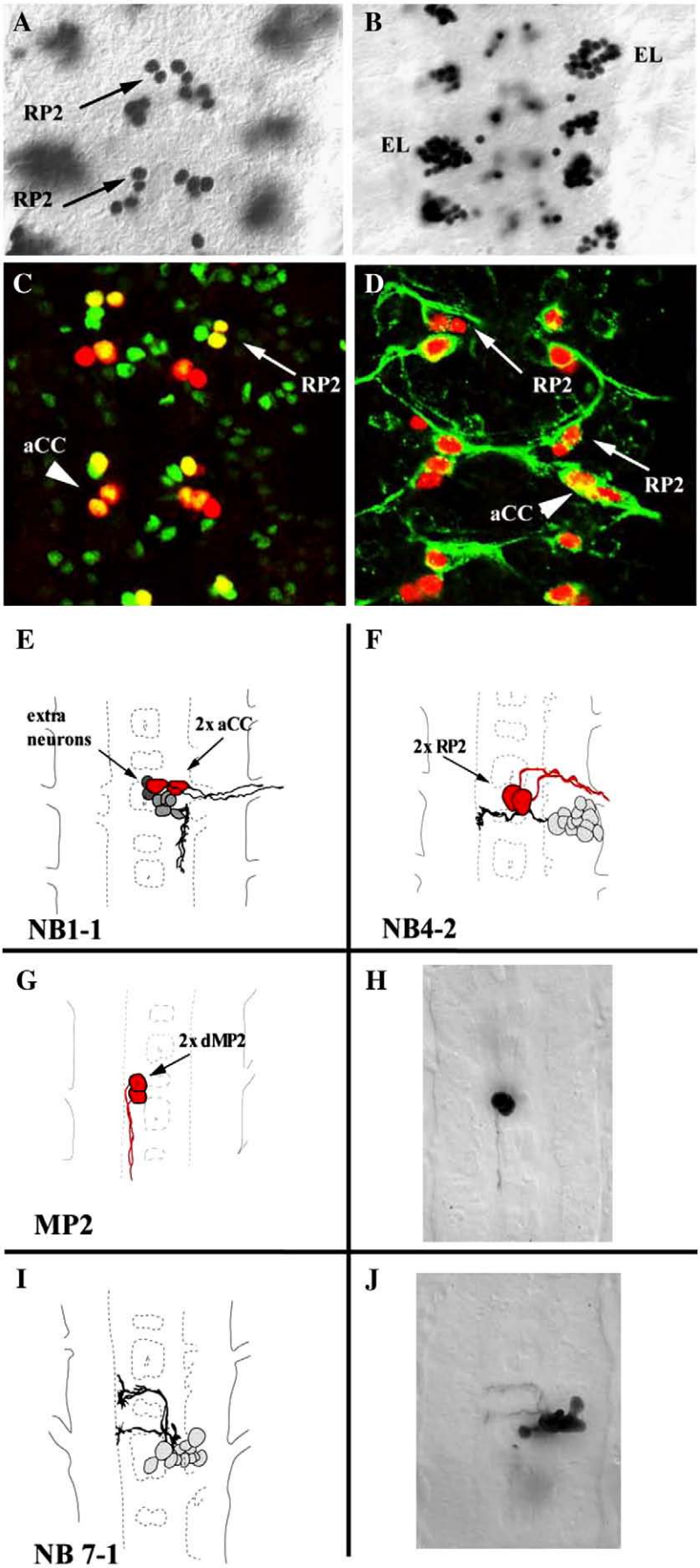


Fig. 3. Analysis of Delta expression during cell fate specification at embryonic stage 11. Anterior is to the left. (A–C) Delta (A, green) and INSC (B, red) double-labelling counterstained with a DNA stain (Topro-3, white). (A) Delta is strongly expressed in the neuroectoderm (solid arrow) and in presumptive mesodermal cells (solid arrowhead). Delta is either very weakly expressed or undetectable in regions where cells of the developing nervous system are located (area between open arrows in A). A rare example of a dividing neuroblast expressing Insc and low level of Delta is shown (open arrowheads in A–C). (C) Merged frame of (A) and (B). Delta is not detectable in INSC-positive GMCs (solid arrowheads). (D–F) Double-labelled specimen of Delta (green) and the mesodermal marker MEF2 (red) from different focal planes of the same embryo. Some mesodermal cells show colabelling (arrows in D–F). Delta is also expressed strongly in repeated groups of cells which are MEF2-negative (arrowheads in D). Similarly, there are MEF2-positive cells which do not coexpress Delta (open arrowhead in F). (G–I) Analysis of Delta (G, red) and *elav-mCD8-GFP* (H, green) expression. (I) Merged frame of (G) and (H). Delta expression is not detectable in *elav-mCD8-GFP* expressing neurons.

Our results revealed that mutant NB clones lacking zygotic *Dl* function developed normal lineages in a genetically *wild type* background although the analysis of *Dl* mutants showed that *Dl* is required for asymmetric cell fate resolution. The data are consistent with the interpretation that *Dl* function is dispensable within neuroblast lineages and that *Dl* has a non-lineage-autonomous function during sibling cell fate specification. To address the question of the source of Delta during developmental stages when sibling cell fates are specified in the ventral nervous system, we first analyzed Delta expression during embryogenesis (Supplementary Fig. 1). We found that Delta is expressed throughout embryogenesis, strongly in the neuroectoderm, and weakly in very few delaminated cells that presumably are neuroblasts which down-regulate *Dl* following delamination. Dl is strongly expressed in some cells located in the ventral midline, in the presumptive mesoderm, in some peripheral neurons at late stage of embryogenesis (stage 13–14 onwards) (Supplementary Fig. 1) as well as in some neurons from late stage 14 onwards (not shown). These data are consistent with previous reports (Haenlin et al., 1990; Kooh et al., 1993). We then studied Delta expression more specifically by analyzing embryos for Delta expression in combination with cell specific markers, such as INSC (a neuroblast and GMC marker), MEF2 (a mesodermal marker), and *elav-mCD8-GFP* (neuronal marker), during the stage of embryogenesis when cell fate specification is mostly occurring (stage 10–12). We found that although Delta was weakly expressed in some neuroblasts (Figs. 3A–C) and some MEF2-positive mesodermal cells (Figs. 3D–F), it was either absent or almost undetectable in the GMCs (Figs. 3A–C) as well as in *elav-mCD8-GFP* positive differentiated neurons (Figs. 3G–I). These data indicate that Delta expression is very weak or undetectable in the nervous system during cell fate specification. However, Delta is strongly expressed in tissues surrounding the developing nervous system suggesting that the source of Delta for cell fate specification in the ventral nerve cord is neighbouring tissues such as the neuroectoderm, mesoderm, and possibly some ventral midline cells.

