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Year: 2011

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Official URL: <http://edoc.unibas.ch/dok/A5844176>

Originally published as:

Viktorin, G. and Riebli, N. and Popkova, A. and Giangrande, A. and Reichert, H.. (2011) *Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in Drosophila brain development*. *Developmental biology*, Vol. 356. S. 553-565.

**Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in *Drosophila* brain development**

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**Keywords**

*Drosophila*; larval brain; neuroblast lineages; intermediate progenitor cells; neuroglioblast; gliogenesis

## Abstract

The neural stem cells that give rise to the neural lineages of the brain can generate their progeny directly or through transit amplifying intermediate neural progenitor cells (INPs). The INP-producing neural stem cells in *Drosophila* are called type II neuroblasts, and their neural progeny innervate the central complex, a prominent integrative brain center. Here we use genetic lineage tracing and clonal analysis to show that the INPs of these type II neuroblast lineages give rise to glial cells as well as neurons during postembryonic brain development. Our data indicate that two main types of INP lineages are generated, namely mixed neuronal/glial lineages and neuronal lineages. Genetic loss-of-function and gain-of-function experiments show that the *gcm* gene is necessary and sufficient for gliogenesis in these lineages. The INP-derived glial cells, like the INP-derived neuronal cells, make major contributions to the central complex. In postembryonic development, these INP-derived glial cells surround the entire developing central complex neuropile, and once the major compartments of the central complex are formed, they also delimit each of these compartments. During this process, the number of these glial cells in the central complex is increased markedly through local proliferation based on glial cell mitosis. Taken together, these findings uncover a novel and complex form of neurogliogenesis in *Drosophila* involving transit amplifying intermediate progenitors. Moreover, they indicate that type II neuroblasts are remarkably multipotent neural stem cells that can generate both the neuronal and the glial progeny that make major contributions to one and the same complex brain structure.

## Introduction

Neural stem cells are the primary progenitor cells at different developmental stages that initiate the neural lineages comprising the differentiated neurons and glia of the brain (Kriegstein and Alvarez-Buylla, 2009). Analysis of stem cell-dependent neurogenesis in several model systems indicates that neural cells are not always produced directly from the primary progenitors, but can also be generated by secondary progenitors of more restricted potential (Götz and Huttner, 2005; Kriegstein et al., 2006). In mammals, neural stem cells can generate neural cells either directly through asymmetric division or indirectly through secondary progenitors which either divide only once to produce two neural progeny or divide more than once to amplify the number of neural progeny further (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004, 2007).

Remarkably similar findings have been obtained in studies of neurogenesis in the developing CNS of *Drosophila*. There, neural stem cell-like primary progenitors called neuroblasts can also generate neurons directly through asymmetric division or, more typically, indirectly through ganglion mother cells (GMC) which divide only once to generate two postmitotic progeny (Skeath and Thor, 2003; Technau et al., 2006; Knoblich, 2008; Doe, 2008). Moreover, recently a third type of neurogenesis has been discovered in the *Drosophila* central brain where identified neuroblasts generate intermediate neural progenitor cells (INPs) which undergo several rounds of asymmetric divisions, each of which results in INP self-renewal and generation of a GMC that produces two neural progeny (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). These INPs have also been referred to as IPs, transit amplifying GMCs, or secondary neuroblasts (Weng and Lee, 2010.) Through the

resulting amplification of proliferation these INP producing “type II” neuroblasts generate large neural lineages that contribute to major neuropile substructures of the brain such as the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Pereanu et al., 2011).

In mammalian brain development, neural stem cells can give rise to both neurons and glial cells, however the neurogenic phase and the gliogenic phase of these primary progenitors are generally separate (Miller and Gauthier, 2007; Miyata et al., 2010). Neural stem cells that can generate both neurons and glia are also found in *Drosophila* (Van De Bor and Giangrande, 2002). These so-called neuroglioblasts have been identified on a single-cell basis in the embryonic ventral nerve cord, and together with glioblasts, which only generate glial cells, they give rise to several subtypes of glial cells in the ventral CNS (Broadus et al., 1995; Bossing et al., 1996; Schmidt et al., 1997; Beckervordersandforth et al., 2008). In the ventral nerve cord, gliogenesis is controlled by the *glial cells missing (gcm)* gene that acts as a key switch-like regulator in the development of all embryonic glial cells except those generated from mesectodermal precursors (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996).

Much less is known about the developmental origins of the glial cells in the *Drosophila* brain. The glial cells of the early larval brain are thought to be generated by a few neuroglioblasts during embryogenesis (Hartenstein et al., 1998). However, most of the glial cells in the adult central brain are generated postembryonically, and during larval development a marked increase in glial cell number occurs (Pereanu et al., 2005). While some of these glial cells apparently arise by mitosis of other glial

cells, the bulk of the postembryonically added glial cells is thought to be generated through the proliferation of neuroglioblasts, which, however, have not yet been identified (Pereanu et al., 2005; Awasaki et al., 2008). This lack of identification of the postulated postembryonic neuroglioblasts has been a major obstacle for understanding the mechanisms by which glial cells are generated during postembryonic development of the central brain.

Here we show that identified type II neuroblasts have neuroglioblast function during postembryonic development of the central brain. We use genetic lineage tracing and clonal analysis to demonstrate that the INPs of type II neuroblast lineages can give rise to glial cells as well as neurons. Our data indicate that two main types of INP-derived lineages are generated postembryonically, namely mixed neuronal/glial lineages and neuronal lineages. Moreover, they show that INP-derived glial cells, like INP-derived neuronal cells from the same type II neuroblast lineages, make major contributions to the developing neuropile of the central complex. They surround the entire developing central complex neuropile, and once its major compartments are formed, they also delimit each of these. During this process, the population of INP-derived glial cells associated with central complex undergoes clonal expansion through local proliferation. Taken together, these findings uncover novel mechanisms for neurogliogenesis in *Drosophila* involving the transit amplifying intermediate progenitors of type II lineages. Thus, type II neuroblasts are remarkably multipotent neural stem cells that can generate both the neuronal and the glial progeny of one and the same complex brain structure.

## Materials and Methods

### *Fly stocks, MARCM, and G-TRACE analysis*

Flies were maintained on standard cornmeal medium at 25°C unless noted otherwise. To visualize the potentially glial offspring of neuroblasts, we used *insc-Gal4<sup>MZI407</sup>*, *UAS-mCD8GFP<sup>LL5</sup>* homozygous flies, or mated them to *gcm-lacZ<sup>rA87</sup>/CyO*, *act-gfp<sup>JMR1</sup>*. For *erm-Gal4* (R09D11; Pfeiffer et al., 2008) G-Trace (flp-out and real-time; Evans et al., 2009) expression patterns we mated *UAS-flp*, *ubi>stop>nGFP*, *UAS-RFP/CyO*, *act-gfp<sup>JMR1</sup>*; *erm-Gal4* to *gcm-lacZ<sup>rA87</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *erm-Gal4*. To generate wild type MARCM (Lee and Luo, 1999) clones, we mated *y w hs-flp<sup>1</sup>*; *tubP-Gal4*, *UAS-mCD8GFP<sup>LL5</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *FRT82B*, *tub-Gal80<sup>LL3</sup>* (Bello et al., 2003) to *gcm-lacZ<sup>rA87</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *FRT82B* males. To generate clones misexpressing *gcm*, we mated *elav-Gal4<sup>C155</sup>*, *hs-flp<sup>1</sup>*; *UAS-mCD8GFP<sup>LL5</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *FRT82B* *tub-Gal80<sup>LL3</sup>* to *UAS-gcm<sup>m24a</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *FRT82B* (*UAS-gcm<sup>m24a</sup>* from Bernardoni et al., 1998). To induce *gcm* loss-of-function clones, we mated *y w hs-flp<sup>1</sup>*; *FRT40A*, *tubP-Gal80<sup>LL10</sup>/CyO*, *act-gfp<sup>JMR1</sup>*; *tubP-Gal4<sup>LL7</sup>*, *UAS-mCD8GFP<sup>LL6</sup>* (Bello et al., 2006) to *FRT40A*, *gcm<sup>N7-4</sup>/CyO*, *act-gfp<sup>JMR1</sup>*. Eggs were collected for 2-4 hours, grown to first larval instar (22-30 hours after egg laying, AEL), plates immersed in a 37°C waterbath for 5 minutes (sparse wild type clones) or up to 30 minutes (other clones), and grown to the desired stage at 25°C. When recovering clones from wandering L3 larvae, some bottles were raised at 18°C during third instar to delay development until dissection. When recovering clones from earlier stages, larvae were grown at 25°C throughout, and kept at a maximum density of 170 larvae per bottle to avoid developmental delay due to food competition and to ensure exact staging.

### ***Immunohistochemistry and in situ hybridization***

Brains were dissected in ice-cold PBS and fixed in 2% Paraformaldehyde for 30-60 minutes at room temperature, washed several times in PBS/0.5% Triton X-100, and preincubated in PBS containing 0.5% Triton X-100 and 10% normal goat serum. Antibodies were incubated overnight at 4°C. We used chicken anti-GFP 1:500 (ab13970, Abcam, Cambridge, UK), rabbit anti-beta-Galactosidase 1:500 (55976, MP Biomedicals, Solon, Ohio, USA), mouse anti-Neurotactin 1:20 (BP106, DSHB, Iowa City, Iowa, USA), rat anti-Elav 1:30 (7E8A10, DSHB), mouse anti-Repo 1:30 (8D12, DSHB), rabbit anti-Repo 1:400 (kindly provided by Veronica Rodrigues), rabbit anti-phospho histone H3 (Ser10, 06-570, Millipore, Temecula, CA, USA), rat anti-Deadpan and rabbit anti-Asense (both kind gifts from Cheng-Yu Lee), and Alexa-conjugated secondary antibodies 1:300 (A11039, A21247, A11036, A11031, A21244, Molecular Probes, Eugene, OR, USA). In situ hybridization was performed as described previously (Soustelle et al., 2007).

### ***Identification of dorsomedial type II lineages***

DM1 was uniquely identified in Nrt/BP106 staining as the dorsoanterior-most large lineage in late third instar brains. DM5 was identified by a unique *gcm-lacZ* and Repo-positive cell cluster within the lineage (Figs 1D, 5E). Other DM lineages were identified by their position relative to DM1 and DM5, their very large cell number (Bello et al., 2008), complex axon branching pattern (Izergina et al., 2009), and location at the posterior/medial brain surface. In younger larvae, DM1-3 lineages were recognized by their similar shapes and positions compared to those in wandering larvae. In addition, they were confirmed to be Type II lineages by weak or absent asense staining in the neuroblast, the presence of numerous asense- or deadpan-



positive cells apart from the neuroblast, or equally numerous prospero- and elav-negative cells throughout the lineage.

### ***Microscopy and image processing***

Fluorescent images were recorded on a Leica TCS SP5 confocal microscope, and processed using Fiji (Schindelin, 2008) or ImageJ (Rasband, 1997-2008). All adjustments were linear and were performed on whole images. Cells were counted using the CellCounter plugin for Fiji/ImageJ (Kurt De Vos).

## Results

### *Postembryonic development of neurons and glial cells in the central brain*

During postembryonic development, the majority of the neurons (95%) that make up the adult central brain are generated by approximately 100 pairs of neural stem cell-like neuroblasts, each of which generate a specific lineage of neural progeny (reviewed in Hartenstein et al, 2008). Figure 1A shows the neuroblast-derived lineages in the central brain of the third larval instar. In addition to neurons, a large number of glial cells are also generated postembryonically in the central brain (Fig. 1B,C). Newly generated glia express both *gcm-lacZ*<sup>rA87</sup> and the glial identity marker Repo, whereas older (or *gcm*-independent) glia express only Repo (Fig. 1B,C).

Among the 100 central brain neuroblasts in each brain hemisphere, there are a total of eight identified type II neuroblasts, and six of these occupy most of the posterior medial edge of the hemisphere (Fig. 1D,E). Because they are easier to identify, most studies of type II neuroblasts have focused on the six posterior medially located lineages. These six lineages have been termed DM1-DM6 by Bello et al. (2008), and tentatively correspond to the DPMm1 (DM1), DPMpm1 (DM2), DPMpm2 (DM3), CM4 (DM4), CM3 (DM5), and CM1 (DM6) lineages (Pereanu and Hartenstein, 2006; Pereanu et al., 2011). A previous analysis of the postmitotic cells generated in these six identified type II neuroblast lineages during postembryonic development indicates that the majority of these are neurons, but glial cells also present (Izergina et al., 2009). This is supported by the observation that some neuroblast lineage-specific Gal4 lines label glial cells in DM lineages (Fig. 1F,G).

These findings suggest that type II neuroblasts may in fact function as neuroglioblasts that produce glia in a novel mode involving INPs. To investigate this, we first required a method for specific labeling of all INP-derived cells in type II neuroblast lineages.

### ***INP progeny are labeled by erm-Gal4-dependent lineage tracing***

In contrast to other progenitor cells in the brain, all mature and proliferating INPs express the *Drosophila Fezf* homolog *earmuff* (*erm*) and can be reliably labeled by an *erm-Gal4* line (Weng et al., 2010; Pfeiffer et al., 2008). Although the *erm-Gal4* line labels all mature INPs of the type II neuroblast lineages, it does not reveal all of their progeny (see below). However, by combining the *erm-Gal4* line with the Gal4/flip-out based cell lineage tracing methodology G-Trace, the INPs and their entire (clonal) set of progeny in the developing brain can be labeled (Fig. 2A,B). In G-Trace, two fluorescent reporters differentially reveal real-time Gal4 expression in precursor cells versus flip-out based expression in all of the cells that are clonally derived from these precursors (Evans et al., 2009).

In experiments in which *erm-Gal4* is coupled with G-Trace for lineage tracing, three populations of labeled cells are seen in the brain hemispheres of the third instar larva (Fig. 2C,D). First, large groups of labeled cells are seen at the dorsomedial midline of each brain hemisphere. These labeled midline cells represent progeny of the six medial type II neuroblasts. Because they are easily identified, and because sparse MARCM clones are recovered frequently enough to allow conclusions about the association with migratory glia derived from them, these dorsomedial cell groups are the focus of this investigation. Second, smaller groups of more laterally located cells

are manifest in each brain hemisphere; they represent the progeny of the two lateral type II neuroblasts. Third, numerous labeled cells are located in the developing optic lobes; their precursors are unknown. Other lineages in the central brain are not labeled by *erm-Gal4*-dependent flip-out, indicating that few, if any, non-type II-derived cells express *erm-Gal4* in the central brain at the stages investigated.

Previous studies have shown that INP cell bodies are clustered in the vicinity of their parent type II neuroblasts near the cortical surface of the brain hemisphere, while the cell bodies of their neural progeny extend in a clustered array away from the INPs (and their neuroblast) towards the developing neuropile (Bello et al., 2008; Weng et al., 2010). Given this spatial array of the *erm*-positive INPs and their *erm*-negative neural progeny, spatially distinct expression of the two labels in *erm-Gal4* G-Trace experiments is expected. This is the case (Fig. 2E,F). Real-time *erm-Gal4* expression (red/orange label) is concentrated in a small group of cell bodies clustered near the cortical surface (all INPs), while flip-out based clonal expression (green label) reveals a much larger group of labeled cell bodies (most INPs and all of their clonal progeny) that extend towards the developing neuropile.

