Control of neuronal cell fate and number by integration of distinct daughter cell proliferation modes with temporal progression

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SUMMARY
During neural lineage progression, differences in daughter cell proliferation can generate different lineage topologies. This is apparent in the Drosophila neuroblast 5-6 lineage (NB5-6T), which undergoes a daughter cell proliferation switch from generating daughter cells that divide once to generating neurons directly. Simultaneously, neural lineages, e.g. NB5-6T, undergo temporal changes in competence, as evidenced by the generation of different neural subtypes at distinct time points. When daughter proliferation is altered against a backdrop of temporal competence changes, it may create an integrative mechanism for simultaneously controlling cell fate and number. Here, we identify two independent pathways, Prospero and Notch, which act in concert to control the different daughter cell proliferation modes in NB5-6T. Altering daughter cell proliferation and temporal progression, individually and simultaneously, results in predictable changes in cell fate and number. This demonstrates that different daughter cell proliferation modes can be integrated with temporal competence changes, and suggests a novel mechanism for coordinately controlling neuronal subtype numbers.

KEY WORDS: Lineage topology, Daughter cell proliferation, Neural progenitor, Proliferation control, Temporal competence changes, Drosophila

INTRODUCTION
During nervous system development, progenitor cells often divide asymmetrically, renewing themselves and budding off daughter cells that typically have a more limited mitotic potential. The daughter cell may in turn display three alternative behaviors: directly differentiating into a neuron or glia; dividing once to generate two neurons and/or glia; or dividing multiple times (i.e. acting as a transit-amplifying or intermediate neural precursor cell) before finally generating neurons and/or glia (Brand and Livesey, 2011; Gotz and Huttner, 2005; Kriegstein et al., 2006; Rowitch and Kriegstein, 2010). Adding to the complexity, recent studies in Drosophila melanogaster have revealed that some lineages display a switch in daughter cell proliferation, from daughters dividing once to daughters that directly differentiate (Baumgardt et al., 2009; Karcavich and Doe, 2005). As a result of these different proliferative behaviors of the daughter cells, lineage topology (the branching pattern of the lineage tree) may differ considerably. In addition to the complexity of different lineage topologies, it is becoming increasingly clear that both vertebrate and invertebrate neural progenitor cells undergo programmed temporal changes in their competence, as evidenced by the generation of different cell types at different developmental time points (Jacob et al., 2008; Okano and Temple, 2009; Pearson and Doe, 2004). Importantly, as temporal competence changes occur simultaneously with changes in daughter cell proliferation, it results in an interplay that can regulate the precise numbers of each unique cell type. Even though this basic principle of ‘topology-temporal interplay’ is likely to be of fundamental importance during nervous system development, it is not well recognized, let alone well understood at the molecular genetic level.

The Drosophila ventral nerve cord (VNC) is derived from a set of neural progenitor cells, termed neuroblasts (NBs), generated in the early embryo (Doe and Technau, 1993). Each neuroblast generates a unique lineage, progressing by multiple rounds of asymmetric cell division, renewing itself and budding off daughter cells denoted ganglion mother cells (GMCs). Each GMC in turn undergoes a final division to generate two neurons and/or glia (Doe, 2008; Knoblich, 2008; Skeath and Thor, 2003; Technau et al., 2006). In most, if not all, cases the GMC division has also been found to be asymmetric (Bardin et al., 2004; Doe, 2008; Knoblich, 2008), and the two postmitotic sibling cells are often generically referred to as cell fate ‘A’ and ‘B’ (Cau and Blader, 2009). Thus, Drosophila embryonic VNC lineages undergo repeated rounds of NB→GMC→A/B divisions, until each neuroblast enters quiescence or undergoes apoptosis (Maurange and Gould, 2005). The homeodomain protein Prospero (Pros) plays a crucial role in limiting GMC proliferation (Doe, 2008; Egger et al., 2008; Knoblich, 2008). Pros is expressed continuously by most, if not all, embryonic VNC lineages, but is blocked from acting in the neuroblast by being sequestered in the cytoplasm by its adaptor protein Miranda (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). As the neuroblast buds off each GMC, Pros is asymmetrically distributed to the GMC, where it enters the nucleus and regulates gene expression (Choksi et al., 2006; Li and Vaessin, 2000). The precise timing of these events allows each GMC to divide once, but prevents its daughter cells from dividing. During larval stages, neuroblasts exit quiescence and proliferate to generate the adult nervous system (Maurange and Gould, 2005). During this postembryonic lineage progression, Pros again plays a role in controlling daughter cell proliferation, and studies have found that Notch signaling is also crucial for limiting daughter cell proliferation postembryonically (Bello et al., 2006; Bowman et al., 2008; Lee et
al., 2006; Weng et al., 2010). However, despite this recent progress, it is unclear how distinct proliferation control mechanisms are integrated to shape different lineage trees.