kuz controls asymmetric cell fate specification cell-autonomously

It has been demonstrated that loss of zygotic *kuz* does not result in any embryonic phenotypes in the nervous system which is possibly a consequence of a strong maternal contribution (Fambrough et al., 1996). To determine whether *kuz* has a function during embryonic neurogenesis, we analyzed EVE expression in germ line clone derived *kuz* embryos (kuz^{GLC}). We also expressed a dominant-negative version of *kuz* (kuz^{DN}) in the nervous system by using the pan-neural driver *scabrous*-Gal4 (*sca*-Gal4). In both experimental conditions, we found moderate defects as indicated by a 2 times overall increase of EVE-positive cells which we attribute to neurogenic effects and a duplication of the RP2 neurons (Fig. 4A). More detailed analysis of *sca-Gal4:UAS-kuz*^{DN} embryos with specific cell fate markers (anti-EVE, anti-ZFH-1, 22C10) revealed sibling cell fate specification defects (Figs. 4C and D). For example, the RP2


A
RP2
RP2
B
EL
EL
C
RP2
aCC
D
RP2
RP2
aCC
E
extra neurons
2x aCC
NB1-1
F
2x RP2
NB4-2
G
2x dMP2
MP2
H
I
NB 7-1
J

neuron was duplicated in the range of 66% to 85% of hemineuromeres ($n=12$) (Fig. 4A). In the remaining hemineuromeres, the number of RP2s was either wt or could not be detected. In 80% of the cases, duplicated RP2 neurons also co-expressed ZFH-1 and 22C10 (Figs. 4C and D, respectively), two markers which are indicative for the RP2 cell fate (Buescher et al., 1998). Since we neither observed fading EVE expressing cells in the vicinity of those RP2s nor did we see any EVE-positive cells which did not stain for ZFH-1, we concluded that the RP2 duplications resulted from a transformation of RP2sibs into RP2s. Scoring for other cell fate changes using anti-EVE revealed a decrease in the number of U-neurons to an average of 1 cell per hemineuromere indicating a transformation of U-neurons into U-sibs. The number of EVE-positive cells in the aCC/pCC position was essentially wild type; however, double-staining with ZFH-1 and 22C10 revealed that the pCC was transformed into its sibling aCC (Figs. 4C and D) in 80% of hemineuromeres. From these data, we concluded that *kuz* is involved in the specification of sibling cell fates during embryonic nervous system development (Suppl. Tab. 5).

It has been reported that KUZ proteolytically processes the ligand Delta as well as the receptor Notch (Lieber et al., 2002; Pan and Rubin, 1997; Qi et al., 1999). We reasoned that with regard to sibling cell fate resolution if KUZ is required for Delta function, then *kuz* mutant NB clones developing in a *wt* background should develop *wt* NB lineages as a result of the non-cell-autonomous function of *Dl* in the transplantation assay (see above). However, if *kuz* is needed for *N* function then a cell-autonomous phenotype should be observed based on the fact that *N* behaves strictly cell-autonomously in the transplantation assay. To test this rationale experimentally, we removed cells from *scaGal4:UAS-kuz^{DN}* donor embryos and implanted them individually into *wt* recipients. Resulting NB clones were analyzed at stage 16 of embryogenesis. Thirty identifiable lineages of NB 1-1 ($n=10$), NB 4-2 ($n=4$), MP2 ($n=11$), and NB 7-1 ($n=5$) were obtained (Figs. 4E–J; Suppl. Tab. 1–4). We observed duplication of the aCC neuron at the expense of the pCC neuron as well as a loss of SPGs in NB1-1 lineages (Fig. 4E), duplication of the RP2 neuron at the expense of the RP2 sib as well as absence of the additional motor neurons in the NB4-2 lineage (Fig. 4F), duplication of the dMP2 cell in the MP2 lineage (Figs. 4G, H), and a complete absence of the U-neurons in the NB7-1 lineage (Figs. 4I, J). In summary, the observed cell fate specification defects in the NB clones which lack *kuz* function suggest that *kuz* works in a cell-autonomous manner. It is also noteworthy that the *kuz* phenotypes are identical to the phenotypes observed in *Notch* transplantation experiments.

Analysis of insc requirement in neuroblast lineages

It has been shown that *insc* is not required in the MP2 lineage for asymmetric cell specification (Rath et al., 2002) and in agreement with this result we did not find any alterations in the MP2 lineages ($n=11$; Suppl. Tab. 2) of *insc* mutant clones. Next, we describe in greater detail the effect of removing *insc* function from NB1-1, NB4-2, and NB7-1 lineages.

NB1-1 (Figs. 5A and B; Suppl. Tab.1): In *insc* mutant lineages, the early born pCC neuron is transformed into an aCC neuron in all clones analyzed. This phenotype is identical to the *Notch* phenotype. However, we observed one important difference between *insc* and *Notch* mutant NB1-1 clones. Approximately one fifth of the *insc* lineages contained at least one subperineural glia indicating that *insc* is not as strictly required for the differentiation of SPGs as *Notch*, in which SPGs could not be detected at all. This finding was further