These findings confirm previous observations on the spatial location of INPs versus their differentiated neural progeny in type II lineages and also demonstrate that (real-time) *erm-Gal4* only labels a subset of the total number of cells that comprise these lineages.

***INP lineages can give rise to glial as well as neuronal progeny***

During postembryonic development, the six type II neuroblasts generate large lineages, and most of the differentiated cells in these lineages are adult-specific neural cells that express the neuronal marker *Elav* (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). The cell bodies of these neurons remain clustered together with their neuroblast of origin in the cortical cell layer. In addition to numerous (400-500) neurons, type II lineages in the late larval brain have also been observed to contain a small number (10-15) of postembryonically generated glial cells that express *Repo* (Izergina et al., 2009). However, the mode of gliogenesis that gives rise to these glial cells in type II neuroblast lineages as well as the mature phenotype of these glial cells are not known. To determine if these glial cells are generated via INPs, we combined *erm-Gal4* G-Trace experiments with anti-*Repo* immunolabeling.

Figure 3 shows the results of this type of experiment in three optical sections taken at different depths through the late third larval instar brain. In all three sections, two large clusters of *erm-Gal4* lineage-labeled cells are visible that represent cells of INP-derived lineages, and a subset of these cells is *Repo*-positive indicating that they are glial cells belonging to INP lineages. These INP-derived glial cells are located closer to the midline commissural region and are more spatially dispersed towards the neuropile than the non-glial cell bodies of the INP lineages that remain peripherally clustered in the cortical cell layer. While the INP-derived glial cells are not the only *Repo*-positive cells in this part of the larval brain, they do represent the majority of the glial cells in this region.

These findings indicate that glial cells as well as neurons can be generated via INPs in type II neuroblast lineages. To confirm this, we generated labeled INP clones in the

six medial type II neuroblast lineages using the MARCM technique (Lee and Luo, 1999). In addition to neuroblast clones, single-cell clones and two-cell clones, INP clones represent a fourth type of MARCM clone which consists of more than two cells but lacks a neuroblast and can be generated if the somatic recombination event takes place in the neuroblast (for details, see Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Mitotic recombination was induced at the first larval instar stage and labeled clones were examined at the mid- and late third larval instar stage.

Several different types of INP clones were found. First, we recovered INP clones that contained both neurons and *gcm-lacZ*-expressing glial cells (Fig. 4A, B, C). (In the type II lineages of the third larval instar, virtually all Repo-expressing glial cells also express *gcm-lacZ*, indicating that they are newly specified; Fig. 1B and data not shown.) These mixed neuronal/glial INP clones usually contained 8-9 cells, of which 1-3 glial cells were found in a given clone. Mixed neuronal/glial cell clones were generally located within the DM1, DM2 and DM3 lineages. Second, we recovered INP clones of similar size in which all of the cells were neurons (Fig. 4D,F). Neuronal INP clones were found predominantly in the region occupied by the lineages DM4 (Fig. 4D) and DM6 (Fig. 4F). In addition to these two types of INP clones, a third multicellular clone type consisting entirely of glial cells was sometimes recovered in the area laterally adjacent to the DM5 lineage (Fig. 4E).

Together with the results of *erm-Gal4*-based lineage tracing, these clonal MARCM experiments demonstrate that INPs can give rise to both neurons and glial cells. While some of the INPs generate neuronal lineages, many other INPs generate mixed lineages composed of both neuronal and glial cells. This indicates that a novel form

of gliogenesis involving transit amplifying secondary progenitors operates in the type II neuroblast lineages. This, in turn, implies that type II neuroblasts have features of multipotent neural stem cells that can generate both neuronal and glial progeny through transit amplifying intermediate progenitors.

### ***Lineage-specific heterogeneity of INP-derived progeny in type II neuroblast lineages***

The relative frequency with which neuronal INP clones versus mixed neuronal/glial INP clones were recovered varied in different regions of the dorsomedial brain. This suggests that the type II neuroblasts might be heterogeneous in the number and/or type of glial cells generated via their INPs. To investigate this, we recovered MARCM labeled clones of entire type II lineages (neuroblast clones) induced at the first larval instar stage, and analyzed the glial cells comprised in each lineage in wandering third instar brains. The cellular resolution obtained with this type of clonal analysis is documented in single optical sections for the DM1 lineage (and for a control type I lineage) in Figure S1. In these single optical sections, the majority of cells MARCM-labeled with GFP are Elav-expressing neurons whose cell bodies are localized together in a cluster extending from the non-neural progenitor region towards the brain midline. In contrast, only a small set of glial cells is GFP labeled and these are localized at the distal end of the labeled clone near the brain midline.

The overall morphology and relative position in the brain of the entire MARCM-labeled clone for each of the six type II neuroblast lineages (DM1-DM6) was highly reproducible, making identification of all six type II lineages in the larval brain possible (Fig. 5A-F, see Materials and Methods). Analysis of the number and relative

position of glial cells within each type II lineage revealed a marked heterogeneity. This is documented in Table 1 which gives the total number of glial cells in each of the six lineages, and in Figure 5A'-F' in which the position of the *gcm*-labeled glial cell bodies comprised in each DM lineage is presented semi-schematically. The DM1, DM2 and DM3 lineages each contain 10-20 glial cells and most of these are clustered together in the part of the lineage that is distal to the neuroblast and close to the brain midline. These glia make up the majority of newly formed glia around the developing commissure that essentially corresponds to the fan-shaped body primordium, as well as the glia around the prospective protocerebral bridge. In contrast, only a small number of glial cells are seen in the DM4 lineage, and the DM6 rarely contains any glial cells at all. The DM5 lineage differs from the others in that it contains a larger number (average of 35) of glial cells, some of which are closely associated with the main cluster of neural cells, while others are more dispersed and positioned more laterally in the brain hemisphere. DM5-derived glia are set apart from the central complex primordia. Taken together, the positions of DM-derived glia cover the entire region of nascent glia in the medial central brain of the third instar larva. These results confirm the specificity of the *erm-Gal4* line and flip-out experiments, suggesting that most of those glia originate from type II lineages.

### ***Progression of glial differentiation in type II neuroblast-derived lineages***

According to previous reports, glia numbers in the larval central brain remain relatively constant during early instars and increase rapidly during the third larval instar (Pereanu et al., 2005). We assessed the first appearance of glia in the dorso-medial region by *gcm* in situ hybridization as well as *gcm-lacZ* and Repo immunolabeling in larval brains (Fig. S2). In late L2 larvae, dorso-medial glia were



visible only by in situ hybridization but not by *gcm-lacZ* expression (Figure S2A,B). The earliest *gcm-lacZ* expression appeared in L3 larvae shortly after the L2/L3 transition (Figure S2C). Most of these glia already expressed Repo at that stage (Fig. S2C').

To investigate how DM lineages form these early glia and how their population expands, we induced MARCM clones in newly hatched larvae and recovered them between late L2 and mid-L3 stages. We focused on the lineages DM1, DM2, and DM3 that form most of the glia in the dorsomedial region and are easily distinguished from surrounding lineages in younger brains. L2 larval brains recovered during the L2/L3 transition (65-71 hours after egg laying, AEL), no DM1-3 MARCM clones or INP clones from the DM1-3 region contained glial cells (Table 2, Fig. 6). In brains from 65-71 hour old L3 larvae recovered during the same experiments, none of the DM clones but 50% of INP clones contained glia. At 75-80 hours AEL, 20% of DM1-3 clones and 70% of INP clones contained glia. From 85 hours AEL on, all DM1-3 lineages and INP clones from the DM1-3 region contained glia. The number of glia per DM1-3 lineage increased from  $6\pm 4$  glial cells at 85 hours AEL to  $14\pm 5$  glial cells at 96 hours AEL, which roughly corresponds to the number of DM1-3 glia in wandering L3 brains ( $15\pm 5$  glial cells, Table 1). Similarly, INP clones increased in size and glial content from  $5\pm 1$  cells including 0-1 glia to  $9\pm 2$  cells including  $3\pm 1$  glial cells (Table 2). Thus the appearance of glia in DM1-3 roughly coincides with the first appearance of glia in the dorso-medial region, indicating that at least some of these glia are postembryonic. Glial numbers increase most rapidly during the first half of the third larval instar.

Next we investigated how gliogenesis is related to the developmental progression of type II lineages. We co-labeled DM and INP MARCM clones with the glial markers *gcm-lacZ* or *Repo*, and with the neuroblast/INP markers *Deadpan* (*Dpn*) or *Asense* (*Ase*). We found no overlap between neuroblast and glial marker expression in MARCM clones, indicating that glial markers appear only in differentiating glia and not in their precursor cells (Fig. S1C, Fig. 6B). Furthermore, of 23 INP clones co-labeled for glial markers and either *Dpn* or *Ase*, only one clone (recovered at 69 hours AEL) contained both a glial cell and a *Dpn*-expressing cell. The other clones either contained 1-2 cells expressing a neuroblast marker but no glial cells, or contained glial cells, but no cells expressing neuroblast markers. This suggests that within an INP lineage, glia start to be formed around the time when the INP stops expressing neuroblast markers.

We were interested in how the formation of type II-derived glia correlated with the expression of cell and neural differentiation markers *Prospero* (*Pros*) and *Elav* in these lineages. Generally, we found that glial markers *Repo/gcm-lacZ* and *Prospero*, or *Elav*, are expressed in a mutually exclusive way (Fig. S1, Fig. 7). However, in younger brains we found several instances of co-expression (Fig. 6C, Fig. 7). During early L3 stage, when glial markers first appear, we recovered 2 of 4 INP clones that contained cells co-expressing *gcm-lacZ* and *Elav* (Figure 7A), and 5 of 7 DM neuroblast and INP MARCM clones that contained cells co-expressing *gcm-lacZ/Repo* and *Prospero* (Figure 7B-D), as well as numerous examples of co-expressing cells outside of clones in the DM region (data not shown). In such co-expressing cells, especially glial markers were expressed more weakly than in neighboring cells (Figs. 6C,7A,C',D'), in accordance with a possible transition to

glial marker gene expression. Notably, not all cells that weakly expressed *gcm-lacZ* also co-expressed Prospero or Elav (Fig. 7A and data not shown), indicating that the transition time is either very short, or that not all glia differentiate from Pros- or Elav-positive precursors. Together these results suggest that at least some type II-derived glial precursors express Elav and/or Prospero when they start to express glial markers, and turn them off rapidly as glial differentiation proceeds.

### ***INP-derived glial cells associate with the developing central complex***

During larval development, the neurons of the six medial type II lineages fasciculate to form several secondary axon tracts and some of these project to the dorsomedial commissure of the larval brain, which is part of the larval primordium of the central complex (Izergina et al., 2009; Young and Armstrong, 2010; Pereanu et al., 2011). During subsequent pupal development, as the central complex primordium grows and differentiates, these commissural projections arborize profusely to form dendrites and axonal arbors that contribute to specific neuropile compartments of the adult central complex such as the fan-shaped body, ellipsoid body, protocerebral bridge, and noduli. Given that many of the neurons in type II neuroblast lineages contribute to the neuropile of the central complex, we wondered if the INP-derived glial cells from these lineages might also be involved in central complex development.

By the end of larval development, the neurotactin-positive axons of several lineages including the six dorsomedial type II lineages form a plexus of commissural fibers that represents the central complex primordium. To determine if INP-derived glial cells are associated with this central complex primordium, we combined *erm-Gal4* G-Trace lineage labeling with anti-neurotactin (BP106) and anti-Repo immunolabeling.

Figure 8 shows the results of this type of experiment in the early pupal central brain (1-2 hours APF, After Puparium Formation) in three optical sections taken through the central commissure primordium. In all cases, Repo-positive glial cells that derive lineally from INPs are closely associated with the neuropile primordia of the fan-shaped body (Fig. 8A-B) and the protocerebral bridge (Fig. 8C). They are seen aggregated at the two lateral entry zones to the fan-shaped body primordium, surrounding almost the entire midline domain of the primordium, and in some cases are intercalated in its fiber tracts. In addition, INP-derived glial nuclei clearly delineate the protocerebral bridge primordium. Although a few glial cells surrounding the primordia are not INP-derived, the clear majority of the glial cells associated with this central complex structure are INP lineal descendants.

The close association of INP-derived glial cells with the developing central complex is also observed during subsequent pupal development in comparable experiments performed at 20 and 40 hours APF. At 20 hours APF, when the central complex has grown significantly in size and shows initial internal compartmentalization, its neuropile is surrounded by numerous INP-derived glial cells, and glial cells are also intercalated within the fiber bundles of the neuropile (Fig. 9A-D; Fig. S3). At 40 hours APF, when the major compartments of the adult central complex, the fan-shaped body, ellipsoid body, noduli and protocerebral bridge, have become apparent, INP-derived glial cells are seen surrounding, delimiting and intercalated into all of these compartments (Fig. 9 E-H, Fig. S3). A comparable arrangement of INP-derived glia was seen in the central complex at 75h APF (data not shown). Indeed most of the glial cell bodies associated with in the neuropile compartments of the central complex throughout its development appear to be INP-derived. Based on their appearance and

location within the central complex neuropile, the INP-derived glial cells are likely to be neuropile glia of the ensheathing (non-astrocyte) type (Awasaki et al., 2008). This is confirmed by labeling the INP-derived glial cells in the developing central complex with membrane-targeted mCD8-GFP, which reveals the typical spindle-like morphology of this type of glia (Fig. S4). We have recovered dorso-medially located astrocyte-like glia as well as interhemispheric ring glia (Simon et al., 1998) in our MARCM experiments (both have much larger nuclei than DM-derived glia and express high levels of Prospero and glial markers), but not in association with neuroblast or INP lineages (data not shown).

Taken together, these findings indicate that INP-derived glial cells of the type II neuroblast lineages differentiate into ensheathing neuropile glia of the central complex. Thus, the type II neuroblasts via their INPs not only give rise to neuronal progeny that contributes to the central complex neuropile they also generate the glial progeny that ensheaths this neuropile.

#### ***INP-derived glial cells proliferate locally in the developing central complex***

Quantification of the number of INP-derived glial cells associated with the developing central complex indicates that a fourfold increase in number occurs during pupal development between 0h (50 glial cells) and 40h APF (200 glial cells). This suggests that the INP-derived glial cells associated with the central complex might be capable of proliferating locally as its neuropile expands and differentiates.

To determine if INP-derived glial cells do proliferate in the developing central complex, we combined *erm-Gal4* G-Trace lineage labeling with anti-pH3 (phospho-

histone H3) and anti-Repo immunolabeling. Figure 10 shows results of these experiments in the pupal central brain at 20 hours APF. Several INP-derived glial cells surrounding the central complex are labeled with anti-pH3 indicating that they are mitotically active. At that stage, up to 12 INP-derived glial cells around the developing fan-shaped body were in mitosis ( $6\pm 5$  cells,  $n=7$  brains). Similarly, at 0-3 hours APF, up to 8 mitotically active cells ( $3\pm 3$  cells,  $n=10$  brains) surrounded the fan-shaped body primordium.