To begin addressing these issues, we are focusing on one particular Drosophila embryonic neuroblast, NB5-6. In the three thoracic (T) segments, NB5-6 generates a lineage of 20 cells (Schmid et al., 1999; Schmidt et al., 1997), including a thorax-specific set of four related neurons that express the Apterous (Ap) LIM-homeodomain protein: the Ap neurons (Fig. 1A,B) (Baumgardt et al., 2007). Ap neurons are born at the very end of the lineage, and NB5-6 undergoes a programmed switch in daughter cell proliferation prior to generating Ap neurons (Baumgardt et al., 2009). Thus, there is a switch from an NB→GMC→A/B to an NB→Ap neuron lineage progression, and the Ap neurons are born directly from the neuroblast (Fig. 1B). Here, we identify two separate daughter proliferation mechanisms acting within the NB5-6T lineage. We find that Pros controls daughter cell (GMC) proliferation in the early lineage. In the late lineage, as Ap cells are generated, the activation of the Notch pathway is superimposed upon Pros activity, acting to further limit daughter cell proliferation and resulting in the programmed proliferation switch. In contrast to their roles in controlling daughter cell proliferation, neither pathway plays any role in controlling the cell cycle exit of the neuroblast. Moreover, the Pros and Notch pathways do not regulate each other and therefore, whereas single mutants show limited overproliferation, double mutants – lacking both of these daughter cell proliferation controls – display extensive overproliferation of the entire NB5-6T lineage, as well as of the entire VNC. Finally, the identification of this daughter cell proliferation switch allowed us to genetically assess the concept of topology-temporal interplay. To this end, we generated double mutants for the Notch pathway and for nab, a subterminal gene that affects Ap neuron specification (Baumgardt et al., 2009) (i.e. a topology-temporal perturbation). These mutants show the predicted combined alteration of both cell fate and cell number, a phenotype not found in either mutant alone.

These results identify Notch signaling as the first bona fide daughter proliferation switch and, moreover, demonstrate a novel role for Notch in Drosophila neuroblasts. Furthermore, we demonstrate that a neural lineage may depend upon two complementary regulatory mechanisms, involving Pros and Notch, for controlling daughter proliferation and thereby achieve a distinct lineage topology. Finally, we find that daughter cell proliferation control is integrated with temporal progression in this lineage, thereby allowing for the generation of the proper numbers of each unique cell type.

**MATERIALS AND METHODS**

Fly stocks

Ibe(K)-EGFP reporter transgenic strains were generated by inserting fragment K (De Graeve et al., 2004) from the ladybird early gene into the pGreen H-Pelican vector (Barolo et al., 2000) and generating transgenes by standard techniques (BestGene, Chino Hills, CA, USA).

Other fly stocks used were: Ibe(K)-lacZ [provided by K. Jagla (De Graeve et al., 2004)]; Ibe(K)-Gal4 (Baumgardt et al., 2009); elav-Gal4 [provided by A. DiAntonio (DiAntonio et al., 2001)]; nak51 [provided by F. Diaz-Benjumea (Terriente Felix et al., 2007)]; E(spl)m6-EGFP [provided by J. Posakony (Castro et al., 2005)]; E(spl)m8-lacZ [provided by F. Schweisguth (Lecourtois and Schweisguth, 1995)]; castr [provided by W. Odendal, NINDS, National Institutes of Health, Bethesda, MD, USA]. UAS-Notch-RNAi stocks used were K1000002 (VDRC) and BL0706 (Bloomington), which were combined in the same fly. To enhance the RNAi, elav-Gal4 was combined with UAS-dcr2 (Baumgardt et al., 2007).

**Immunohistochemistry**

Antibodies were generated in rat against Dpn expressed in E. coli from a plasmid provided by J. B. Skeath (Washington University School of Medicine, St Louis, MO, USA). Immunohistochemistry was performed as previously described (Baumgardt et al., 2009). Primary antibodies were: guinea pig anti-Hey (1:500; provided by C. Deldiakis (Monastirioti et al., 2010)); guinea pig anti-Deadpan (1:1000; provided by J. B. Skeath); rabbit anti-phospho-histone H3 Ser10 (ph3) (1:250; Upstate-Millipore, Billerica, MA, USA); rabbit anti-β-gal (1:5000; ICN-Cappel, Aurora, OH, USA); rabbit anti-cleaved Caspase 3 (1:100; Cell Signaling Technology, Danvers, MA, USA); chicken anti-β-gal (1:1000; Abcam, Cambridge, UK); rabbit anti-GFP (1:500; Molecular Probes, Eugene, OR, USA); guinea pig anti-Col (1:1000), guinea pig anti-Dmm (1:1000), chicken anti-proNplp1 (1:1000) and rabbit anti-profMFRa (1:1000) (Baumgardt et al., 2007); rat anti-Grh (1:1000) (Carlsson et al., 2010); rabbit anti-Nab (1:1000; provided by F. Diaz-Benjumea (Terriente Felix et al., 2007)); rabbit anti-Cas (1:250; provided by W. Odendal (Kambadur et al., 1998)); rat mAb anti-GsbN (1:10; provided by R. Holmgren (Buenzow and Holmgren, 1995)); mouse mAb anti-Dac 2.3-2 (1:25); mAb anti-Antp (1:10), mAb anti-ProS MR1A (1:10), mAb anti-Eya 10H6 (1:250) and mAb anti-NICD (1:10) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA); rabbit anti-Inscutable (1:1000; provided by F. Yu and W. Hongyan, Temasek Lifesciences Laboratory (TLL), National University of Singapore, Singapore); rat anti-Miranda (1:100; provided by F. Matsuzaki, Center for Developmental Biology, RIKEN, Kobe, Japan); and guinea pig anti-Numb (1:1000; provided by J. B. Skeath).

**EdU labeling**

Embryos at St11-13 were injected with 5-ethyl-2′-deoxyuridine (EdU) solution (0.2 mM EdU, 0.1 mM KCl) (Click-IT Alexa Fluor 488 Imaging Kit, Invitrogen) by standard procedures and placed at 26°C overnight. At 18 hAEF, the central nervous system was dissected, attached to poly-l-lysine-coated slides and fixed with 4% paraformaldehyde for 15 minutes. Slides were immunostained as previously described (Baumgardt et al., 2009). The Click-IT reaction was carried out according to the manufacturer’s instructions.