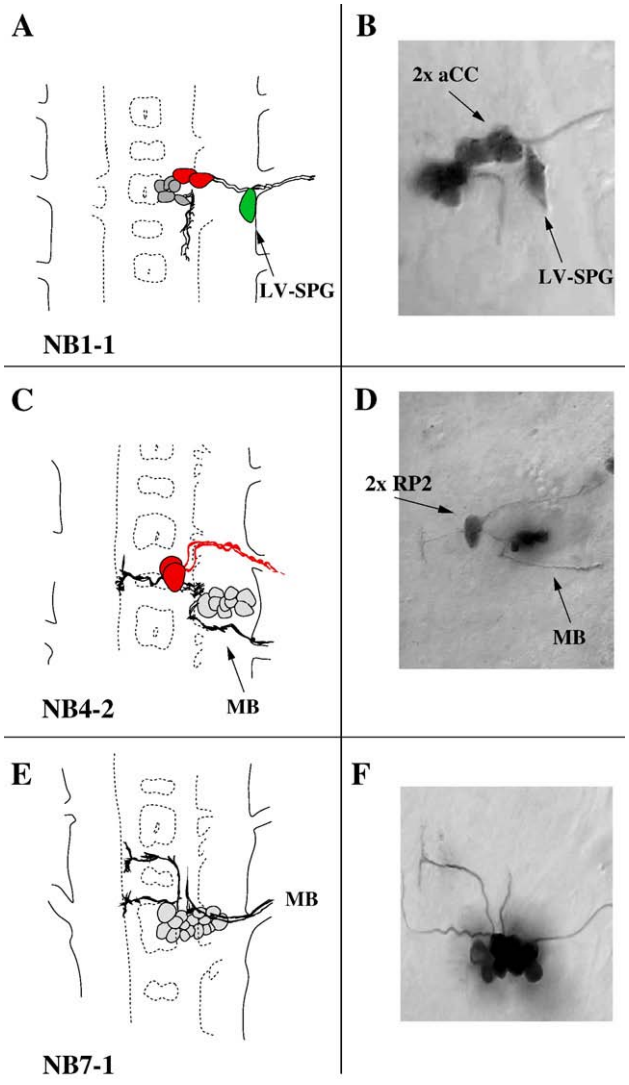


Fig. 5. Schematic representations and photographs of *insc* mutant neuroblast clones of NB1-1, NB4-2, and NB7-1. Representative lineages of NB1-1 (A and B), NB4-2 (C and D), and NB7-1 (E and F). (A and B) *insc* NB1-1 lineages duplicate the aCC neuron (red) at the expense of the pCC neuron. The interneuronal cluster is largely unaffected; however, in contrast to *Notch* mutant lineages some *insc* clones develop subperineural glia cells (green; see also Suppl. Tab. 1). (C, D) *insc* mutant NB4-2 lineages duplicate the RP2 cell (red) and do not show any RP2 sib neurons. However, in contrast to *Notch* clones, the motoneuronal bundle (MB) emanating from the lateral cluster of neurons is still present in *insc* clones. (E and F) *insc* mutant NB7-1 lineages still show the motoneuronal bundle (MB) originating from the U-neurons indicating that most of the U-neurons are present. This is in contrast to *Notch* mutant clones where the MBs are absent (see Fig. 1H).

Fig. 4. *kuz* controls sibling cell fate specification in the embryonic CNS. (A–D) Expression of *UAS-kuz^{DN}* in the embryonic nervous system driven by *sca-Gal4*. (A, B) EVE staining in *sca-Gal4*: *UAS-kuz^{DN}* (*kuz^{DN}*) embryos. (A) Two EVE-positive cells can be detected in the RP2 position, indicating a duplication of the RP2 cells (arrows). (B) The EVE–lateral neurons (EL) are increased in numbers, possibly due to a mild neurogenic effect of *kuz*. (C and D) Analysis of molecular markers indicate cell fate duplication of RP2 and aCC. (C) Double-labelling with EVE (red) and ZFH-1 (green) showing duplication of RP2 and aCC neurons. Two RP2s as well as two aCCs co-expressing EVE and ZFH-1 can be seen (arrow and arrowhead, respectively). (D) Cell fate duplication as revealed by EVE and 22C10 colabelling of RP2 (arrow) and aCCs (arrowhead). (E–J) Neuroblast lineages derived from *kuz^{DN}* transplanted cells. (E and F) Schematic representation of NB1-1 (E) and NB4-2 (F) *kuz^{DN}* clones. (E) The aCC neuron is duplicated at the expense of its sibling pCC. Furthermore, the SPG glial cells are missing and additional cells can be found in or in the vicinity of the neuronal cluster. (F) RP2 is duplicated at the expense of RP2sib. Note that the motoneuronal bundle (MB) is missing in *kuz^{DN}* clones. (G and H) A *kuz^{DN}* MP2 lineage is presented. The dMP2 is duplicated and the vMP2 neuron is missing. (I and J) A *kuz^{DN}* mutant lineage of NB7-1 is shown. Note that the motor neuronal bundle emanating from the cluster of neurons in *wt* clones is missing in *kuz* clones.

supported by the expression of the M84/P101 enhancer trap line (Klämbt and Goodman, 1991) in the background of *insc* mutant embryos. M84/P101 exclusively labels the subperineural glia, including the A and B glia derived from NB1-1. In *insc* mutants, M84/P101 expression was reduced to 1–2 cells in 70% of the hemisegments ($n=350$), and in about 30% of the hemisegments, glial cells were retained in the *wt* number (data not shown).

NB4-2 (Figs. 5C and D; Suppl. Tab. 3): *insc* mutant clones ($n=6$) showed a duplication of the RP2 fate at the expense of the RP2sib neuron. In contrast to *N* mutant clones, however, the motor neurons in the cluster were present and showed *wt* morphology.

NB7-1 (Figs. 5E and F; Suppl. Tab. 4): In contrast to *Notch* NB7-1 clones, 12 clones derived from *insc* cells (80%; $n=15$) contained the wild type motoneuronal fascicle comprising of the U-neurons. It is technically challenging to exactly quantify U-motor neurons in NB7-1 lineages but it is possible to identify them through their motoneuronal projections that leave the nervous system into the periphery of the embryo. We observed that in some clones the U-motoneuronal fascicle appeared thinner possibly indicating that fewer U-neurons are present. Additionally, we quantitated the EVE-positive U-neurons in *insc* embryos. We observed a reduction of the number of U-neurons to an average of 3.9 ± 1 (AVG $\pm$ STD) U-neurons per hemineuromere ($n=150$) in *insc* embryos as compared to 4.9 ± 0.4 U-neurons in *wt* embryos ($p<0.001$; one-tail *t*-test). Therefore, on average, a highly statistically significant loss of about 1 EVE-positive cell in the U-neuron position was observed per hemineuromere in *insc* embryos which is equivalent to the loss of 1 U-neuron per NB7-1 lineage. In summary, in contrast to *Notch*, loss of *insc* does not seem to have a profound effect on the specification of later born cells in these lineages.