These findings imply that the population of INP-derived glial cells associated with the central complex expands during pupal development through local proliferation. Thus, gliogenesis in these lineages occurs in two distinct modes both of which amplify proliferation. While glial cells are initially generated by type II neuroblasts via transient amplifying INPs, they subsequently amplify their number through local mitotic activity as the central complex neuropile differentiates and forms the compartments characteristic of its adult structure.

### ***The gcm gene controls the development of INP-derived glial cells***

Given that the glial cells studied here differ developmentally from all other glia in the brain in that they derive from INP generating type II neuroblasts, we investigated if their generation required *gcm*, a key regulator in the development of many (but not all) glial cell types (Jones et al., 1995; Hosoya et al., 1995; Vincent et al., 1996; Awasaki et al., 2008). For this, we carried out clonal gain-of-function (misexpression) and loss-of-function experiments using MARCM. Clones were induced at first larval instar and recovered in late third larval instar.

For *gcm* gain-of-function experiments, either *elav-Gal4* or *tub-Gal4*, both of which drive reporter expression throughout secondary neuroblast lineages (Dumstrei et al., 2003; Bello et al., 2006), was used to misexpress *UAS-gcm* in MARCM-labeled neuroblast clones. In all cases, misexpression of *gcm* in type II neuroblast clones resulted in a marked increase of Repo-positive cells (Fig. 11A,B and data not shown). 30-40% of the cells in these MARCM-labeled clones expressed Repo ( $33\pm 5\%$ , n=8 clones), implying that *gcm* misexpression leads to a marked increase in the number of glial cells in type II neuroblast clones. Similar findings were obtained by driving misexpression of *gcm* in INPs with *erm-Gal4* (data not shown).

In most type I neuroblast lineages of the central brain that do not give rise to Repo-positive cells, misexpression of *gcm* also leads to formation of few Repo-positive cells (Fig. 11C,D). Thus, *gcm* is gliogenic in both type I and type II lineages, but the latter appear to be more sensitive to misexpression of *gcm*.

For *gcm* loss-of-function experiments, we used MARCM to generate mutant DM1, DM2, or DM3 neuroblast lineages that were homozygous for the *gcm*<sup>N7-4</sup> allele. The resulting mutant neuroblast clones consistently showed a loss of glial cells. Repo-positive glial cells were completely absent within *gcm*<sup>N7-4</sup> mutant clones (n=7), and all distal clonal progeny expressed the neuronal marker Elav (Fig. 11E-H). Axonal patterns in those clones appeared normal, which indicates that glia were not required for the establishment and proper fasciculation of secondary axon tracts. These findings show that *gcm* is necessary and sufficient for INP-derived glial cell formation in type II lineages, and that glia from DM1-3 mutant for *gcm* assume a neural fate.

## **Discussion**

In this report we use a combination of genetic lineage tracing, clonal MARCM techniques and molecular labeling to study the developmental mechanisms that give rise to the glial cells of the amplifying type II neuroblast lineages. Our findings uncover a novel mode of neurogliogenesis in these lineages that involves transit amplifying INPs, which can generate glial cells as well as neurons. Our analysis also shows that lineal INP-derived glial and neuronal cells both make major contributions to the central complex. INP-derived neurons project into the neuropile compartments of the central complex and INP-derived glial cells surround and delimit these compartments while undergoing clonal expansion through local proliferation. In the following we discuss the implications of these findings.

### ***Gliogenesis in type II neuroblast lineages involves transit amplifying progenitor cells***

The majority of the glial cells in the adult brain are generated postembryonically. Hitherto unidentified neuroglioblasts have been postulated to give rise to the bulk of these adult-specific glial cells, although some of these arise via proliferative glial cell divisions (Pereanu et al., 2005; Awasaki et al., 2008). Here we report the identification of type II neuroblasts as neural stem cells with neuroglioblast function in postembryonic development of the central brain. Previous work has shown that these amplifying type II neuroblasts augment proliferation through the generation of INPs resulting in the generation of remarkably large neuronal lineages (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Our data indicate that type II



neuroblasts also generate glial progeny through INPs and, hence, reveal a novel form of CNS neurogliogenesis that involves transit amplifying cells (Fig. 12).

Although type II neuroblasts represent a new type of neuroglioblast in *Drosophila* brain development, the six identified type II neuroblasts are heterogeneous in terms of their gliogenic activity. While four of these neuroblasts generate comparable numbers of glial cells, a fifth neuroblast (DM4) gives rise to only a few glial cells and the sixth (DM6) rarely generates glia during larval development. This heterogeneity in gliogenic activity is also reflected in the INPs of these type II lineages. Thus, while mixed glial/neuronal INP clones were recovered for most type II lineages, all of the INP clones that contained glia in the DM5 lineage were purely glial, and no glial INP clones were recovered for DM6. Despite this heterogeneity, the generation of INP-derived glial cells in all of these type II lineages appears dependent on the *gcm* gene. Indeed, this seems to be feature common to most glial progenitors in the embryonic and postembryonic CNS (Jones et al., 1995; Hosoya et al., 1995; Vincent et al., 1996; Awasaki et al., 2008).

While glia can derive from the very first larval INPs produced by DM neuroblasts, glia differentiate late in the progression of the lineage, at a time when neuroblast markers are no longer present in the INP. At that time, lineages already contain large amounts of differentiating cells, positive for Prospero and/or the neural differentiation marker Elav. Interestingly, both Prospero and Elav are also found in many young glia, some of them verified to be part of Type II-derived lineages. This co-expression is reminiscent of embryonic glia that have been reported to transiently express Elav (Berger et al., 2007), which may thus be a common feature of glial cells also in the

larval brain. In addition, there may be Prospero-positive, gliogenic ganglion mother cells that could divide symmetrically to contribute to the variable number of glia observed in late larval INP lineages.

Neural stem cells in the mammalian brain, notably the radial glia of the cortex, also represent mixed progenitors that can give rise to both neuronal and glial cells in different proliferative modes, and one of these proliferative modes involves transit amplifying INPs (Kriegstein and Alvarez-Buylla, 2009; Miyata et al., 2010). Moreover, some of the amplifying INPs involved in mammalian neocortex development are thought to give rise to neuronal progeny while others give rise to glial progeny. The amplification of proliferation through INPs has been postulated to be fundamental for the increase in cortical size during evolution (Kriegstein et al., 2006). The fact that a very comparable mode of INP-dependent proliferation operates in the generation of complex brain architecture in *Drosophila* suggests that this might represent a general strategy for increased size and complexity in brain development and evolution.

### ***Multipotent neural stem cells generate neurons and glial cells in central complex development***

Previous work has shown that a large subset of the neurons generated by type II neuroblasts contributes to the development of the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Peraanu et al., 2011). The data presented here indicate that the INP-derived glial cells from the type II lineages are also involved in central complex development. INP-derived glial cell bodies associate with the larval primordium and, throughout pupal development; they surround and delimit all of the

compartments of the differentiating central complex neuropile. Thus neural and glial cells from the same neuroblast lineage participate in the development of the same complex brain neuropile. This reveals a remarkable multipotential nature of the type II neuroblasts; they are neural stem cells that have the potential to generate both neural and glial cells of one and the same complex brain structure.

Neuroblast lineages have been viewed as “units of projection” in that the neurons of a given lineage often project their axons along a common trajectory and contribute to the formation of a common neuropile; this is exemplified by the four neuroblast lineages that give rise to the intrinsic cells of the mushroom body neuropile (Hartenstein et al., 2008; Ito and Awasaki, 2008). The neurons of the type II neuroblast lineages do not strictly conform to this notion, since subsets of neurons in the lineage project to different parts of the brain (Izergina et al., 2009). However, for the large subset of neurons in the type II lineages that project to the developing central complex, the notion of a lineal “unit of projection” is valid. Indeed this concept can be expanded to include the lineally related glial cells that also contribute to the development of the same neuropile compartment.

### ***Local proliferation of INP-derived glial cells during postembryonic brain development***

In type II neuroblast clones, most of the INPs, as well as the cell bodies of the neurons that they produce, remain clustered together in the peripheral cell body layer of the brain (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Weng et al., 2010). Although a few of the INP-derived glial cells are also found in these clusters, most are not. During larval and pupal development, the majority of the INP-derived

glial cells are found in or near midline commissural structures such as the central complex precursor (late larva) or the developing central complex neuropile (pupa).

One reason for this is that INP-derived glial cells probably migrate away from their site of origin to a different site of final differentiation. Migration of glial cells during CNS development is a common feature in many species and has been studied in detail in the developing ventral nerve cord and optic lobes of *Drosophila* (Klämmt, 2009). Migration of glial cells has also been reported in postembryonic development of the central brain, and in some cases the migrating cells appear to form clusters suggesting that they might derive from common progenitors (Hartenstein et al., 1998; Awasaki et al., 2008).

Another reason for the fact that so many INP-derived glial cells are found in or near the developing central complex is that they proliferate locally. This implies that INP-derived glial cells can undergo clonal expansion in the neuropile. Clonal expansion has been described for the perineurial glial cells localized on the surface of the brain and has also been postulated to take place during the postembryonic development of neuropile glial cells (Pereanu et al., 2005; Awasaki et al., 2008). Since INP-derived glial cells undergo substantial (at least fourfold) proliferative clonal expansion, it will be important to determine what controls their mitotic activity. In view of the vulnerability of the amplifying type II neuroblast lineages to overproliferation and brain tumor formation, a tight control of self-renewing glial cell proliferation in these lineages is likely to be essential.

## **Acknowledgements**

We thank Utpal Banerjee, Bruno Bello, Cory Evans, Cheng-Yu Lee, Barrett Pfeiffer, Veronica Rodrigues, Jerry Rubin, the Bloomington Stock Center and the DSHB for strains and antibodies, Volker Hartenstein for assistance in matching type II lineages with current nomenclature, and Susanne Flister for excellent technical help. This work was supported by SNSF grant 31003A12488/1 and SNSF/NFP63 “Neural stem cells and regenerative medicine” grant 406340128006/1 to HR. Work in the laboratory of AG was supported by INSERM, CNRS, UDS, Hôpital de Strasbourg, ARC, INCA and ANR; AP was supported by FRM.

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## Figure Legends

### **Fig. 1. Central brain neuroblast lineages give rise to glial and neuronal progeny.**

**(A-E)** Wandering third instar larval brain (genotype *insc-Gal4, UAS-mCD8GFP / gcm-lacZ<sup>A87</sup>*, 80  $\mu\text{m}$  maximum intensity projection) labeled with Repo (blue) and *gcm-lacZ* (red). **(A-C)** Within the region of central brain neuroblast lineages (**A**; *insc>mCD8GFP*), many newly formed glia are present (**B,C**; magenta overlap) expressing both Repo and *gcm-lacZ*. They are positioned in the commissure (arrowheads) as well as medially (short arrows) and laterally (long arrows) in the central brain. Dotted line indicates border between central brain (CB) and optic lobe (OL). **(D,E)** DM1-DM6 lineages in the same brain; numbers indicate identified DM lineages. **(F,G)** *insc-Gal4, UAS-mCD8GFP* homozygous larvae have Repo-positive cells (magenta) in GFP-labeled DM lineages (arrows). Numbers indicate tracts of identified DM lineages. Other glia (presumably surface, cortex, or astrocyte-like glia) are not GFP-labeled (asterisks). All figures present anterior to the top. Scale bars, 20  $\mu\text{m}$ .

### **Fig. 2. *erm-Gal4*-dependent lineage tracing labels INP-derived progeny of type II neuroblast lineages.**

**(A)** Schematic of the type II neuroblast lineages in the larval central brain. **(B)** Expression of *erm-Gal4* in G-Trace flies (*UAS-flp, ubi>\*>nGFP, UAS-RFP*) leads to *erm-Gal4* independent GFP expression in *erm-Gal4* expressing cells and their progeny. Real-time expression of *erm-Gal4* is reported by RFP expression. **(C)** *erm-Gal4* driven lineage tracing labels the six dorsomedial type II lineages, the two lateral type II lineages (asterisk) and cells in the optic lobe (OL; delimited by dashed lines). **(D)** Close-up of the DM lineages. **(E,F)** Close-up of the

DM1 region. Real-time expression (red) is located near the cortical surface, flip-out based clonal expression (green) is located in cells that extend further from the cortical surface towards the commissure. Scale bars, 25  $\mu\text{m}$ .

**Fig. 3. *erm-Gal4* expressing INPs give rise to glial cells.** (A-C) Three optical sections at different depths in the third larval instar brain of *erm-Gal4* flip-out (*UAS-flp, ubi>\*>nGFP*) driven nGFP expression show the DM1 regions of both hemispheres. Most glia (Repo, magenta) are flip-out progeny of *erm-Gal4* expressing INPs (green; overlap white). *erm-Gal4*-derived glia are indicated with white dotted outlines, glia not derived from *erm-Gal4* with orange dotted outlines. Scale bar, 25  $\mu\text{m}$ .

**Fig. 4. Progeny of single INPs are composed of neurons and/or glia in a lineage-specific manner.** *tub-Gal4 UAS-mCD8GFP / gcm-lacZ<sup>A87</sup>* - labeled MARCM clones of INPs within identified DM lineages. (A-C) INP clones derived from DM1-3 contain 8-9 cells in total, including one or two glial cells. (D,F) INP clones from DM4 and DM6 contain a similar number of cells but no glia. (E) DM5-derived subclones are positioned in the center of the hemisphere and are composed of only glial cells. Scale bar, 20  $\mu\text{m}$ .

**Fig. 5. Lineage-specific heterogeneity of INP-derived glia.** (A-E; upper panels) Maximum intensity projections of MARCM clones from lineages DM1-DM6, labeled with *tubulin-Gal4 UAS-mCD8GFP* (white), and co-labeled with *gcm-lacZ* (green) identify glial progeny of the DM neuroblasts. Clones were induced at hatching and recovered in wandering third instar. Insets in (A-E) show single confocal slices of

*gcm-lacZ* positive nuclei (green) within the clone. **(F)** The *gcm-lacZ* positive nuclei (magenta) were associated with DM6 axon tracts but not part of the clone. **(A'-F'**; lower panels) Semi-schematic representation of glial cell bodies located within the respective clones (green) or outside of the clones (magenta). All *gcm-lacZ* positive nuclei of each particular brain in all confocal sections that are part of the corresponding maximum projection are shown. Brain hemispheres are outlined with dotted lines for orientation. Scale bar, 50  $\mu$ m.

**Fig. 6. DM1/2 neuroblast lineages contain cells positive for Dpn, Prospero, and Elav when the first glia start to differentiate.** **(A-A''')** DM1 or DM2 lineage in an L3 brain from the L2/L3 transition at 70 hours AEL, containing numerous Elav-positive cells but no glia. **(B-B''')** DM1 lineage from an L3 brain at 80 hours AEL containing numerous Deadpan-positive nuclei (example outlined in cyan) and one glial cell (magenta dotted outline). **(C-C''')** DM1 lineage at from an L3 brain at 85 hours AEL containing several glial cells (magenta dotted outline) and Prospero-expressing cells (example outlined in cyan), as well as 1-2 cells coexpressing both (white dotted outline).