**Chemical mutagenesis**

Brieﬂy, the previously identiﬁed 450 bp FMRFa T enhancer (Schneider et al., 1993) was inserted into the pGreen H-Pelican vector (Barolo et al., 2000). A total of 127 FMRFa-EGFP transgenic lines were generated, yielding two robust reporter lines, one on the second and one on the third chromosome, in which Ap4/FMRFa neurons could be visualized in the living late embryo and larvae (Fig. 1D). A total of 9781 mutant lines were generated in an F3 screen, mutated on the second or third chromosome, with ethyl methanesulfonate. Mutants were mapped by deletions and generated in an F3 screen, mutated on the second or third chromosome, with ethyl methanesulfonate. Mutants were mapped by deletions and finally to known alleles.

**Confocal imaging and data acquisition**

A Zeiss LSM 700 or a Zeiss META 510 confocal microscope was used for fluorescent images; confocal stacks were merged using Adobe Photoshop. Statistical calculations were performed using GraphPad Prism software (v4.03). To address statistical signiﬁcance, Student’s t-test was used, or, in the case of non-Gaussian distribution of variables, a nonparametric Mann-Whitney U test or Wilcoxon signed rank test, was used. Images and graphs were compiled in Adobe Illustrator.
RESULTS

A genetic screen for extra Ap4/FMRFa neurons identifies the Notch pathway

NB5-6T and its lineage can be readily identified by reporter genes under the control of an enhancer fragment from the ladybird early (lbe) gene [lbe(K)] (Baumgardt et al., 2007; De Graeve et al., 2004). NB5-6T delaminates at late stage (St) 8 and generates a lineage of 20 cells until it exits the cell cycle at St15 and undergoes apoptosis at St16 (Baumgardt et al., 2007; De Graeve et al., 2004). The four Ap neurons, identifiable by Eyes absent (Eya) (Miguel-Aliaga et al., 2004), are born sequentially and directly from the neuroblast at the end of this lineage (Fig. 1A) (Baumgardt et al., 2009). In the T1 segment, a modified NB5-6T lineage is generated, displaying five Ap cells, and we have therefore focused on the T2-T3 segments. The Ap neurons can be subdivided into three subtypes: the Ap1/Nplp1 and Ap4/FMRFa neurons, which express the Nplp1 and FMRFamide (FMRFa) neuropeptides, respectively, and the Ap2/Ap3 interneurons (Fig. 1A,B). Expression of Nplp1 and FMRFa commences 18 hours after egg laying (hAEL).

Drosophila embryonic neuroblasts express a sequential cascade of transcription factors that act to determine competence; this temporal gene cascade (Brody and Odenwald, 2002; Jacob et al., 2008; Pearson and Doe, 2004) consists of Hunchback (Hb), Kruppel (Kr), Nubbin (also known as Pdm1) and Pdm2 (collectively denoted Pdm herein), Castor (Cas) and Grainy head (Grh), in the order Hb→Kr→Pdm→Cas→Grh. NB5-6T displays the typical progression of these factors, and Ap neurons are generated within a late Cas/Grh temporal window, in which Cas in particular plays a key role in controlling Ap neuron specification (Fig. 1C) (Baumgardt et al., 2009). In addition, the ‘subtemporal’ regulators Squeeze and Nab act downstream of Cas to subdivide the Ap window into the three Ap neuron subtypes (Fig. 1C) (Baumgardt et al., 2009).

In a genetic screen scoring for FMRFa-EGFP expression, we identified a group of mutants with additional Ap4/FMRFa cells (Fig. 1D-F). Of these ‘double Ap4’ mutants, seven were genetically mapped to the kuzbanian (kuz) gene and one to the neuralized (neur) gene, two positive regulators in the Notch signal transduction pathway (reviewed by Kopan and Ilagan, 2009).
Perturbation of the Notch pathway results in extra Ap neurons via two different mechanisms

Further characterization of *kuz* and *neur*, and of other Notch pathway perturbations, revealed not only extra Ap/FMRFa cells but also other additional Ap neurons, as evident by Eya and Nplp1 expression (i.e. an increase in the number of all late-born NB5-6T cells) (supplementary material Fig. S1A-D,M). What is the origin of these extra Ap cells? Notch signaling is well known for its early role in controlling the generation of neuroblasts in the ectoderm – in Notch pathway mutants too many neuroblasts are generated (Artavanis-Tsakonas et al., 1983). Thus, we anticipated that early Notch pathway perturbation might indeed lead to the formation of additional NB5-6T neuroblasts, and hence entire NB5-6T lineages. This was evident in *neur* and *Delta (Dl)* alleles, where additional Ap neurons were indeed accompanied by an increase in the overall number of NB5-6T lineage cells (supplementary material Fig. S1A-C,E-G,M,N). This overall increase of NB5-6T cells indeed stemmed from formation of extra NB5-6T neuroblasts, as evident by staining for Deadpan (Dpn), a basic-helix-loop-helix (bHLH) protein expressed in neuroblasts and transiently in GMCs (Bier et al., 1992) (supplementary material Fig. S1I-K,O). Thus, as anticipated, early perturbation of the Notch pathway can result in excess Ap neurons simply as an effect of the generation of additional NB5-6T neuroblasts during neuroblast selection. However, *kuz* is expressed maternally (see www.FlyBase.org), thereby allowing for proper Notch signaling at early embryonic stages in *kuz* mutants. In line with this, in zygotic *kuz* mutants we found that supernumerary Ap cells were generated without evidence of additional NB5-6T neuroblasts or of additional complete NB 5-6T lineages (supplementary material Fig. S1D,H, L-O).

These findings reveal that supernumerary Ap cells are generated by at least two mechanisms in Notch pathway mutants: additional entire NB5-6T lineages and additional Ap neurons from within a single NB5-6T lineage.