Discussion

In this study, we attempted to get further insight into mechanisms of neural cell fate specification throughout lineage progression of four neuroblasts (NB1-1, MP2, NB4-2, and NB7-1) mediated by several members of the Notch signalling pathway (*N*, *Dl*, *kuz*) as well as Inscuteable, a major regulator of asymmetric cell division. We found that loss of *N*, *kuz*, or *insc* in neuroblast lineages, but not loss of *Dl* function, led to cell fate transformations. Our analysis revealed that *N* and *kuz* affected cell fate specification in a cell-autonomous manner probably throughout the lineages studied whereas *Dl* acted non-cell-autonomously. Unlike *N* and *kuz*, *insc* requirement seemed to be limited to the early born cells of these lineages. This is probably true also for other neuroblast lineages not studied here. The function of the four genes during cell fate specification is discussed in further detail below.

Notch function in NB lineage progression

The transplantation assay we used involves the controlled temporal and spatial removal of single labelled cells from donor embryos and their likewise implantation into unlabelled host specimen. As a consequence, the entire lineage of any implanted cell will be traceable with high structural resolution in the host (Udolph et al., 1993). The major stronghold of the transplantation technique is that heterogenetic transplantations usually from a mutant donor into *wt* genetic background can be performed resulting in genetic mosaicism. This allows studying autonomy of gene function in identifiable and complete embryonic cell lineages (Prokop and Technau, 1994; Udolph et al., 1998). Such studies are not possible in the embryo using any other technique. Using this approach, we were able to show that *Notch*-deficient neuroectodermal cells gave rise to stereotypic NB lineages, which include lineage-specific indicator neurons and abnormalities were detectable for identified postmitotic neurons/glia. In most of the analyzed lineages, one of the GMC-derived daughter cells was duplicated at the expense of its sibling. Hence, our results show that although *Notch* is pivotal in lateral inhibition during NB formation, it is not involved in specifying the identity and lineage progression of NBs. Furthermore, *Notch* does not seem to affect the proliferative capacity of embryonic NBs as mutant NBs in average produced the correct number of progeny cells in each of the lineages studied. This implies that *Notch* neither affects the asymmetric division of embryonic neural stem cells (neuroblasts) nor the subsequent division of GMCs into two postmitotic daughter cells. This seems to be different in the adult olfactory sensory system of *Drosophila* where *Notch* is also involved in asymmetric determination of olfactory receptor neuron identity on the level of early progenitors in the lineage (Endo et al., 2007). Similar findings have been reported in the murine system where differential Notch signalling was shown to distinguish between neural stem cells and progenitor cells (Mizutani et al., 2007) as well as between hematopoietic stem cells and unipotent progenitors (Duncan et al., 2005). Our data indicate that for the *Drosophila* embryonic CNS, differential Notch signalling seems to be only required for cell fate specification at the level of postmitotic cells derived from individual GMCs.

It has been reported that in the neuroectoderm, *Notch* can function in a cell-autonomous or a non-cell-autonomous manner (Hoppe and Greenspan, 1986, 1990; Technau and Campos-Ortega, 1987). However, to our knowledge, it has not been formally demonstrated that *Notch* acts cell-autonomously or non-cell-autonomously in the process of sibling cell fate specification in the embryonic nervous system. By analyzing four complete NB lineages generated from individual *Notch* mutant cells, we observed cell fate defects which are in line with the interpretation that *Notch* is required during cell fate specification in one of the two daughters of GMCs in an autonomous manner. We did not address a possible non-autonomous role of *N* as the reciprocal experiment of transplanting *wt* cells into *N* mutants is technically challenging if not impossible as the morphology of *N* embryos is severely disrupted which would make sensitive assays to measure effects by the *wt* clone on the mutant host tissue unfeasible.

Our clonal analysis also shows that the requirement for *Notch* in sibling cell fate specification is not limited to the progeny of early born GMCs but is likely to be extended throughout the embryonic NB lineage. However, due to the lack of specific and differential markers for most sibling cell pairs, we cannot entirely rule out the possibility that in some cases *Notch* might also be required for other aspects of differentiation such as cell survival and/or axonal outgrowth and pathfinding.

Intra-lineage Dl signalling is not required for cell fate specification

Dl is required for nervous system development (Alton et al., 1988; Hartenstein et al., 1992) and sibling cell fate resolution in the nervous system (Skeath and Doe, 1998; Zeng et al., 1998). One study has reported a non-cell-autonomous function of *Dl* during early neuroblast formation (Technau and Campos-Ortega, 1987). It has also been demonstrated that lateral signalling between direct siblings is not a prerequisite for sibling cell fate resolution between vMP2/dMP2 and RP2/RP2 sibling pairs (Buescher et al., 1998; Spana and Doe, 1996); however, the role of *Dl* during NB lineage progression is still unclear. Here, we analyzed *Dl* function at the level of single neuronal/glial cells in neuroblast lineage progression during embryonic nervous system development. When *Dl*-deficient cells were transplanted into wild type background, the NB lineages which developed from the mutant implants show complete wild type characteristics. This demonstrates that *Dl* is not required within the mutant lineage itself to resolve correct cell fates. However, when *Dl* was removed from the entire embryo, sibling cell fate resolution defects were detectable indicating a requirement for *Dl* in this process per se. Hence, Dl is required for sibling cell fate specification but not within the cell to be specified indicating that *Dl* function is non-autonomous during sibling cell fate

specification. As for the *Notch* cell transplantations, we did not consider reciprocal transplantations of *wt* cells into *Dl* mutant background for the same reasons pointed out above. Nevertheless, our conclusion of a non-autonomous function of *Dl* still holds as it is widely accepted that in a genetic mosaic a gene function is considered non-autonomous if a phenotype can be rescued by the surrounding *wt* tissue.