**Fig. 7. Glia differentiate from precursors that express Elav or Prospero.** **(A-A''')** A single cell within an INP lineage from DM1 or DM2 coexpresses *gcm-lacZ*<sup>A87</sup> and Elav weakly (white dotted line), indicating a transition. Other cells that express *gcm-lacZ* either strongly (magenta dotted line) or weakly (red dotted line) do not express Elav. **(B-C''')** A single cell within an INP lineage from DM2 or DM3 coexpresses *gcm-lacZ* and Pros (white dotted line), indicating a transition. **(D-D''')** Another cell from the same MARCM clone that strongly expresses *gcm-lacZ* does not

express Pros (magenta dotted outline). (C) and (D) represent single focal planes of the clone shown in (B) as a maximum intensity projection. Scale bars, 20  $\mu\text{m}$ .

**Fig. 8. INP-derived glial cells are closely associated with the central complex primordium.** (A-B) Two optical sections through the central commissure primordium in 1-2 hour old pupae show that *erm-Gal4* derived glia (*UAS-flp, ubi>\*>nGFP*, green; Repo, magenta) are located in close association with the fiber tracts (BP106/Neurotactin, cyan) that constitute the fan-shaped body primordium (FB, orange outline in A'). (C) 30  $\mu\text{m}$  posterior to the fan-shaped body, *erm-Gal4* derived glia outline the primordium of the protocerebral bridge (PB, orange outline in C'). Scale bar, 25  $\mu\text{m}$ .

**Fig. 9. INP-derived glial cells are associated with the developing central complex throughout pupal development.** (A-D) Single optical sections through the central complex primordium in a 20 hour old pupa are labeled with *erm-Gal4 G-trace* (green), Repo (magenta), and Nrt/BP106 (cyan). INP-derived glia make up most of the glia surrounding the fan-shaped body at the level of its anterior surface (A,A'), the noduli (B,B'), the posterior chiasma (C,C'), and surround the protocerebral bridge (D,D'). (E-H) Single optical sections through the central complex primordium in a 40 hour old pupa are labeled with *erm-Gal4 G-trace* (green), Repo (magenta), and nc82 (cyan). INP-derived glia are found on the anterior surface of the fan-shaped body up to the level of the ellipsoid body (E,E'), surrounding and in between fan-shaped body and noduli (F,F'), on the posterior surface of the fan-shaped body (G,G'), and outlining the protocerebral bridge (H,H'). EB, ellipsoid body; FB, fan-shaped body, NO, noduli, PB protocerebral bridge, pch, posterior chiasm. Scale bars, 20  $\mu\text{m}$ .

**Fig. 10. INP-derived glia surrounding the pupal fan-shaped body are mitotically active.** (A-C) Single confocal section with three Repo-positive *erm-Gal4*-derived central complex glia expressing the mitotic marker pH3 at 20 hours APF; inset and arrows denote dividing cells. (A) INP progeny (white) positive for pH3 (magenta); (B) INP progeny (green) positive for Repo (magenta); (C) Repo (white). Scale bar, 20  $\mu\text{m}$ .

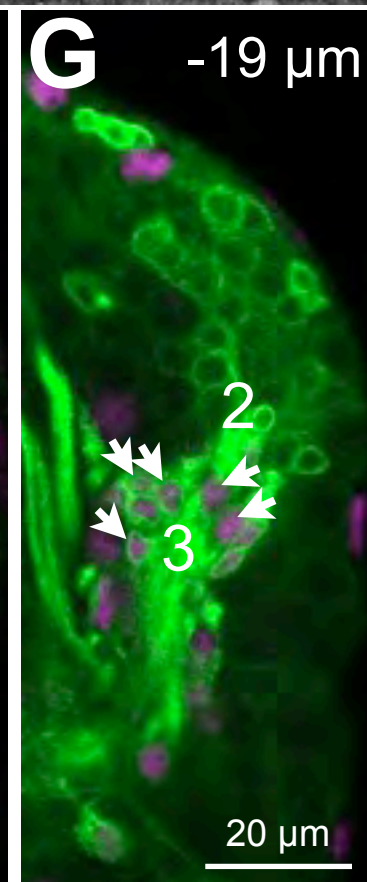
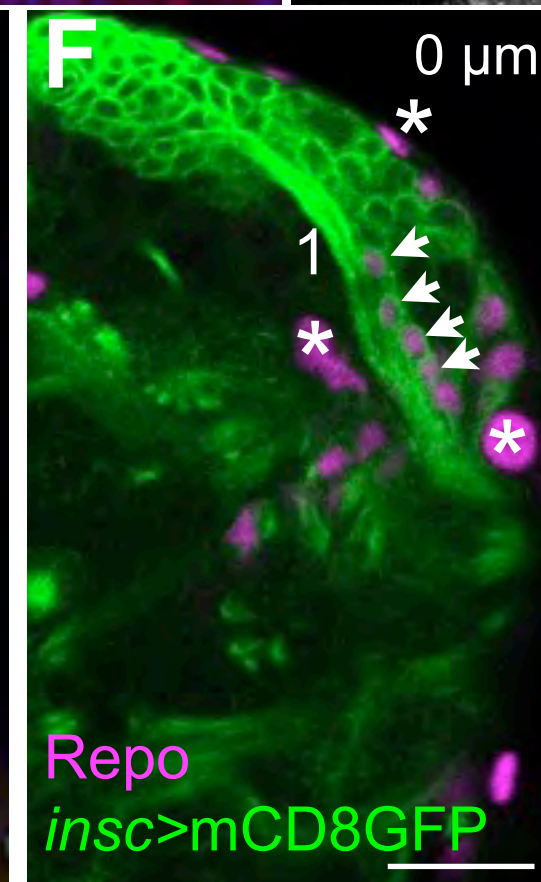
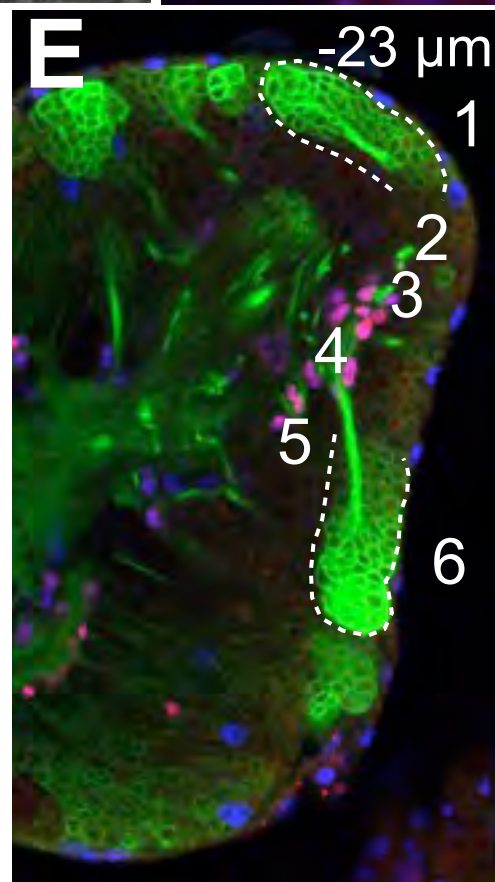
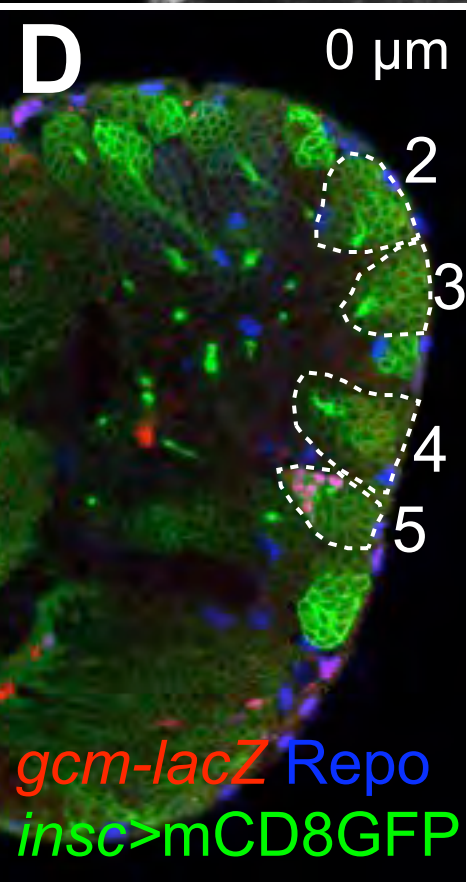
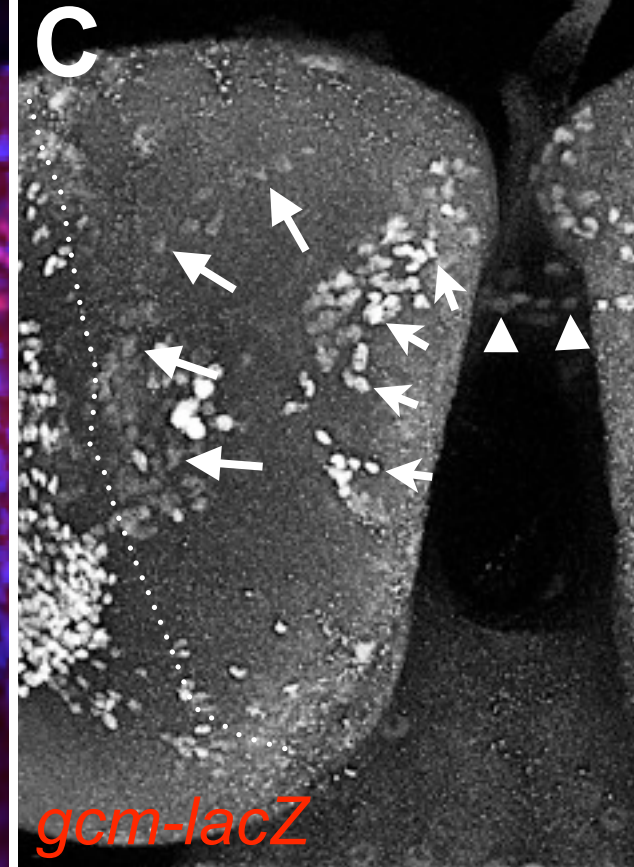
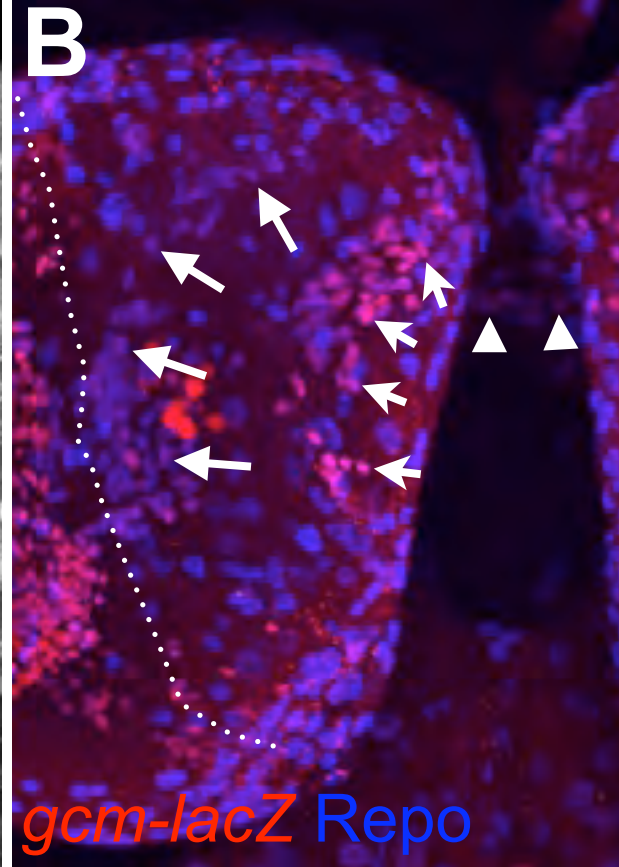
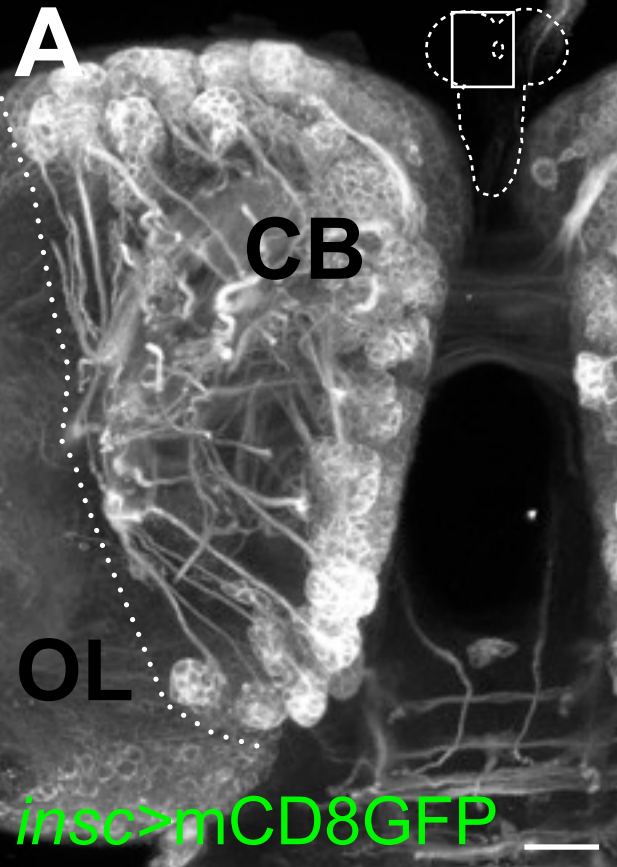
**Fig. 11. Type II lineages generate glia in a *gcm*-dependent manner.** (A-D) Ectopic glia caused by misexpression of *gcm* in a type II (DM1) MARCM clone (A,B) and a type I clone (C,D). (A,B) A maximum intensity projection of the complete bilateral DM1 lineages shows numerous additional Repo-expressing cells in an *elav-Gal4, UAS-mCD8GFP, UAS-gcm<sup>m24a</sup>* MARCM clone (right side, white dotted line) compared to its wild type counterpart (left side, orange dotted line). (C,D) A single confocal slice of a type I *tub-Gal4, UAS-mCD8GFP, UAS-gcm<sup>m24a</sup>* MARCM clone shows ectopic Repo expression (magenta, single channel in D). (E-H) A *gcm<sup>N7-4</sup>* mutant DM MARCM clone labeled with *tub-Gal4 UAS-mCD8GFP* lacks Repo-positive cells. (E,F) Optical projection of all cells in the clone reveals no Repo positive cell (magenta) within the clone (dotted line). In the opposite hemisphere, glial cells in this region are present (orange dotted outline). (G,H) A single section of the same clone shows that all mutant cells express Elav (blue), indicating that loss of *gcm* causes a transformation of glial (Repo, magenta) into neuronal fate. Scale bars, 20  $\mu\text{m}$ .