The Notch pathway is crucial for proper cell numbers, but not for cell fate, in the late NB5-6T lineage

To further validate our findings that the Notch pathway controls Ap cell numbers also within a single NB5-6T lineage, we analyzed a number of other Notch pathway perturbations selected to specifically enable study of the Notch pathway in this lineage after neuroblast delamination (Fig. 2A). These late Notch pathway perturbations all resulted in an increase in the number of Ap neurons (Fig. 2B-M). However, there was no apparent effect upon

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**Fig. 2. Late Notch pathway perturbations reveal an increased number of Apterous neurons in NB5-6T.** (A) The canonical *Drosophila* Notch pathway (Bray, 2006). (B) In the control, the four Ap neurons express Eya, and the Ap1 and Ap 4 neurons express Nplp1 and FMRFa, respectively. (C-J) Notch pathway perturbations result in extra Ap neurons in thoracic hemisegments. However, the number of Ap cluster cells never exceeds eight per hemisegment, and the neuropeptide-expressing cells never exceed two. (K-M) Data are represented as mean number of Eya-, Nplp1- or FMRFa-expressing cells per thoracic T2/T3 Ap cluster (± s.e.m.; n≥30 clusters). Asterisks denote significant difference compared with control (P<0.05). Genotypes: (B) OregonR; (C) *lbe(K)*-Gal4/+; UAS-Tom, UAS-Tom/+; (D) *elav-Gal4/UAS-Tom, UAS-Tom*; (E) *UAS-dcr2/UAS-N.dsRNABL7076, elav-Gal4;* (F) *kuz+294*; (G) *sanpodoG104*; (H) *elav-Gal4/UAS-mamDN*; (I) *elav-Gal4/UAS-shmiR-Su(H)2056*; (J) *E(spl)-C*+/+.
the differentiation of Ap neurons, evident by the expression of the Nplp1 and FMRFα neuropeptides, as well as nine previously identified Ap neuron determinants (Fig. 2B-M; supplementary material Fig. S2) (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Hewes et al., 2003; Karlsson et al., 2010; Lundgren et al., 1995; Miguel-Aliaga et al., 2004). Hence, Notch signaling is not involved in the specification of Ap neurons.

To address whether Notch also controls cell numbers during the earlier stages of NB5-6T lineage progression, we counted the number of cells arising early in the N5-6T lineage in kuz mutants, but found no evidence of additional cells at St13 (Fig. 3V). Moreover, in Dl mutants, which in all cases displayed additional NB5-6T neuroblasts in each hemisegment, we did not observe an increase in the numbers of NB5-6T lineage cells beyond that anticipated from the presence of multiple lineages (supplementary material Fig. S1C,K,M-O).

These results demonstrate that the Notch pathway acts to control cell numbers in the latter part of the NB5-6T lineage by preventing proliferation of Ap cells and furthermore indicate that the Notch pathway is not involved in daughter proliferation control in the early lineage.

Notch signaling triggers the switch in daughter proliferation in the NB5-6T lineage

Notch pathway perturbations that do not result in extra NB5-6T neuroblasts still result in an excess of Ap neurons generated from within a single lineage. We postulated three possible mechanisms underlying this effect: (1) a premature switch in the temporal cascade, resulting in the premature specification of Ap neurons; (2) a failure of the neuroblast to exit the cell cycle, resulting in a continuous birth of Ap neurons; or (3) a failure of the switch from GMC to direct neuron (Fig. 3W). To distinguish between these possibilities, we determined the time points at which Ap neurons

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**Fig. 3.** Notch controls the daughter cell proliferation switch. (A-H) In both control and kuz, expression of Col and Eya is absent at St13 (A,B) and commences at St14 (C,D) in similar numbers of cells (E,F). However, at St15 and St16 additional Ap cells are apparent in kuz (G,H). (I,J) At early St13, both control and kuz show expression of Cas but not Grh in the neuroblast (Dpn+ cell). (K,L) Onset of Grh, at late St13, is observed in both control and kuz. (M) In control, only one cell can be observed undergoing cell division in the late lineage at this stage (0% double divisions; n=21 T2/T3 hemisegments). (N) In kuz, additional dividing cells can be often observed (37%; n=19 T2/T3 hemisegments). (O,P) The NB5-6T neuroblast undergoes typical apoptosis in both genotypes. (Q-U) In control (Q) and nab (R), Ap cells can be separately labeled by EdU, in line with their sequential generation, and with the cell fate change in Ap2 neurons to an Ap1/Nplp1 fate in nab. By contrast, in kuz, elav>Notch-RNAi and elav>Tom embryos, the ectopic pairs of FMRFα or Nplp1 cells cannot be separately labeled by EdU (see supplementary material Fig. S3 for details). (V) Quantification of the number (± s.e.m.) of lbe(K)-EGFP-expressing cells in control and kuz at St13 (n=22 T2/T3 hemisegments). Note that at this stage, prior to the switch, there is no sign of additional cells generated in kuz when compared with control. (W) The wild-type NB5-6T lineage and three possible mechanisms to explain the Notch pathway phenotypes observed. The normal temporal progression and appearance of Ap cells, the ectopic cell divisions in the late lineage, the death of the neuroblast at St16, and the failure to distinguish the pairs of Ap1/Nplp1 and Ap4/FMRFα cells rule out mechanisms I and II. Genotypes: (A,C,E,G,I,K,M,O) lbe(K)-EGFP, (Q) OregonR; (B,D,F,H,J,L,N,P) kuz29-4; (R) nab852; (S) kuz29-4; (T) UAS-dcr2/UAS-N.dsrNA27076, UAS-N.dsrRNAkk100002; elav-Gal4+/; (U) elav-Gal4/UAS-Tom, UAS-Tom.
were generated and the temporal progression in the neuroblast. We also identified the mitotic events in this lineage and performed DNA labeling to determine sibling cell relationships.