Since our results showed that Delta is not required within the NB lineages, we examined the possible sources of Delta during embryonic phases of sibling cell fate specification in the nervous system. We found that Delta is expressed mainly in the neuroectoderm and the mesoderm whereas it was only weakly expressed or undetectable in the developing nervous system such as in NBs, GMCs, and neurons. Noticeably, the development of the nervous system seemed to take place in pockets of Delta-negative domains surrounded by the Delta-positive neuroectoderm and mesoderm. Although the exact source of Delta requires further studies, it is conceivable that neighbouring tissues outside the neuroblast lineages are the source of Delta ligand needed for the specification of sibling cell fates. This is supported by cell culture studies involving the MP2 lineage where it has been shown that high cell densities as well as cell contact with cells outside the lineage were required to correctly specify the vMP2 cell fate (Spana and Doe, 1996). Our clonal analysis provides additional insight into Delta signalling between developing cells in the CNS. Firstly, signalling from the *Notch*-independent sibling via Dl is not required for the specification of the *Notch*-dependent sibling throughout the lineage. Thus, our results extend previous *in vivo* findings which were based on an individual GMC and the resulting pair of siblings RP2 and RP2sib (Buescher et al., 1998). Secondly, the correct resolution of cell fates throughout the complete lineages also demonstrates that lineage-related Delta signalling between other components of the lineage (e.g. NBs → GMCs; GMCs → siblings; and among non-sibling cells of the same lineage) is at least dispensable for sibling cell fate resolution. In contrast to our observations, it was shown that during adult development of the peripheral nervous system, Delta signalling is required within the sensory organ precursor lineage itself (Frise et al., 1996; Jan and Jan, 1995; Posakony, 1994; Zeng et al., 1998). Therefore, the mechanisms of providing the ligand Delta to activate Notch signalling for sibling cell fate resolution seem to vary between different tissues. Another ligand for Notch is Serrate (Artavanis-Tsakonas et al., 1995). However, zygotic *Serrate* mutations do not produce any visible embryonic phenotype suggesting that *Serrate* is dispensable for embryonic development (Thomas et al., 1991). As such, it is unlikely that *Serrate* could rescue any loss of *Dl* function within the clones. In addition, zygotic *Dl* mutants resulted in strong neurogenic and sibling cell fate resolution phenotypes, all of which indicate a critical requirement for *Dl* independent from *Serrate*.

kuz is cell-autonomously required for sibling cell fate specification in the nervous system

The metalloprotease KUZ is involved in the proteolytic processing of either Notch or Delta or both (Pan and Rubin, 1997; Qi et al., 1999). In this study, we showed that *kuz* function is required during two distinct phases of embryonic nervous system development which are also controlled by *Notch* function, i.e., in the process of lateral inhibition during neuroblast formation and during sibling cell fate specification in NB lineage progression. By overexpressing a kuz^{DN} transgene in a pan-neural fashion (Pan and Rubin, 1997), we observed cell fate transformations in pairs of indicator sibling neurons. These transformations are phenotypically identical to those observed in *Notch* mutants suggesting that *kuz* genetically acts in the Notch signalling pathway in this context. *kuz* has been genetically and biochemically linked to Notch signalling (Sotillos et al., 1997). From our *kuz*-deficient genetic mosaics generated by transplanting single kuz^{DN} cells into *wt* recipients, we observed sibling cell fate transformations in cells lacking *kuz* function demonstrating cell-autonomy of *kuz*. Furthermore, defects were only found in those cells which also depend on *Notch* function. Conversely, we did not detect any phenotypes in the *N*-independent sibling cells, although kuz^{DN} should be expressed there based on the pan-neural expression pattern of the driver used. This suggests that *kuz* was functionally only required in the cells which also strictly depend on *Notch*. The cell-autonomous requirement for *Notch* and *kuz* in the same cell supports the notion that KUZ has a function down-stream of ligand binding to Notch. Although our data do not rule out the possibility that KUZ might also process Delta to produce a functional ligand for (non-lineage-related) neighbouring *Notch*-dependent cells, we favour a model in which KUZ functions in facilitating Notch activation cell-autonomously during sibling cell fate specifications in the nervous system. Such function of *kuz* has been demonstrated during wing development (Klein, 2002) and biochemical data showed that processing of Notch by KUZ resulted in a biologically active and functional Notch receptor (Lieber et al., 2002). Our study provides evidence that, during embryonic central nervous system development, KUZ, possibly via regulating Notch activity, controls correct resolution of sibling cell fates during neuroblast lineage progression.

insc function during neuroblast lineage development

Although *insc* has been described as a master regulator of intrinsic cell asymmetry (Kraut et al., 1996), it has also been reported that *insc* is not required for sibling cell fate resolution in the MP2 lineage (Rath et al., 2002). From our analysis of three additional neuroblast lineages, we observed a strict requirement for *insc* in sibling cell fate pairs which are derived from the first GMCs of two of the lineages studied (i.e., aCC/pCC from NB1-1, RP2/RP2 sib from NB4-2) and for 1 U-neuron derived from an unidentified GMC of the NB7-1 lineage. However, we did not observe any fate changes in cells born later in the NB1-1 and NB4-2 lineages as well as in most of the U-neurons from the NB7-1 lineage, all of which normally require *Notch* function. In *insc* mutants, the glial cells of the NB1-1 lineage are partly present indicating that cell fate specification is at least partially correct. Furthermore, in *Notch* NB4-2 clones, we observed a complete loss of the motoneuronal fascicle formed by the U-neurons, whereas in *insc* clones, the U-neuronal fascicle generally could be found. Further quantification of the number of EVE-positive U-neurons revealed that on average 1 U-neuron was missing in the NB7-1 lineages. The loss of 1 U-neuron is consistent with the interpretation that daughter cells of one GMC of NB7-1 are misspecified, and as such, this GMC requires *insc* function for generating correct sibling cell fates. In analogy to the *insc* requirement in the first GMCs of NB1-1 and NB4-2, it is possible that the first GMC of NB7-1 is also dependent on *insc* although this has to be demonstrated by further experiments. It has been reported that in wild type, the first 5 GMCs of NB7-1 give rise to 5 pairs of U-neurons and U-sibs; thus all these GMCs create asymmetric cell fates (Pearson and Doe, 2003). However, it was still unclear if for this NB lineage the asymmetric cell fates are the result of an intrinsic asymmetric division of the respective GMCs. Our results indicate that *Notch* is required in all 5 GMCs, but *insc* is only required in 1 of the 5 GMCs of NB7-1, and it is therefore dispensable for the asymmetric division for the 4 remaining GMCs giving rise to U-neurons. A possible explanation for the observed differential *insc* requirement could be that *insc* loss-of-function might be rescued by other members of the apical complex components. Such a scenario has been reported for the MP2 lineage. It is possible that later born GMCs in the studied lineages still depend on intrinsic mediators of asymmetric cell division such as *bazooka* but do not require *insc* function (Rath et al., 2002).