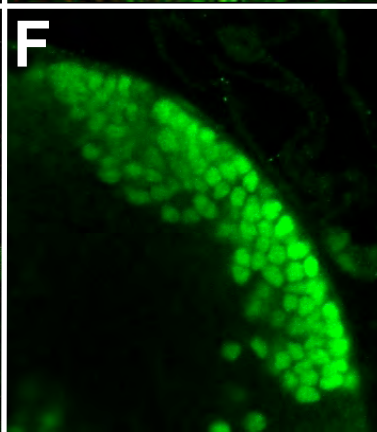
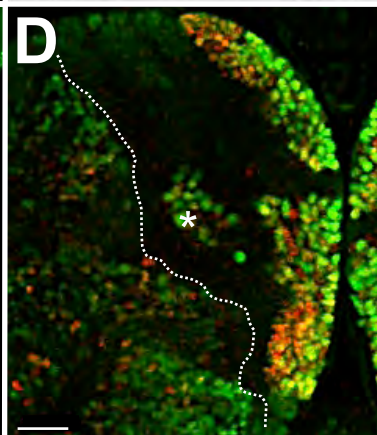
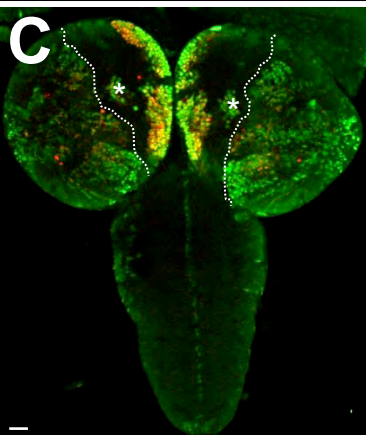
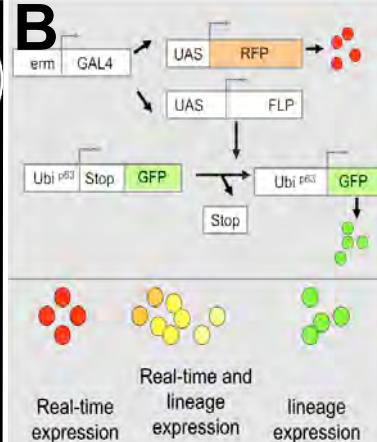
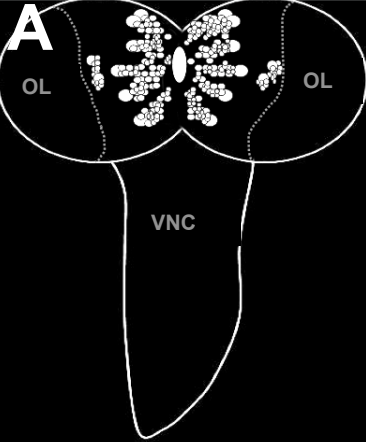
**Fig. 12. Simplified summary scheme of glia formation in type II lineages.** Type II neuroblasts generate INPs that give rise to either mixed neuronal and glial lineages (A), neuronal lineages (B), or glial lineages (C). *gcm* is necessary and sufficient for gliogenesis in these lineages and begins to be expressed late in the progression of the lineage.

## Table legends

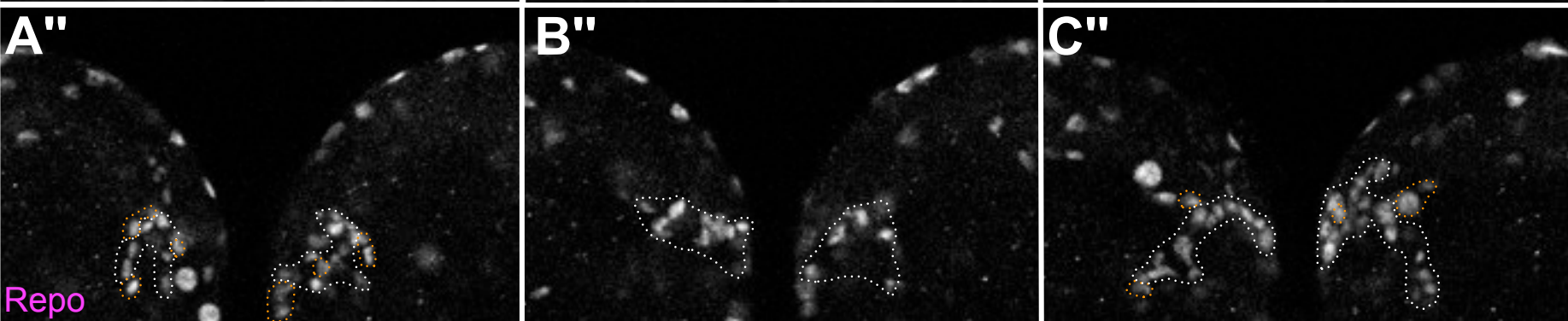
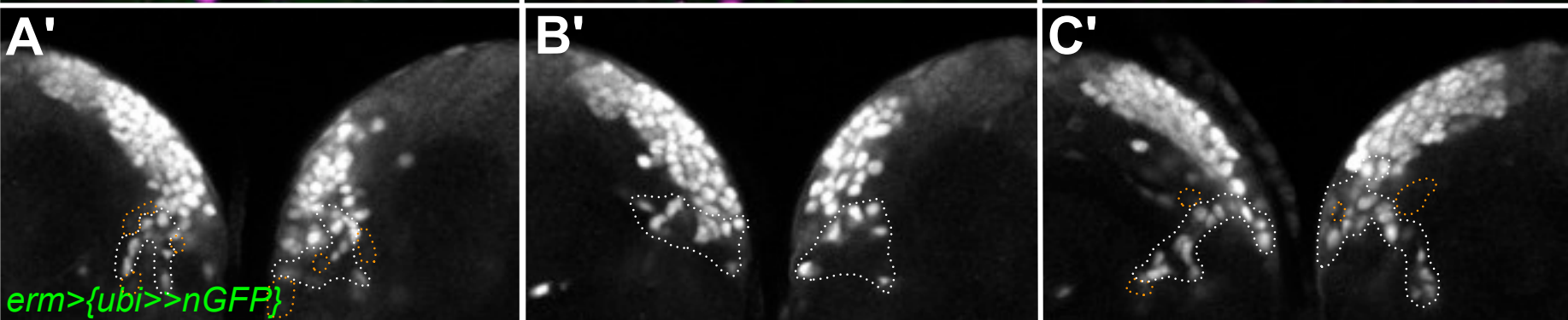
**Table 1. Each type II DM lineage produces a specific number of glial cells.** Glial cells were counted from identified MARCM labeled DM neuroblast clones. DM5-derived glia located near the midline and located laterally were counted separately. s.d., standard deviation; n, number of lineages counted.

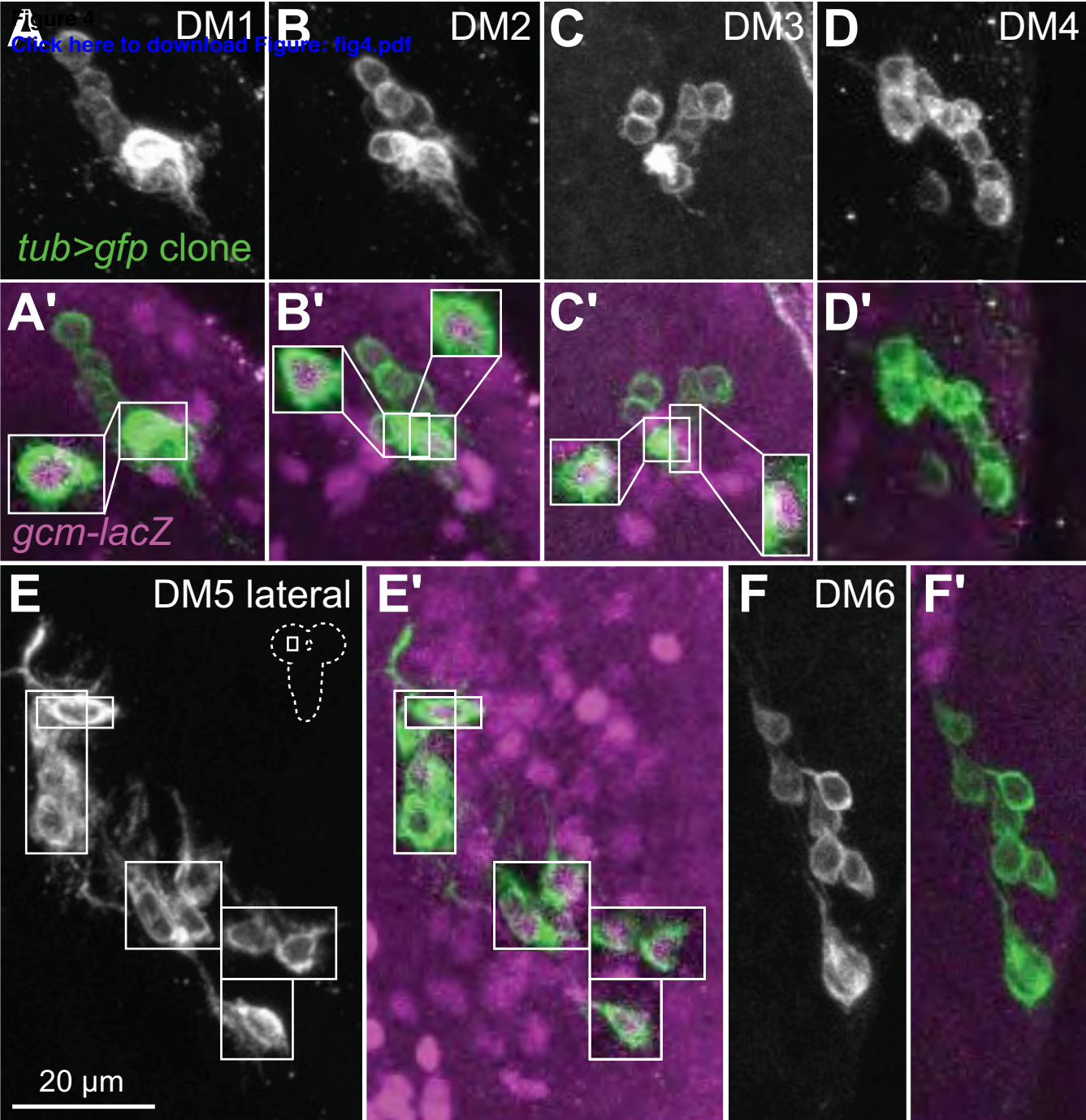
**Table 2. Type II–derived glia form during early third instar.** Glial cells were counted from MARCM labeled DM neuroblast clones identified as DM1, DM2, or DM3, and INP clones from the DM1-3 region. **Rows 1-2:** The percentage of lineages with glia identifies the beginning of glial production in lineages around 65-80 hours AEL in early third instar. Lineages from second instar larvae of the same age did not contain glia. Neuroblast clones differentiate glia later than their INP sister clones induced at the same time. **Row 3:** The number of glia located within DM1-3 neuroblast clones increases rapidly until mid-L3, when the approximate number counted in wandering larvae is reached (compare with Table 1). **Rows 4-5:** In INP clones, glial numbers increase concomitantly. Cell counts are given in mean±s.d.