We first addressed the timing of Ap neuron generation. In both wild type and kuz, Ap neurons were identifiable at St14 by the onset of Col (Col; Knot – FlyBase) and Eya expression (Fig. 3A-D). In line with the late effects on the lineage in kuz mutants, we did not observe supernumerary Col and Eya cells until St15-16 (Fig. 3E-H). Next, we addressed the temporal competence progression in NB5-6T. Analyzing the expression of Cas and Grh in wild type and kuz, we found that expression of these factors in the NB5-6T neuroblast commences at their normal time points: late St11 (Cas) and late St13 (Grh) (Fig. 3I-L; not shown) (Baumgardt et al., 2009). Hence, Notch pathway perturbation does not lead to the premature appearance of Ap neurons, nor to shifts in NB5-6T temporal progression.

Previous studies using phospho-histone H3 Ser10 (pH3) antibodies and DNA labeling (BrdU/EdU) of the NB5-6T lineage revealed that Ap neurons are born sequentially at the end of this lineage (Baumgardt et al., 2009). We confirmed this in wild type, as demonstrated by the finding of only one mitotic cell, the neuroblast, in the latter part of the lineage (St14; Fig. 3M; 0% of double divisions). By contrast, in kuz mutants we frequently found evidence of abnormal cell divisions, including the presence of two mitotic cells in the Ap window, or of dividing Ap cells (Fig. 3N; 37% of double divisions). However, pH3 analysis of the neuroblast (Dpn+ cell) at late St16 revealed no evidence of ectopic divisions (0% divisions; n=42 neuroblasts). Moreover, the neuroblast underwent its stereotyped apoptosis at late St16 (Fig. 3O-P). Thus, although we find clear evidence of ectopic cell divisions in the late lineage, we find no evidence for extended neuroblast divisions past the normal stage (St15).

Finally, to determine sibling cell relationships, we performed DNA labeling by injecting St11-13 embryos with EdU, allowing embryos to develop until 18 hAEL, and staining for EdU combined with Eya, Nplp1, and FMRFa. In wild type, Ap neurons are generated sequentially (Baumgardt et al., 2009) and can hence be separately labeled by EdU (Fig. 3Q; supplementary material Fig. S3). Similarly, in mutants affecting the temporal progression of the lineage, such as nab, in which the Ap2 neuron is misexpressed into an Ap1/Nplp1 neuron without the generation of any additional Ap neurons (Baumgardt et al., 2009), we found that the two Ap1/Nplp1 neurons could be distinguished by EdU labeling (Fig. 3R; supplementary material Fig. S3). As outlined above, in Notch pathway mutants the temporal progression in NB5-6T is unaffected (Fig. 3A-L). As anticipated from these findings, the sequential birth of the three Ap neuron cell types (Ap1/Nplp1, Ap2/3 and Ap4/FMRFa) was unaltered in Notch pathway mutants (Fig. 3S-U; supplementary material Fig. S3). However, the two Ap1/Nplp1 and Ap4/FMRFa neurons observed in Notch pathway mutants could not be distinguished from each other by EdU staining (Fig. 3S-U; supplementary material Fig. S3). Thus, these cells must be generated as consecutive sibling pairs. This presumably applies to the Ap2 and Ap3 cells as well, but we are currently unable to determine this owing to a lack of markers for distinguishing between these two cell types.

In summary, in Notch pathway mutants we find that the temporal competence progression (Cas→Grh) is unaltered and that the temporal birth order of the three types of Ap neurons is normal. The onset of Col and Eya expression is also unaltered. However, additional Ap neurons appear late during lineage progression, and this is accompanied by ectopic mitotic events late in the lineage in Ap cells. In addition, double Ap1/Nplp1 and Ap4/FMRFa cells cannot be distinguished by DNA labeling, demonstrating that they are generated as sibling pairs. Finally, the neuroblast exits the cell cycle at St15 and undergoes apoptosis at St16, as in wild type. These findings are in agreement with a model in which perturbation of the Notch pathway results in a failure of the neuroblast to undergo the GMC-to-direct neuron switch, resulting in the aberrant division of Ap cells (Fig. 3W).

The NB5-6T lineage progresses via typical asymmetric cell divisions

During Drosophila VNC development, Notch signaling is off in delaminating neuroblasts – a prerequisite for ectodermal cells to assume a neuroblast identity (reviewed by Skeath and Thor, 2003). Notch signaling is subsequently controlled in each lineage by the asymmetric distribution of Numb, a membrane-associated protein that blocks Notch signaling (reviewed by Fortini, 2009). Numb asymmetrically distributes from the neuroblast to the GMC, and finally asymmetrically to one of the postmitotic daughter cells – the ‘B’ cell – whereas Numb is absent from the ‘A’ cell. Thus, in the NB5-6T lineage only the ‘A’ cell lacks Numb, and a number of genetic analyses have demonstrated a role for Notch in specifying the ‘A’ cell fate (Fuertsenberg et al., 1998). By contrast, our analysis suggests that the Notch pathway is activated in the NB5-6T neuroblast itself. This could indicate alterations in the apical-basal asymmetric protein distribution machinery.