The NB1-1, NB4-2, and NB7-1 lineages are derived from early delaminating S1 NBs (Bossing et al., 1996) and early born neurons in the NBs 1-1 and 4-2 lineages have pioneering function. Pioneer neurons have been described to exert a crucial role in establishing neuronal fibre tracts during development (Hidalgo and Brand, 1997; Sanchez-Soriano and Prokop, 2005). It is conceivable that *insc* plays a role as a safeguard to assure that neurons with pivotal developmental functions are generated correctly. Later lineage components are born into a preexisting context of differentiating neurons and glial cells. This raises the possibility that extrinsic signals might become more dominant for cell fate specification during later phases as compared to early lineage decisions when intrinsic mechanisms might predominate. However, the fact that most of the later born cells still require *Notch* function indicates that *Notch* is involved in the specification of cell fates either as a down-stream component of *insc* independent asymmetric cell division or alternatively, it could be that extrinsic mediators such as Notch, may by themselves be sufficient to effect distinct sibling cell fates for the daughters of the later born GMCs.

Taken together, the 4 lineages studied here show a differential requirement for *Notch* and *insc* in sibling cell fate specification. *insc* requirement is dependent on the stage of neuroblast lineage progression in NB1-1, NB4-2, and probably NB7-1. Early-born cells derived from early GMCs are dependent on both *N* and *insc* to resolve their fates. An exception to this, however, is the MP2 which does not require *insc* although it divides only once to generate two asymmetric siblings. The MP2 division pattern is not neuroblasts like and resembles more a typical GMC or midline cell division pattern (Bossing et al., 1996). This and other unknown intrinsic differences

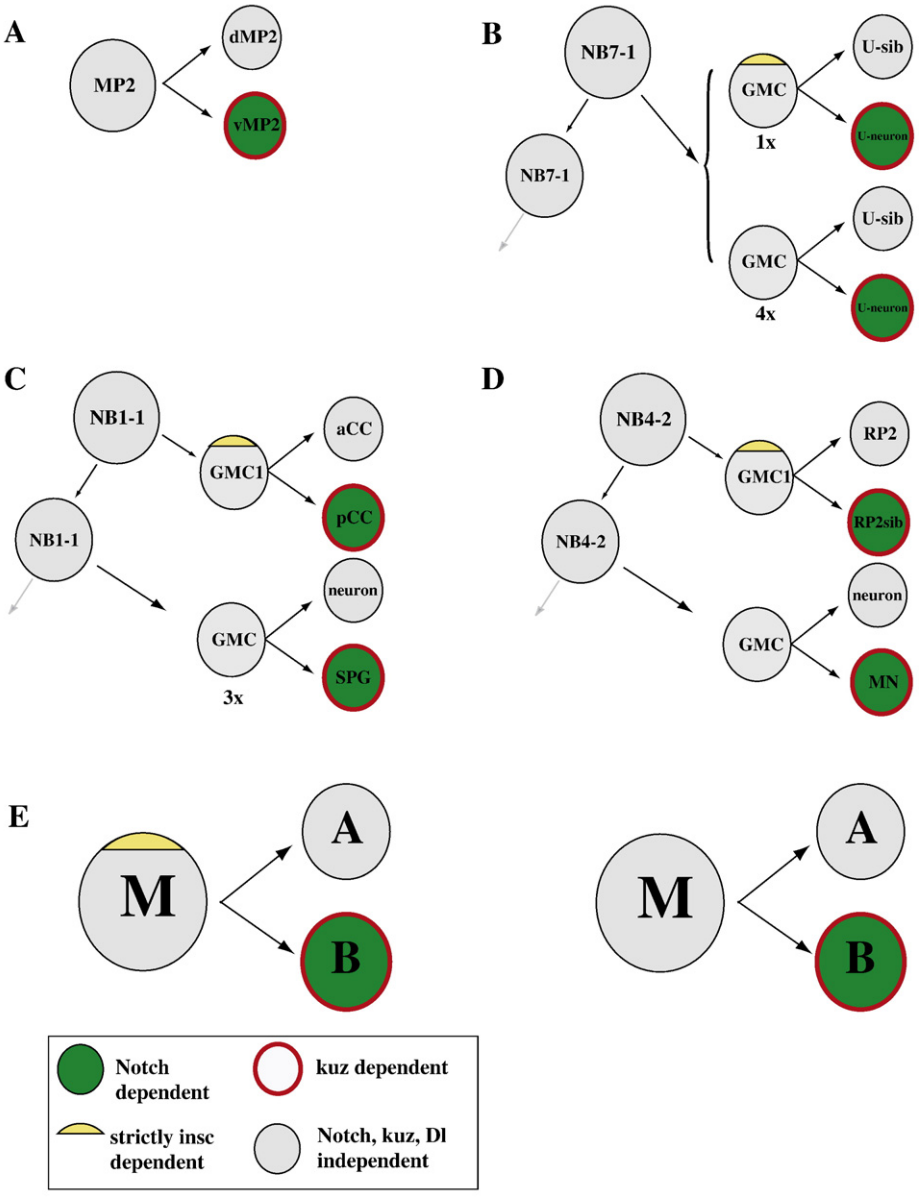


Fig. 6. Summary of cell fate transformations observed in the 4 studied neuroblast lineages, MP2 (A), NB7-1 (B), NB1-1 (C), NB4-2 (D). *insc*-mediated asymmetric cell division is strictly required in the first GMC of NB1-1, NB4-2 as well as in one GMC from NB7-1 for correct cell fate specification of one of the two postmitotic sibling neurons. *insc* is not required for cell fate specification of sibling cells of the MP2 and later GMCs of NB1-1, NB4-2 as well as 4 GMCs of the NB7-1 lineage. *Notch* and *kuz* are cell-autonomously required for sibling cell fate specification in MP2, NB1-1, Nb4-2, and NB7-1 beyond cells derived from the first GMCs of the respective neuroblasts. (E) Summary of the role of *N*, *Dl*, *kuz*, and *insc* during asymmetric cell division and sibling cell fate specification. Some cells strictly require *insc* during asymmetric cell division to assure correct sibling cell fate specification (left). Other cells do not require *insc* for asymmetric cell division but still require the Notch signalling pathway for cell fate specification (right) suggesting that such cells either still divide asymmetrically in the absence of *insc* or utilize other unknown mechanisms to differentially activate Notch in one of the siblings.

of MP2 might explain why MP2 does not require *insc* for cell fate specification. However, MP2 requires *baz* for sibling cell fate specification indicating that *insc* might be redundant in MP2 (Rath et al., 2002). It is also possible that the time point of the first division plays a role for *insc* requirement. Although MP2 delaminates in the S1 wave (early stage 9), its division takes place only at the end of late stage 10. This seems to be delayed compared to other neuroblasts which start dividing relatively soon after their delamination (Bossing et al., 1996) and this could contribute to MP2 behaving differently to first-born cells from other NB lineages.