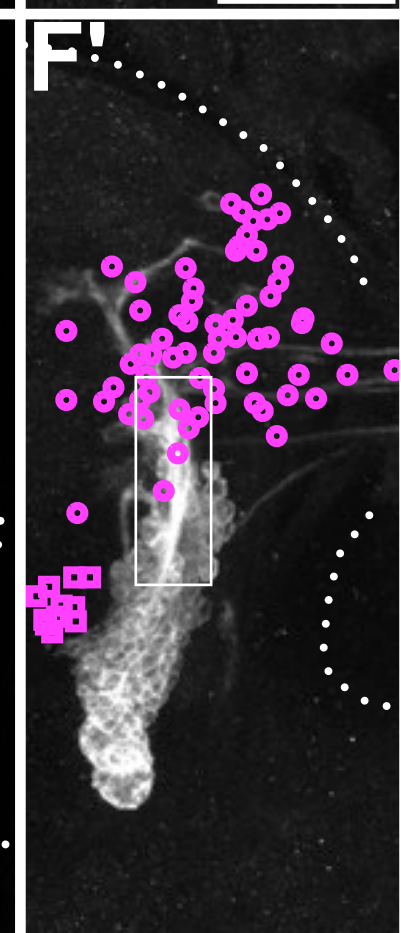
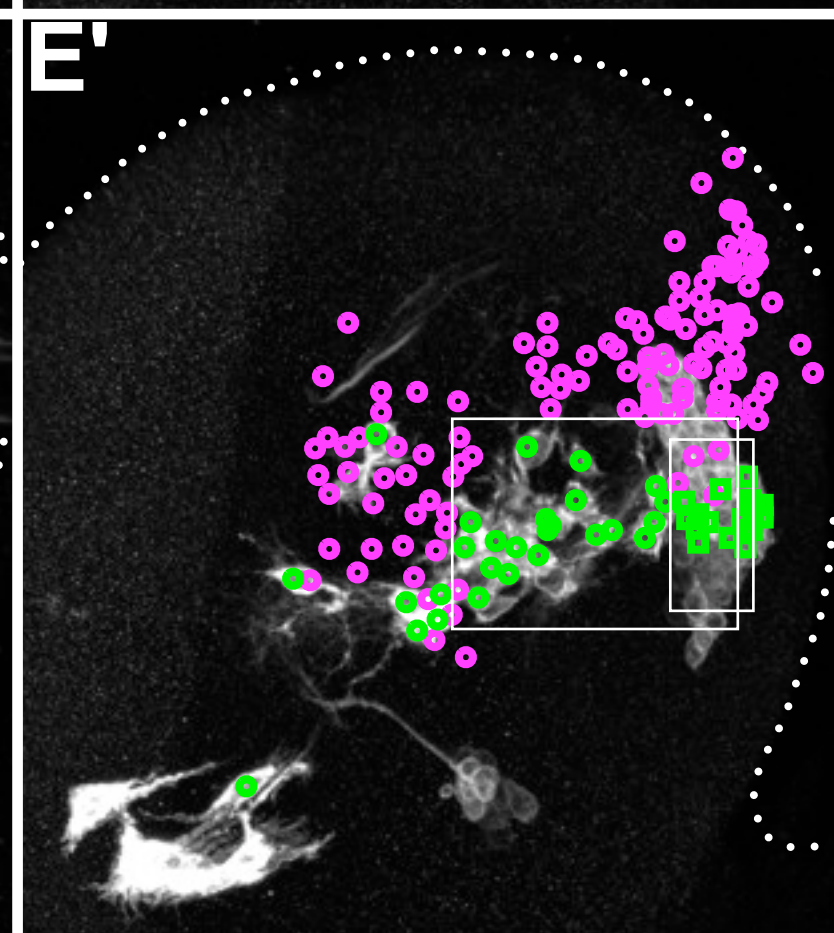
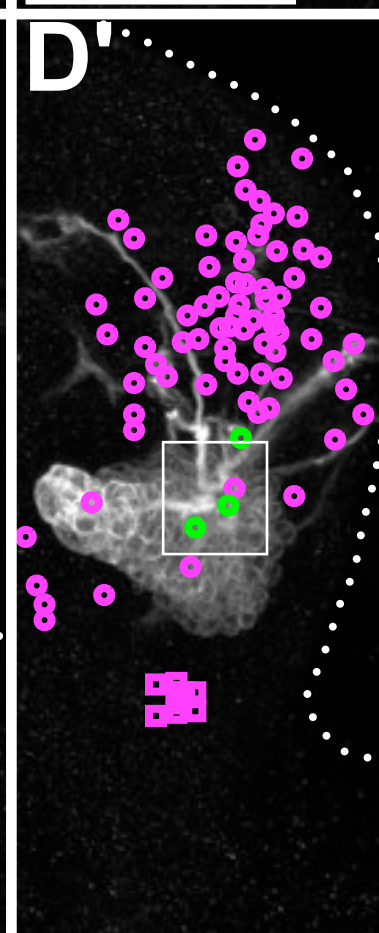
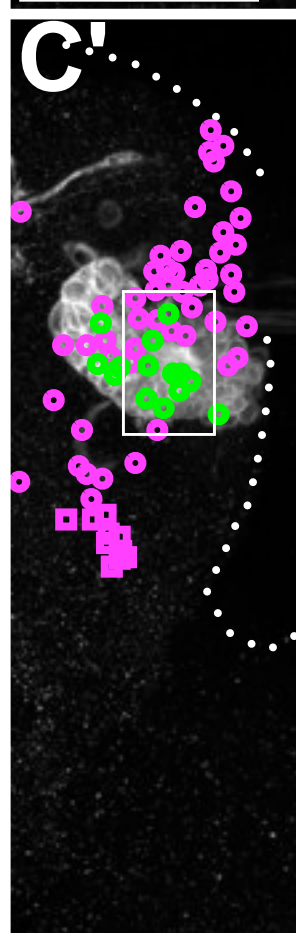
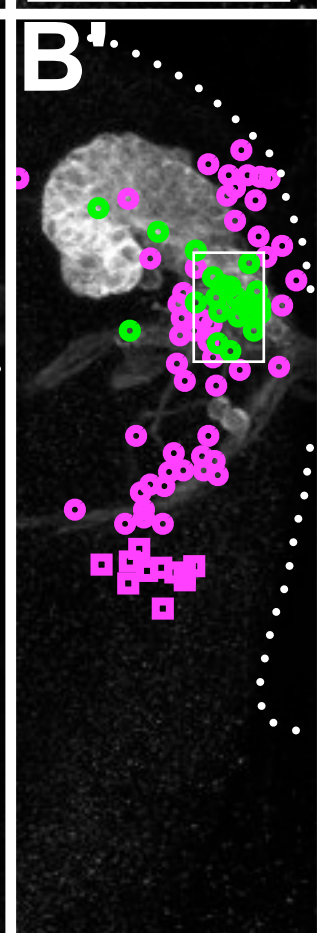
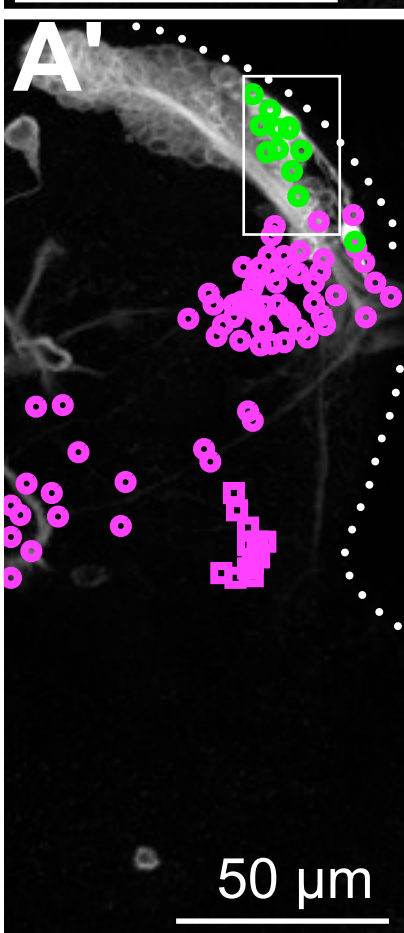
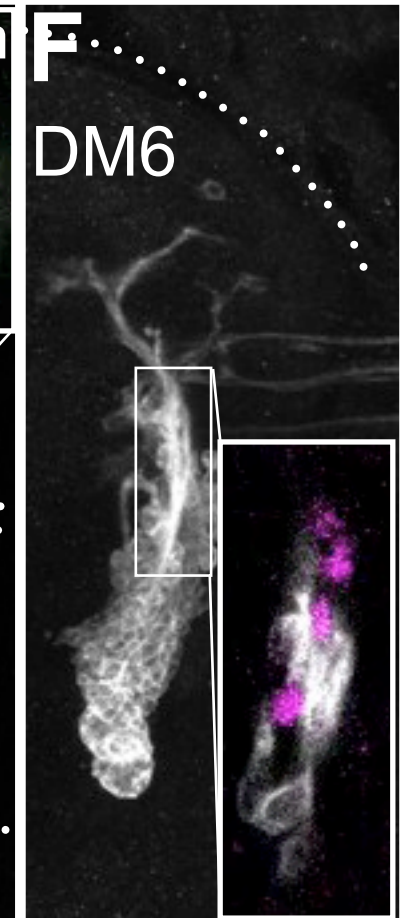
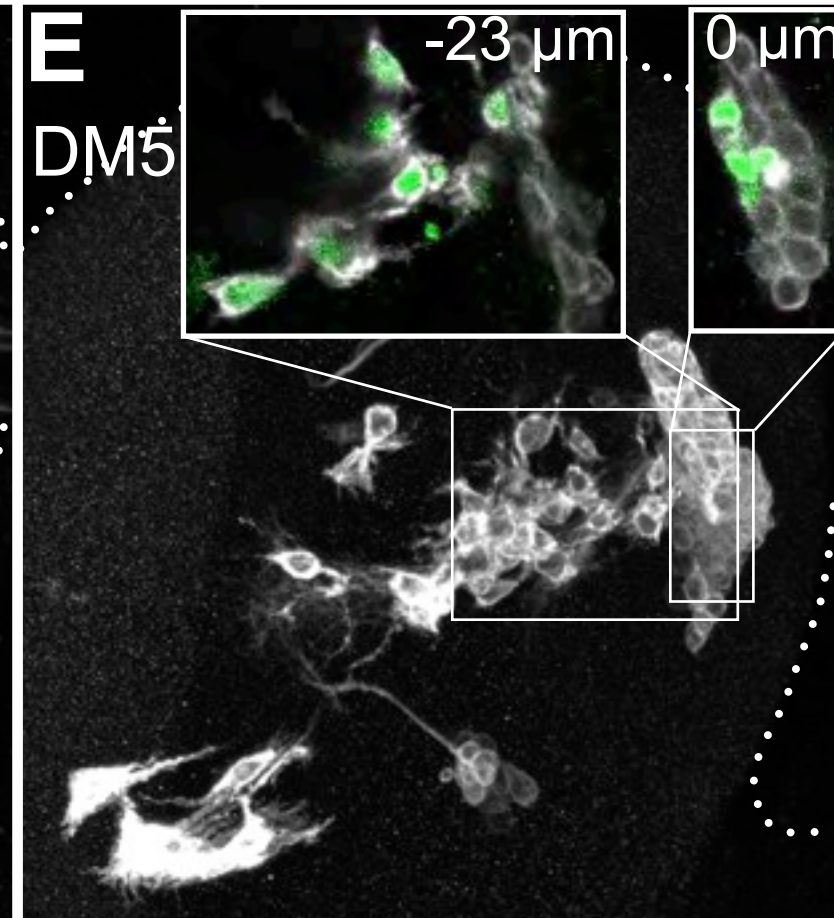
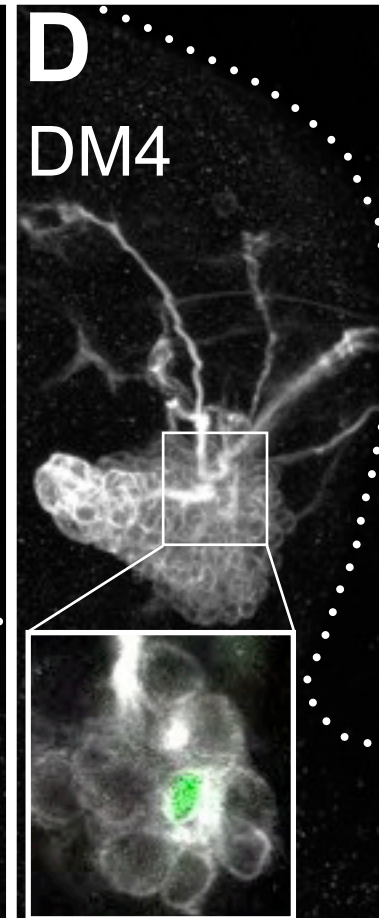
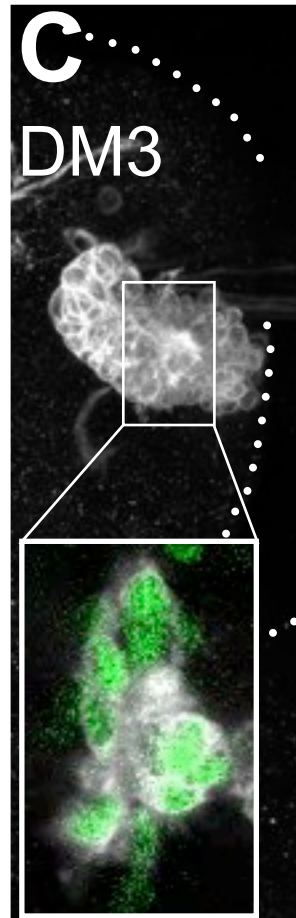
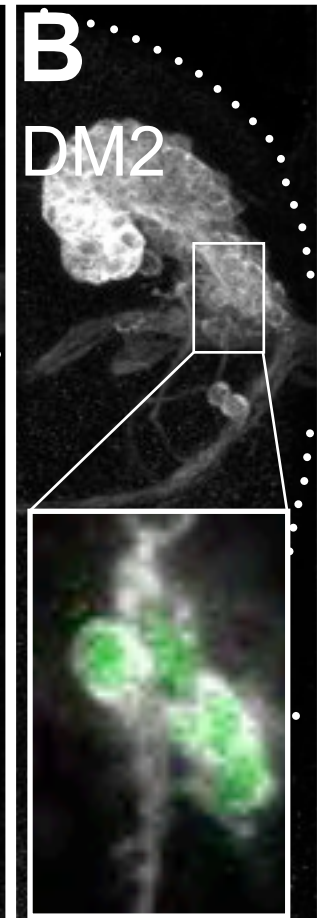
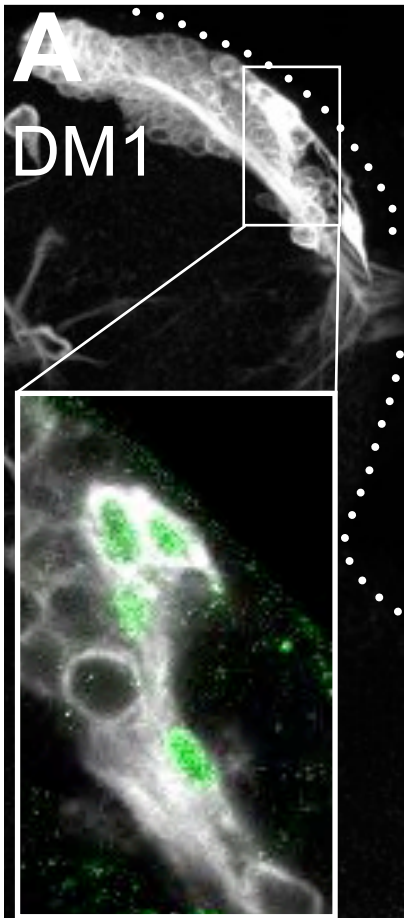