To address this we analyzed the expression and localization of Inscutable (Insc) and Miranda (two apical proteins) (Knoblich, 2008), as well as Pros and Numb. We find that all four proteins display the typical apical-basal cellular distribution in the NB5-6T neuroblast during late stages (supplementary material Fig. S4A-C). We furthermore find no evidence that Numb itself alters the asymmetric machinery, as evident by the normal expression and distribution of the asymmetric proteins in kuz (supplementary material Fig. S4D-I). To determine whether Numb is involved in modulating Notch signaling in the later part of this lineage, we analyzed numb. We found only a minor effect upon Ap neuron generation, with a partial loss of Eya and FMRFa, but not of Nplp1, at 18 hAEL (supplementary material Fig. S5A,B,G). Analysis of neuroblast divisions using pH3 revealed that the neuroblast was undergoing divisions during St13-15, and prior to St16 we did not observe any evidence of apoptosis (supplementary material Fig. S5C-F; not shown). This indicates that all four Ap cells are generated in numb and are only partially affected in their terminal differentiation.

The distribution of Inscutable, Miranda, Pros and Numb reveals that the NB5-6T lineage displays the typical apical-basal asymmetric lineage progression. The genetic analysis further reveals that numb only plays a minor role in the latter part of this lineage and acts to keep Notch off in the Ap neurons, thereby allowing them to terminally differentiate.

The Notch pathway is activated in the NB5-6T neuroblast prior to the proliferation switch

When and where is the Notch pathway activated in the NB5-6T lineage? To address this we analyzed Notch pathway activation directly by staining with an antibody against the Notch intracellular domain (NICD), aiming to track the cleavage and intracellular relocalization of Notch. This revealed progressively stronger Notch activation in the NB5-6T neuroblast as the lineage progressed (Fig. 4A,B). In addition, we assayed Notch pathway activation in an indirect manner by analyzing the expression of well-known Notch
target genes: members of the HES (Hairy, Enhancer of Split) family of bHLH transcription factors (reviewed by Bray, 2006), as well as the HES-related Notch target Hey (Monastirioti et al., 2010). Hey has recently been found to be expressed in many ‘A’ cells (Notch-activated sibling neurons and/or glia) and to depend upon Notch signaling for its expression (Monastirioti et al., 2010). In line with this, we found that postmitotic cells in the early part of the lineage express Hey (Fig. 4C). However, analysis of Hey expression in the late NB5-6T lineage revealed expression neither in Ap neurons nor in the neuroblast (Fig. 4D). Next, we analyzed the expression of E(spl)m8-EGFP (Castro et al., 2005) and E(spl)m8-lacZ (Lecourtois and Schweisguth, 1995). Expression was weak and variable in the neuroblast at St10-11 but became more robust at St12, just prior to the daughter cell proliferation switch (Fig. 4E-J). To validate the use of E(spl)m8 reporters as a bona fide readout of Notch activation specifically in the NB5-6T lineage, we analyzed E(spl)m8-EGFP expression in kuz mutants and found complete loss of reporter expression within the NB5-6T neuroblast (Fig. 4K).

The expression analysis of Notch itself and of the Notch targets among the HES gene family indicates that during early stages NB5-6T undergoes multiple rounds of the typical NB→GMC→A/B progression, with Notch activation in the ‘A’ cell and specific activation of the HES factor Hey in ‘A’ cells as an effect thereof. At St10-11, expression of E(spl)m8-EGFP is consistent with the notion that Notch is also activated in the NB5-6T neuroblast, prior to the GMC-to-direct neuron switch (Fig. 4L). This pattern of Notch activation is also supported by the Notch pathway genetic analysis. The expression of Hey in ‘A’ cells but not in the neuroblast, and the activation of E(spl) reporter gene expression in
the neuroblast, demonstrate that Notch signaling results in context-dependent HES gene activation within various parts and at various stages of progression of this one VNC lineage.

**Prospero controls daughter cell proliferation within early stages of NB5-6T lineage development but is not involved in the proliferation switch**

As outlined above, Pros has also been shown to control daughter cell proliferation, i.e. of GMCs in the embryonic VNC. We addressed the possible role of Pros in the NB5-6T lineage. In line with previous studies, we found that pros mutants displayed a clear increase of NB5-6T cells in the early parts of the lineage (Fig. 5A,B,J). In the latter part of the NB5-6T lineage, in the Ap window, we also observed expression of Pros, with the typical weaker, cytoplasmic staining in the neuroblast versus stronger, nuclear staining in the Ap neurons (supplementary material Fig. S6A,F). Strikingly, despite the typical expression and distribution of Pros, we found no evidence of additional Ap neurons being generated in pros mutants (Fig. 5A,B,H,I). In addition, in pros mutants we found no evidence of continued division of the neuroblast past St15 (supplementary material Fig. S6D) and observed the scheduled apoptosis of the neuroblast at late St16 (supplementary material Fig. S6E).

These results reveal that pros controls daughter cell (GMC) proliferation in the early NB5-6T lineage. However, although Pros is expressed in the late lineage, pros is involved neither in the proliferation switch nor in neuroblast cell cycle exit.

**Prospero, the Notch pathway and the temporal factor Castor do not regulate each other**

To address the possible regulatory connection between Pros and the Notch pathway, we analyzed Pros expression in kuz mutants and, conversely, E(spl)m8-GFP expression in pros mutants. As anticipated, in neither case did we find any evidence for cross-regulation of one pathway upon the other (supplementary material Fig. S6A-C,L).

The cas temporal gene is crucial for cell specification in the latter part of NB5-6T development and activates a number of genes (grh, dac, sqz, nab, col), thereby triggering a cascade of regulatory events that ultimately results in the proper specification of the different Ap neurons (Baumgardt et al., 2009). Cas is expressed immediately prior to the daughter proliferation switch in NB5-6T (Fig. 1C). We therefore addressed the regulatory interplay between cas, pros and the Notch pathway. We did not observe any loss of Pros or E(spl)m8-EGFP in cas mutants, nor of Cas in pros mutants (supplementary material Fig. S6F-K). Above, we already noted that Cas is not affected in kuz mutants (Fig. 3).