Later born cell types, such as the SPGs of NB1-1, the motor neurons of NB4-2 and the majority of the U-neurons of NB7-1 still show strict dependence on *Notch* but seem to at least partially bypass the requirement for intrinsic asymmetry mediated by *insc*. As such, our findings show that *insc*-mediated asymmetry is uncoupled to *Notch*-mediated sibling cell fate specification in MP2 as well as in late born cells from other NB lineages. A schematic summary of specification of cell types in the 4 studied NB lineages is provided in Fig. 6. Further studies on temporal aspects of NB lineage development and the asymmetric localization of apical as well as basal components in NBs and GMCs beyond the first division would provide further insight into mechanisms of differential cell fate specification from neural stem cells during lineage progression.

Acknowledgments

We thank N. Perrimon, G. Rubin and R. Kraut and the Bloomington Drosophila Stock Center at Indiana University for providing fly stocks. The 22C10 monoclonal antibody developed by S. Benzer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This study was supported in part by a Wellcome Trust Programme Grant to W.C. and by the Institute of Medical Biology, Singapore to G.U.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.030.

References

Albrecht, S., Wang, S., Holz, A., Bergter, A., Paululat, A., 2006. The ADAM metalloprotease Kuzbanian is crucial for proper heart formation in *Drosophila melanogaster*. Mech. Dev. 123, 372–387.

Alton, A.K., Fechtel, K., Terry, A.L., Meikle, S.B., Muskavitch, M.A., 1988. Cytogenetic definition and morphogenetic analysis of *Delta*, a gene affecting neurogenesis in *Drosophila melanogaster*. Genetics 118, 235–245.

Artavanis-Tsakonas, S., Matsuno, K., Fortini, M.E., 1995. Notch signaling. Science 268, 225–232.

Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*: I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. 179, 41–64.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., Nguyen, H.T., 1995. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. Genes Dev. 9, 730–741.

Broadus, J., Skeath, J.B., Spana, E.P., Bossing, T., Technau, G.M., Doe, C.Q., 1995. New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. Mech. Dev. 53, 393–402.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R., Israel, A., 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin–metalloprotease TACE. Mol. Cell. 5, 207–216.

Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., Tear, G., Chia, W., 1998. Binary sibling neuronal cell fate in the *Drosophila* embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. Genes Dev 12, 1858–1870.

Chou, T.B., Perrimon, N., 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. Genetics 131, 643–653.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N., Reya, T., 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat. Immunol. 6, 314–322.

Endo, K., Aoki, T., Yoda, Y., Kimura, K., Hama, C., 2007. Notch signal organizes the *Drosophila* olfactory circuitry by diversifying the sensory neuronal lineages. Nat. Neurosci. 10, 153–160.

Famborough, D., Pan, D., Rubin, G.M., Goodman, C.S., 1996. The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in *Drosophila*. Proc. Nat. Acad. Sci. U. S. A. 93, 13233–13238.

Fortini, M.E., 2001. Notch and presenilin: a proteolytic mechanism emerges. Curr. Opin. Cell Biol. 13, 627–634.

Frasch, M., Hoey, T., Rushlow, H., Levine, M., 1986. Characterisation and localisation of the even-skipped protein of *Drosophila*. EMBO 6, 749–759.

Frise, E., Knoblich, J.A., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1996. The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell–cell interaction in sensory organ lineage. Proc. Natl. Acad. Sci 93, 11925–11932.

Greenwald, I., 1989. Cell–cell interactions that specify certain cell fates in *C. elegans* development. Trends Genet 5, 237–241.

Guo, M., Jan, L.Y., Jan, Y.N., 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17, 27–41.

Haenlin, M., Kramatschek, B., Campos-Ortega, J.A., 1990. The pattern of transcription of the neurogenic gene *Delta* of *Drosophila melanogaster*. Development 110, 905–914.

Hartenstein, A.Y., Rugendorff, A., Tepass, U., Hartenstein, V., 1992. The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. Development 116, 1203–1220.

Hidalgo, A., Brand, A.H., 1997. Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drsosophila*. Development 124, 3253–3262.

Hoppe, P.E., Greenspan, R.J., 1986. Local function of the *Notch* gene for embryonic ectodermal pathway choice in *Drosophila*. Cell 46, 773–783.

Hoppe, P.E., Greenspan, R.J., 1990. The Notch locus of *Drosophila* is required in epidermal cells for epidermal development. Development 109, 875–885.

Ito, K., Urban, J., Technau, G.M., 1995. Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. Roux's Arch. Dev. Biol 204, 284–307.

Jan, Y.N., Jan, L.Y., 1995. Maggot's hair and bug's eye: role of cell interactions and intrinsic factors in cell fate specification. Neuron 14, 1–5.

Jan, Y.N., Jan, L.Y., 2000. Polarity in cell division: what frames thy fearful asymmetry? Cell 100, 599–602.

Klämbt, C., Goodman, C.S., 1991. The diversity and pattern of glia during axon pathway formation in the *Drosophila* embryo. Glia 4, 205–213.

Klein, T., 2002. kuzbanian is required cell autonomously during Notch signalling in the *Drosophila* wing. Dev. Genes Evol. 212, 251–255.

Kooh, P.J., Fehon, R.G., Muskavitch, M.A., 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. Development 117, 493–507.

Kraut, R., Campos-Ortega, J.A., 1996. *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adapter protein. Dev. Biol. 174, 65–81.

Kraut, R., Chia, W., Jan, L.Y., Jan, Y.N., Knoblich, J.A., 1996. Role of *inscuteable* in orienting asymmetric cell division in *Drosophila*. Nature 383, 50–55.

Kuwada, J.Y., Goodman, C.S., 1985. Neuronal determination during embryonic development of the grasshopper nervous system. Dev. Biol 110, 114–126.

Lai, Z.C., Fortini, M.E., Rubin, G.M., 1991. The embryonic expression patterns of *Zfh-1* and *Zfh-2*, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. Mech. Dev. 34, 123–134.

Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.

Lieber, T., Kidd, S., Young, M.W., 2002. kuzbanian-mediated cleavage of *Drosophila* Notch. Genes Dev. 16, 209–221.

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A., Gaiano, N., 2007. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature 449, 351–355.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., Kopan, R., 2000. A ligand-induced extracelllualr cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol. Cell 5, 197–206.

Pan, D., Rubin, G.M., 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. Cell 90, 271–280.

Parmentier, M.L., Woods, D., Greig, S., Phan, P.G., Radovic, A., Bryant, P., O'Kane, C.J., 2000. Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in *Drosophila*. J. Neurosci. (Online) 20, RC84 (1–5).

Pearson, B.J., Doe, C.Q., 2003. Regulation of neuroblast competence in *Drosophila*. Nature 425, 624–628.

Petronczki, M., Knoblich, J.A., 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat. Cell Biol 3, 43–49.

Posakony, J.W., 1994. Nature versus nurture: asymmetric cell divisions in *Drosophila* bristle development. Cell 76, 415–418.

Prokop, A., Technau, G.M., 1993. Cell transplantation. In: Hartley, e. D. (Ed.), Cellular Interactions in Development: A Practical Approach. Oxford Univ. Press, Oxford, pp. 33–57.

Prokop, A., Technau, G.M., 1994. Early tagma specific commitment of *Drosophila* progenitor NB1-1. Development 120, 2567–2578.

Qi, H., Rand, M.D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., Artavanis-Tsakonas, S., 1999. Processing of the Notch ligand Delta by the metalloprotease Kuzbanian. Science 283, 91–94.

Rath, P., Lin, S., Udolph, G., Cai, Y., Yang, X., Chia, W., 2002. Inscuteable-independent apicobasally oriented asymmetric divisions in the *Drosophila* embryonic CNS. EMBO Rep. 3, 660–665.

Sanchez-Soriano, N., Prokop, A., 2005. The influence of pioneer neurons on a growing motor nerve in *Drosophila* requires the neural cell adhesion molecule homolog FasciclinII. J. Neurosci. 25, 78–87.

Schaefer, M., Shevchenko, A., Knoblich, J.A., 2000. A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in *Drosophila*. Curr. Biol. 10, 353–362.

Schmid, A., Chiba, A., Doe, C.Q., 1999. Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. Development 126, 4653–4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., Technau, G.M., 1997. The embryonic central nervous system lineages of *Drosophila melanogaster*: II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. Dev. Biol. 189, 186–204.

Schmidt-Ott, U., Technau, G.M., 1992. Expression of *en* and *wg* in the embryonic head and brain of *Drosophila* indicates a refolded band of seven segment remnants. Development 116, 111–125.

Schober, M., Schaefer, M., Knoblich, J.A., 1999. Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. Nature 402, 548–551.

Siller, K.H., Doe, C.Q., 2009. Spindle orientation during asymmetric cell division. Nat. Cell Biol. 11, 365–374.

Skeath, J.B., Doe, C.Q., 1998. Sanpodo and Notch act in opposition to distinguish sibling neuron fates in the *Drosophila* CNS. Development 125, 1857–1865.

Sotillos, S., Roch, F., Campuzano, S., 1997. The metalloprotease–disintegrin Kuzbanian participates in *Notch* activation during growth and patterning of *Drosophila* imaginal discs. Development 124, 4769–4779.

Spana, E.P., Doe, C.Q., 1996. Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron 17, 21–26.

Spana, E.P., Kopczynski, C., Goodman, C.S., Doe, C.Q., 1995. Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. Development 121, 3489–3494.

Technau, G.M., Campos-Ortega, J.A., 1987. Cell autonomy of expression of neurogenic genes of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 4500–4504.

Thomas, U., Speicher, S.A., Knust, E., 1991. The *Drosophila* gene *Serrate* encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. Development 111, 749–761.

Tio, M., Udolph, G., Yang, X., Chia, W., 2001. cdc2 links the *Drosophila* cell cycle and asymmetric division machineries. Nature 409, 1063–1067.

Udolph, G., Prokop, A., Bossing, T., Technau, G.M., 1993. A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. Development 118, 765–775.

Udolph, G., Urban, J., Ruesing, G., Luer, K., Technau, G.M., 1998. Differential effects of EGF receptor signalling on neuroblast lineages along the dorsoventral axis of the *Drosophila* CNS. Development 125, 3291–3299.

Udolph, G., Rath, P., Chia, W., 2001. A requirement for Notch in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions. Development 128, 1457–1466.

Wang, X., Adam, J.C., Montell, D., 2007. Spatially localized Kuzbanian required for specific activation of Notch during border cell migration. Dev. Biol. 301, 532–540.

Ward, E.J., Coulter, D.E., 2000. odd-skipped is expressed in multiple tissues during *Drosophila* embryogenesis. Mech. Dev. 96, 233–236.

Wodarz, A., 2005. Molecular control of cell polarity and asymmetric cell division in *Drosophila* neuroblasts. Curr. Opin. Cell Biol. 17, 475–481.

Wodarz, A., Ramrath, A., Kuchinke, U., Knust, E., 1999. Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. Nature 402, 544–547.

Wodarz, A., Ramrath, A., Grimm, A., Knust, E., 2000. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. J. Cell Biol. 150, 1361–1374.

Yao, K.M., White, K., 1994. Neural specificity of elav expression: defining a *Drosophila* promoter for directing expression to the nervous system. J. Neurochem. 63, 41–51.

Yu, F., Morin, X., Cai, Y., Yang, X., Chia, W., 2000. Analysis of *partner of inscuteable*, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell 100, 399–409.

Yu, F., Cai, Y., Kaushik, R., Yang, X., Chia, W., 2003. Distinct roles of Galphai and Gbeta13F subunits of the heterotrimeric G protein complex in the mediation of *Drosophila* neuroblast asymmetric divisions. J. Cell Biol. 162, 623–633.

Yu, F., Wang, H., Qian, H., Kaushik, R., Bownes, M., Yang, X., Chia, W., 2005. Locomotion defects, together with Pins, regulates heterotrimeric G-protein signaling during *Drosophila* neuroblast asymmetric divisions. Genes Dev. 19, 1341–1353.

Zeng, C., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1998. Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage. Genes Dev. 12, 1086–1091.