*tub>mCD8gfp* MARCM *gcm-lacZ* in clone / outside of clone



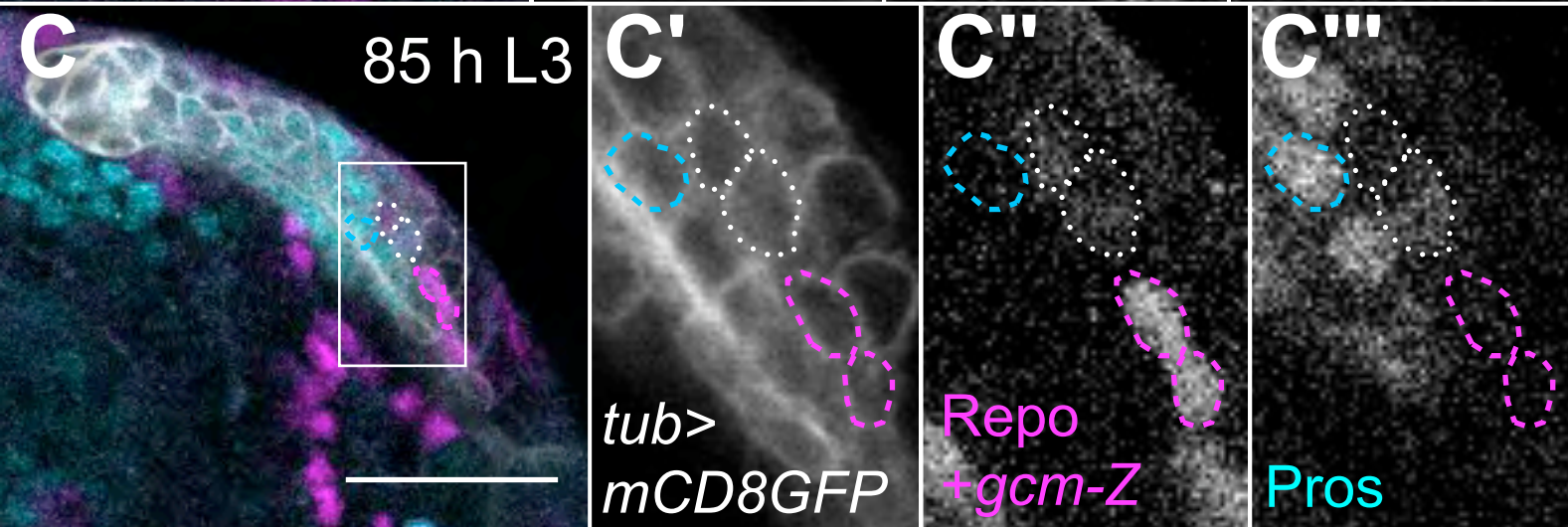
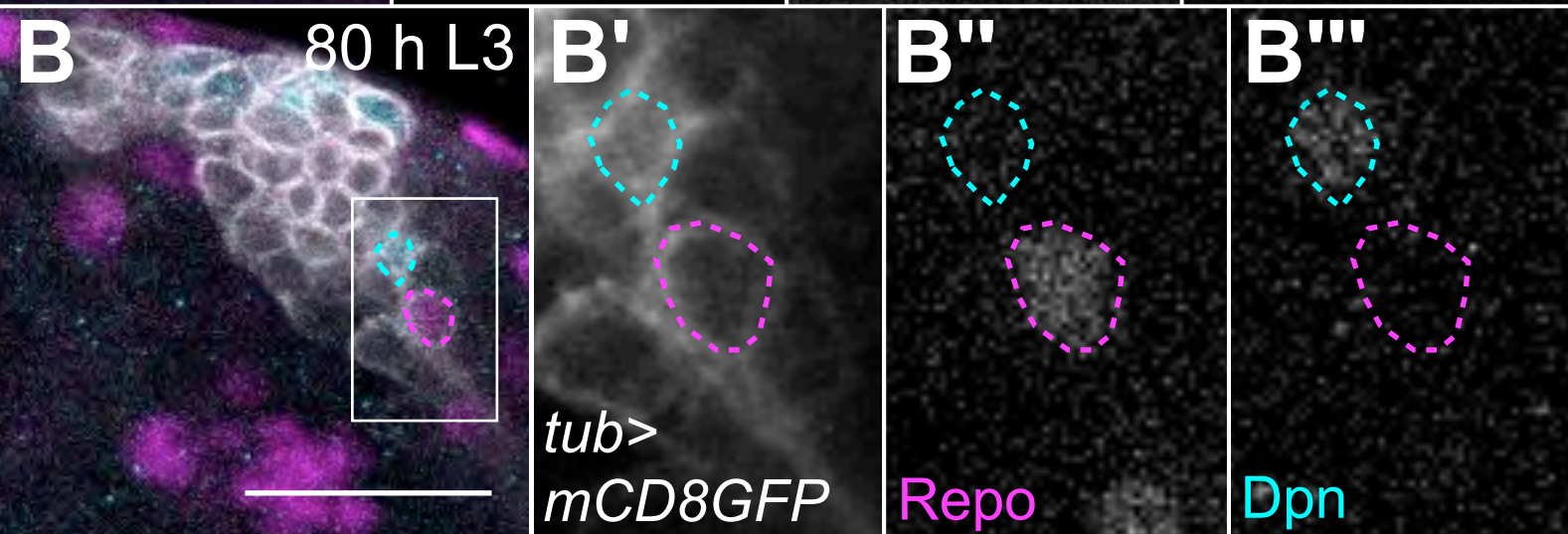
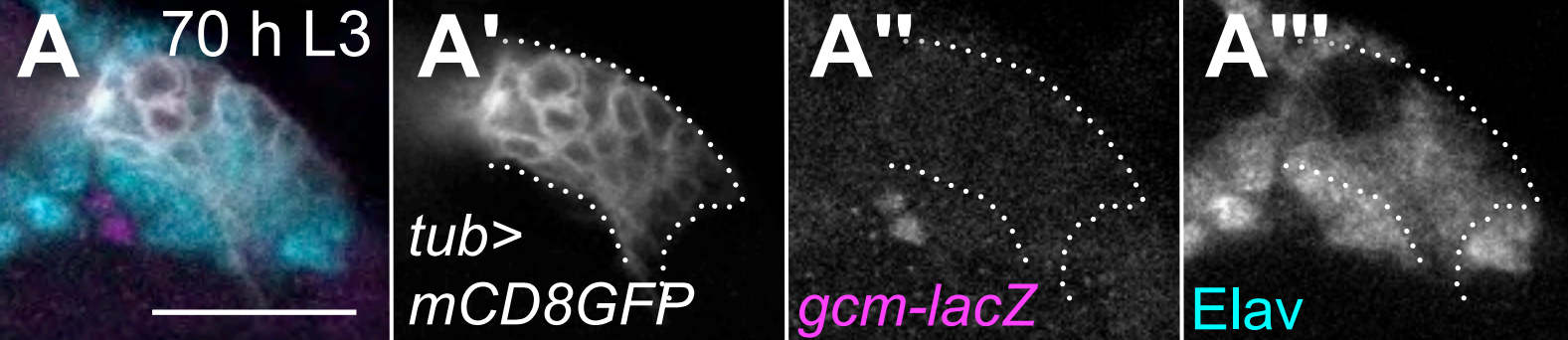
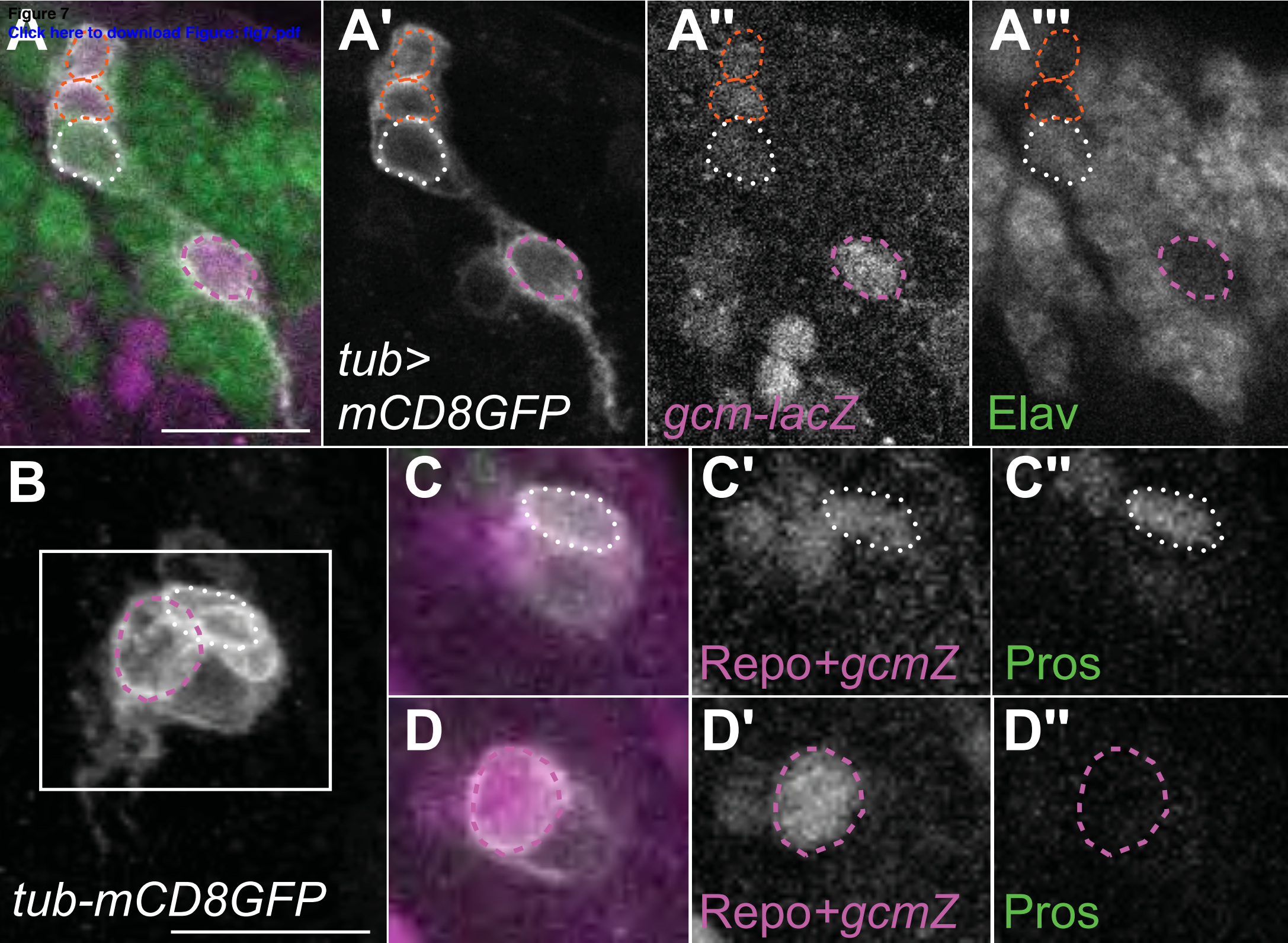
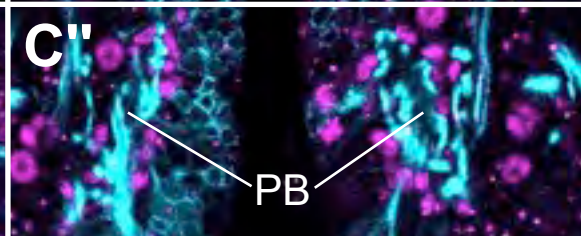
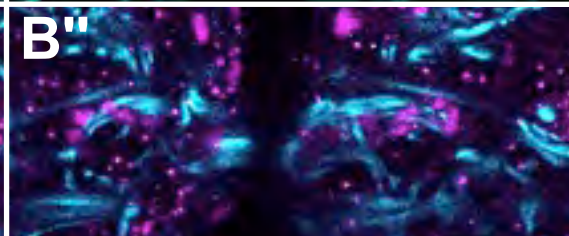
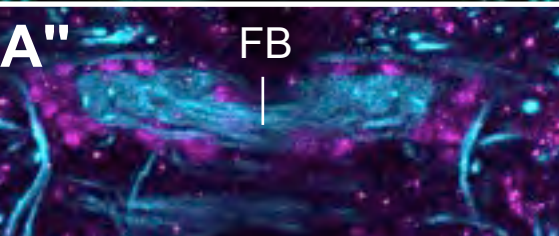
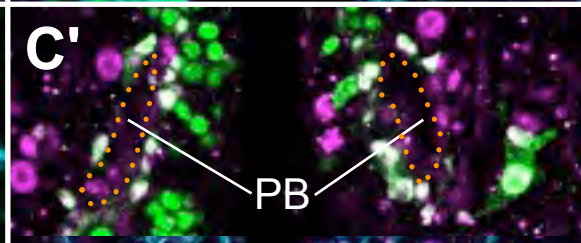
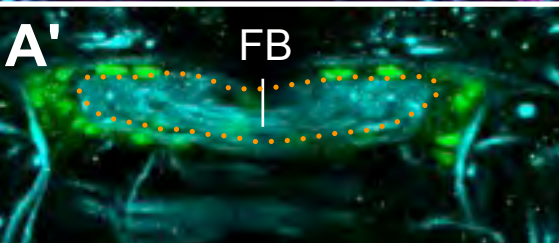
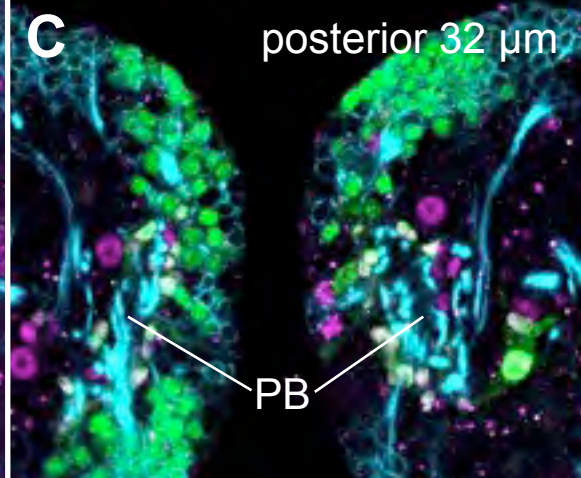
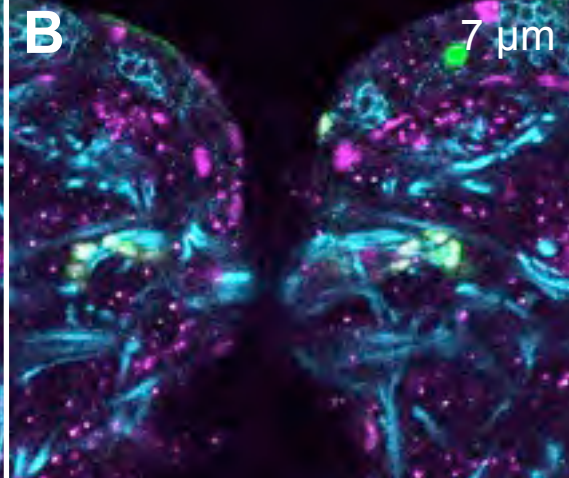
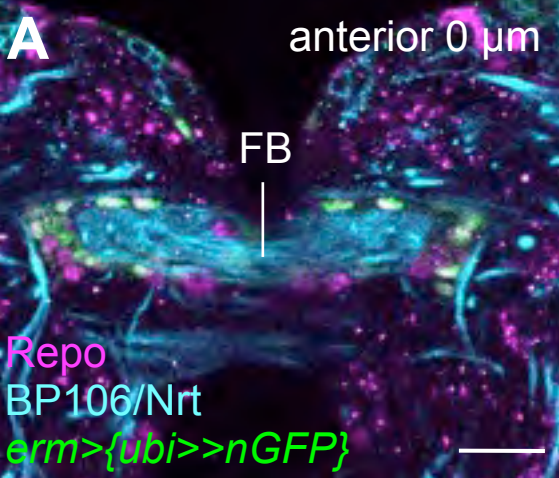