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**Fig. 5. Prospero is crucial for controlling GMC and ‘Ap GMC’ divisions. (A)** In the control, four Ap neurons are visible and the normal number of cells is observed in the lineage. **(B)** In pros mutants, there are additional cells in the lineage, but Ap cell numbers are normal. **(C)** In kuz, there are additional Ap cells, but no additional early-born cells. **(D)** In kuz;pros, there are additional early- and late-born cells. **(E)** In kuz;pros mutants at St15 display a number of actively dividing cells within the NB5-6T lineage. **(F,G)** Expression of lbe(K)-EGFP, Col and Grh (F) or Dac (G) reveals that the majority of Col cells also express Grh and Dac. **(H)** Quantification of lbe(K)-EGFP-, Eya- or Col-expressing cells (± s.e.m.; n≥9 T2/T3 hemisegments) counted at St16 (Eya) or St15 [Col, lbe(K)-EGFP]. Asterisks denote significant difference compared with control (P<0.05). **(K)** Model of the NB5-6T lineage, showing the activity of the Pros and Notch pathways, as well as the phenotypes in different genetic backgrounds. Genotypes: (A,C,F) lbe(K)-EGFP; (B,G) lbe(K)-EGFP;pros17; (J) E(spl)m8-GFP+; pros17; (D,H) lbe(K)-EGFP; kuz294-4; (I,K,L) lbe(K)-EGFP; kuz294-4; pros17; (E) lbe(K)-EGFP; pros17/DF(3R)Exel7308.
These results show that the two daughter cell proliferation mechanisms – Pros and the Notch pathway – and the key late temporal factor Cas do not regulate each other.

**Prospero limits the division of Apterous cells in Notch pathway mutants**

In Notch pathway perturbations we observe a maximum of eight Ap cells per NB5-6T lineage, resulting from one aberrant division of each of the four Ap cells. We postulated that this was due to Ap cells being converted to GMC-like cells as a result of Notch pathway perturbations (Fig. 5K,L). Because Pros, which is a crucial controller of GMC divisions in the early lineage (Fig. 5K,M), is expressed in the latter part of the lineage, in both wild type and kuz mutants, the possibility was raised that Ap cells are prevented from dividing more than once by the action of Pros. To test this idea, we generated kuz;pros double mutants.

**kuz;pros** double mutants were malformed, displaying extensive overgrowth of the entire VNC, and did not develop past ~St16. Owing to developmental halt at St16, we did not detect expression of the Nplp1 or FMRFa neuropeptides (not shown). However, using the lbe(K)-EGFP marker, we were able to identify the NB5-6T lineage and to address its development. As anticipated, these double mutants displayed an increase in Ap cells beyond that observed in kuz, with an average of 16 Col-expressing cells in the NB5-6T lineage (Fig. 5A-D,I). To confirm the identity of these Col-expressing cells as Ap neurons, we used Eya, Grh and Dac as additional markers for Ap neurons, revealing the frequent co-expression of these markers (Fig. 5D,F,G).

In addition to the increase in Ap cells, double mutants displayed an extensive overproliferation of the entire NB5-6T lineage, with an average of 69 cells (Fig. 5D,J). As anticipated from the generation of such high numbers of cells within this relatively short time frame (late St8 to St16), we observed a number of mitotic cells within each NB5-6T lineage (Fig. 5E).

In **kuz;pros** double mutants both daughter proliferation controls are absent, and we find an increase in the number of Ap neurons generated when compared with kuz mutants alone (Fig. 5N). That we observe an average of only 16 Ap cells is likely to reflect the fact that **kuz;pros** mutants do not develop past St16, and as Ap neurons are born between St13 and St15 this precludes more extensive overproliferation. In addition, the entire NB5-6T is overgrown, and multiple cells throughout the lineage are undergoing continuous proliferation.

**Simultaneous disruption of Notch signaling and temporal coding reveals a topology-temporal interplay**

Mutations in the subtropical gene nab result in a misspecification of the Ap2 neuron into an Ap1/Nplp1 neuron, without the generation of any additional Ap neurons (Fig. 3R; supplementary material Fig. S3) (Baumgardt et al., 2009). Conversely, late disruption of the Notch pathway results in one division of each Ap neuron, but without any alteration in temporal progression or Ap cell fate (Figs 2, 3). Thus, both nab and kuz mutants display two Ap1/Nplp1 cells, but as a result of different mechanisms (Fig. 6A-C,F). Importantly, the Notch pathway does not regulate Nab, and the crucial temporal gene cas – an activator of nab – does not regulate the Notch pathway (see above). These findings allowed us to genetically test the concept of topology-temporal interplay. We reasoned that by simultaneously perturbing the daughter cell proliferation control and the temporal progression, we should be able to generate additional Ap1/Nplp1 neurons beyond those observed when each system is affected individually. To this end, we generated **kuz;nab** double mutants and analyzed them for Eya and Nplp1 expression at 18 hAEL. Strikingly, these double mutants revealed a combined effect, with extra Ap1/Nplp1 neurons beyond those observed in **kuz** or **nab** alone. Specifically, whereas **kuz** or **nab** single mutants never displayed more than two Ap1/Nplp1 neurons, double mutants displayed up to four Ap1/Nplp1 neurons (Fig. 6A-E). Thus, daughter cell proliferation and temporal progression can be independently or combinatorially interfered with, leading to predictable outcomes of cell fate and cell numbers (Fig. 6F).

**DISCUSSION**

We find that the NB5-6T lineage utilizes two distinct mechanisms to control daughter cell proliferation. In the early part of the lineage, pros limits daughter cell (GMC) proliferation, whereas in...
the late part canonical Notch signaling in the neuroblast further restricts daughter cell proliferation, resulting in a switch to the generation of neurons directly (Fig. 5K). The switch in daughter cell proliferation is integrated with temporal lineage progression and enables the specification of different Ap neuron subtypes and the control of their numbers.

A programmed switch in daughter cell proliferation

Our data on Notch activation in the NB5-6T lineage, using both antibodies and reporters, indicate progressive activation in the neuroblast: weak at St10-11 and more robust from St12 onward. Thus, Notch activity coincides with the proliferation mode switch. How is this gradual activation of Notch in the neuroblast controlled? NB5-6T undergoes the typical progression of the temporal gene cascade, with Cas expression preceding strong Notch activation. Thus, one possible scenario is that the late temporal gene cas activates the Notch pathway. However, our analysis of the E(spl)m8-EGFP reporter shows that this Notch target is still activated at the proper stage in cas mutants. Although this does not rule out the possibility that other, unknown, temporal factors might regulate Notch signaling, it rules out one obvious player, cas. Alternatively, as Notch signaling is off when neuroblasts are formed – a prerequisite for neuroblast selection – Notch activation in the neuroblast at later stages might simply reflect a gradual reactivation of the pathway. Although such a reactivation might at a first glance appear too imprecise, it is possible that the specificity of this particular Notch output – proliferation control – might be combinatorially achieved by the intersection of Notch signaling with other, more tightly controlled, temporal changes.

Overlapping daughter cell proliferation controls: a cooperative tumor-forming mechanism

Pros and Notch control daughter proliferation in different parts of NB5-6T and we find no evidence of cross-regulation between these pathways. The limited overproliferation of the lineage when each pathway is separately mutated results not from redundant functions, but rather stems from the biphasic nature of this lineage. Specifically, in pros mutants, Notch signaling is likely to be on in all ‘A’ type sibling daughter cells, as Numb continues to be asymmetrically distributed between daughter cells. Thus, Notch signaling in ‘A’ cells may preclude each ‘A’ cell from dividing even once (Fig. 5M, blue circles). This notion is in line with recent studies showing that postmitotic Notch activated cells (‘A’ cells) within the Drosophila bristle lineages are particularly resilient to overexpression of cell cycle genes (Simon et al., 2009). Similarly, in Notch pathway mutants, as Ap cells now divide (in essence becoming GMC-type cells), Pros will still play its normal role in these ‘GMCs’ and limit their proliferation to a single extra cell division. However, in kuz;pros double mutants, Ap cells are relieved of both types of daughter cell proliferation control and can thus divide for many additional rounds (Fig. 5N). This notion also applies to early parts of the NB5-6T lineage and probably to the majority of other VNC lineages, as indicated by the extensive overproliferation of the entire NB5-6T lineage, and to the general overproliferation of the VNC. However, based on our findings that neither the Notch pathway nor pros controls neuroblast identity or its progression, we postulate that these large clones contain a single, normally behaving NB5-6T neuroblast. In fact, the neuroblast is likely to exit the cell cycle and undergo apoptosis on schedule, as neither of these decisions depends upon pros or the...
Notch pathway. Of interest with respect to cancer biology is that our findings point to a novel mechanism whereby mutation in two tumor suppressors (e.g. Pros and Notch) cooperate to generate extensive overproliferation: not by acting in the same progenitor cell at the same time, but by playing complementary roles controlling daughter cell proliferation.

The topology-temporal interplay: a novel developmental intersection

As an effect of alternate daughter cell proliferation patterns, both vertebrates and invertebrates display variability in neural lineage topology (Brand and Livesey, 2011; Gotz and Hutten, 2005; Kriegstein et al., 2006; Rowitch and Kriegstein, 2010). Similarly, progenitors in these systems undergo temporal changes in competence, as evident by changes in the types of neurons and glia generated at different time points (Jacob et al., 2008; Okano and Temple, 2009; Pearson and Doe, 2004). Hence, the temporal-topology interplay described in this study is likely to be extensively used and to be conserved in mammals. As a proof of principle of this novel developmental intersection, we analyzed single and double mutants for kuz and nab, thereby independently versus combinatorially affecting temporal progression and daughter cell proliferation. Strikingly, these mutants show the predicted combined effect, with the appearance of additional Ap1/Nplp1 neurons beyond those found in each individual mutant.

If programmed proliferation switches are conserved, how might such a topology-temporal interplay become utilized in mammals? There are several examples in which different clusters/pools/nuclei of neurons of distinct cell fate are generated from the same progenitor domain in the developing mammalian nervous system. Such pools often contain different numbers of cells, but the proliferation. Strikingly, these mutants show the predicted combined effect, with the appearance of additional Ap1/Nplp1 neurons beyond those found in each individual mutant.

Acknowledgements

We thank C. Deldiakis, S. Cohen, F. Schweisguth, F. Diaz-Benjumea, R. Holmgren, W. Odenwald, M. Levine, J. B. Skeath, J. Posakony, the Developmental Studies Hybridoma Bank at the University of Iowa, the Drosophila Genetic Resource Center at Kyoto and The Bloomingston Stock Center for sharing antibodies, fly lines and DNAs; T. Edlund, D. van Meyel, I. Miguel-Alia and J. B. Thomas for critically reading the manuscript; and H. Ekman, A. Angel and A. Starkenberg for technical assistance. FlyBase provided http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.074500/-/DC1

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Supplemental Figure 1

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15hAEL images show the expression patterns of different genes in control and kuz^{29-4} conditions.
Supplemental Figure 3

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nab r52

kuz

Notch-RNAi

elav>Tom

E(spl)/+

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