Figure 7  
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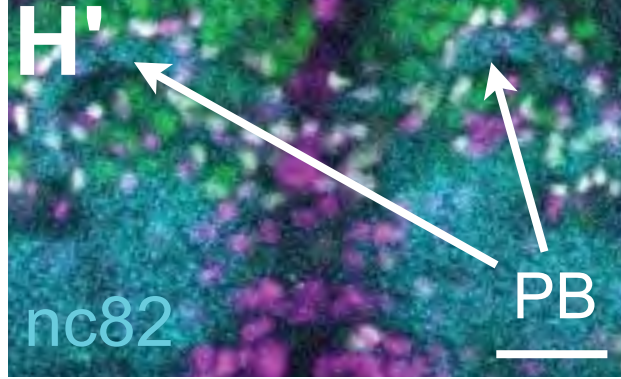
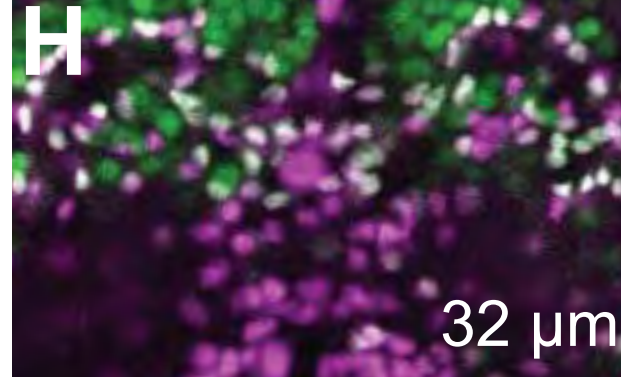
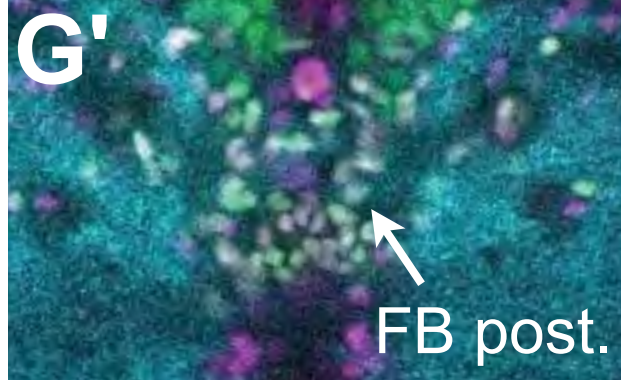
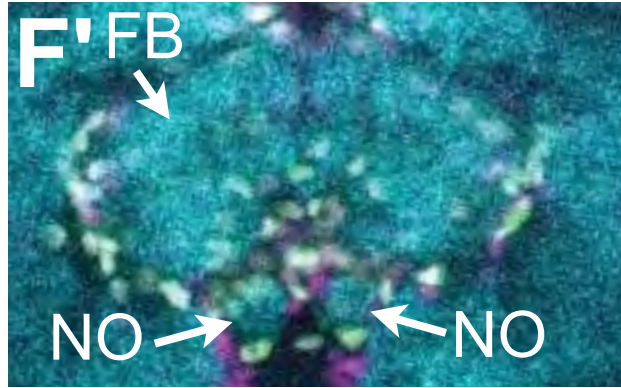
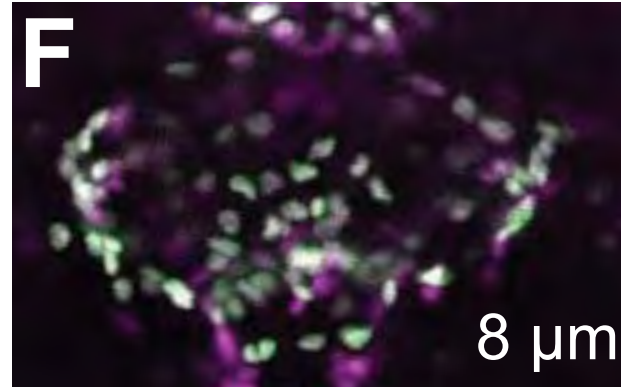
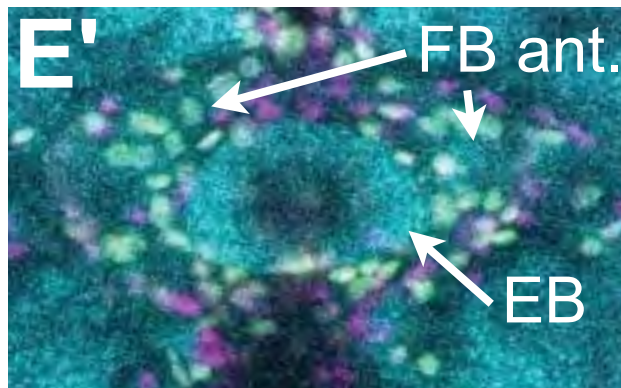
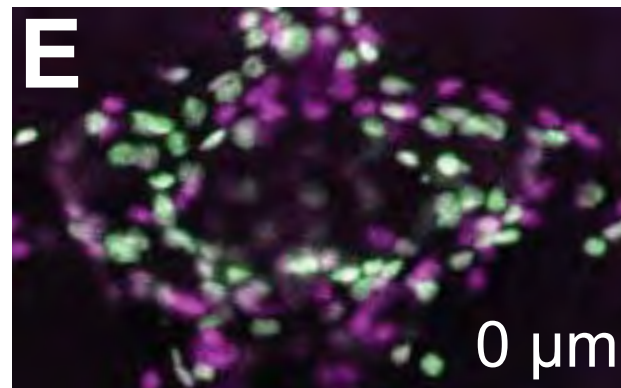
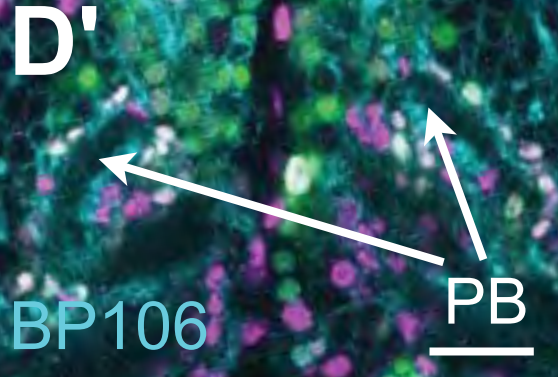
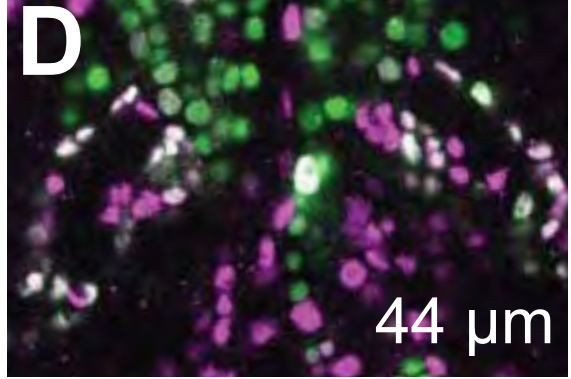
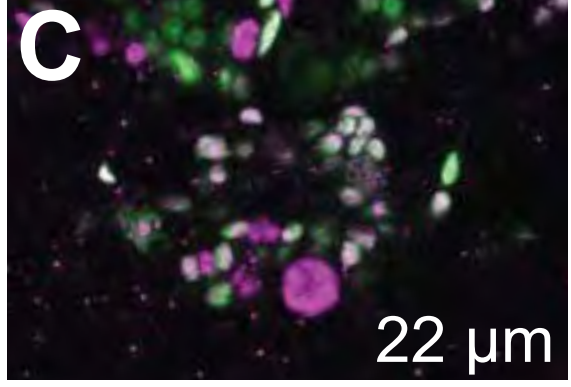
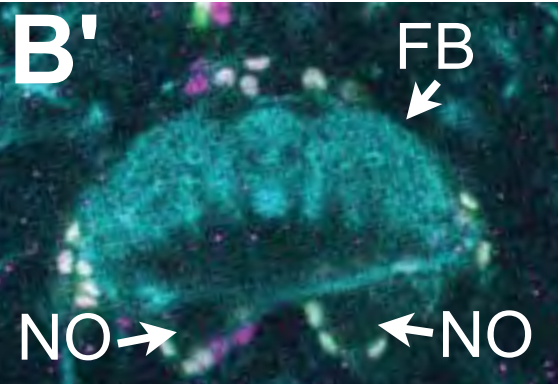
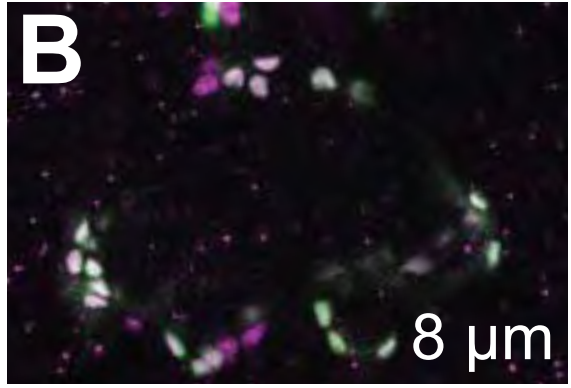
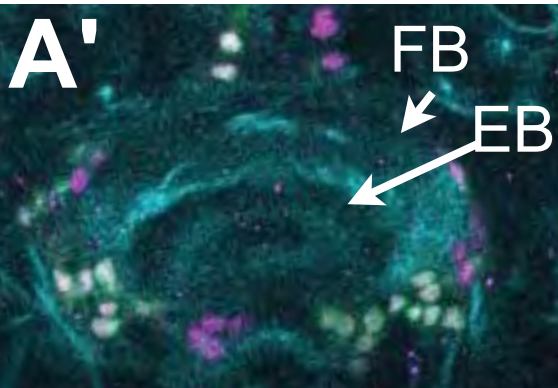
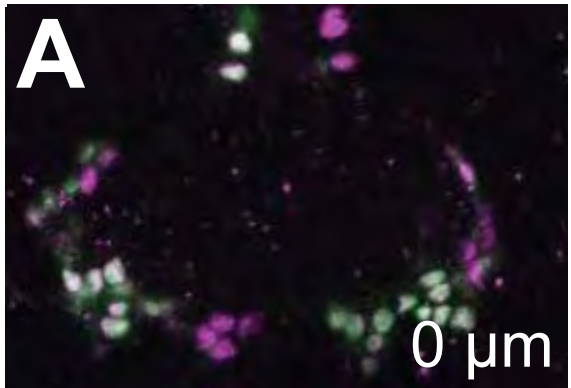




20 h APF *erm>{ubi}>nGFP* Repo BP106

40 h APF *erm>{ubi}>nGFP* Repo nc82

anterior



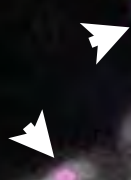
posterior

**A**

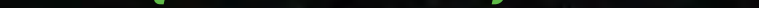
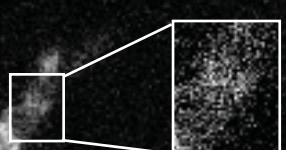
20 h APF

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pH3

*erm>{ubi}>>nGFP***B**

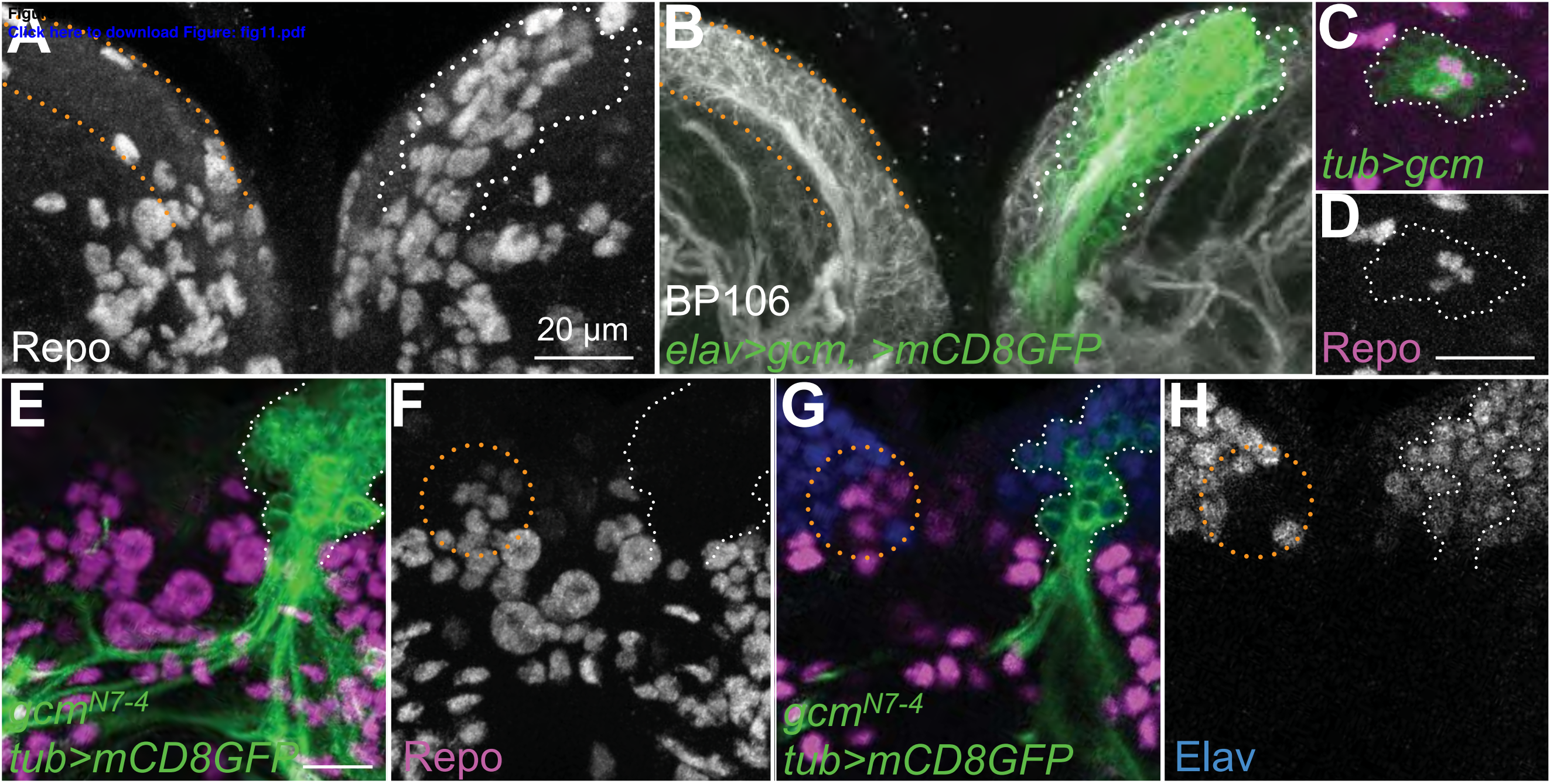
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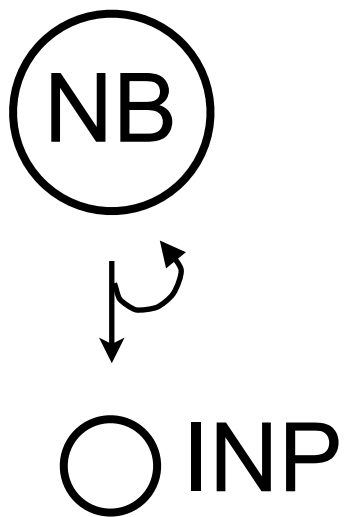
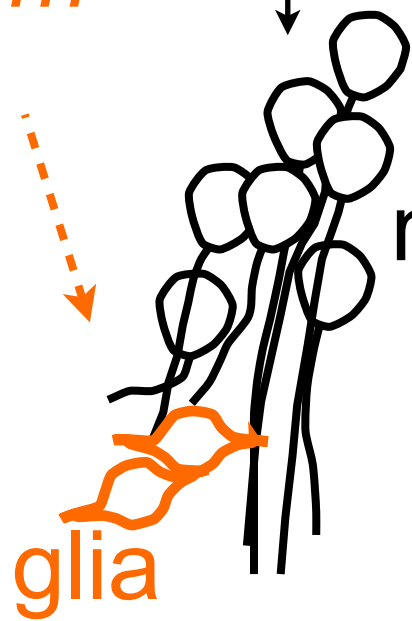
*erm>{ubi}>>nGFP***C**

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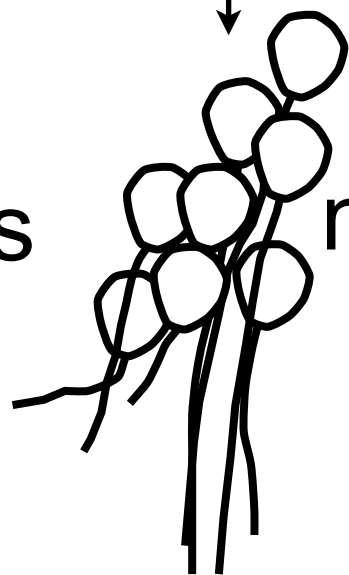
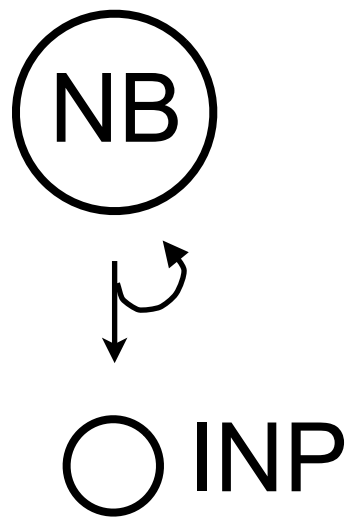




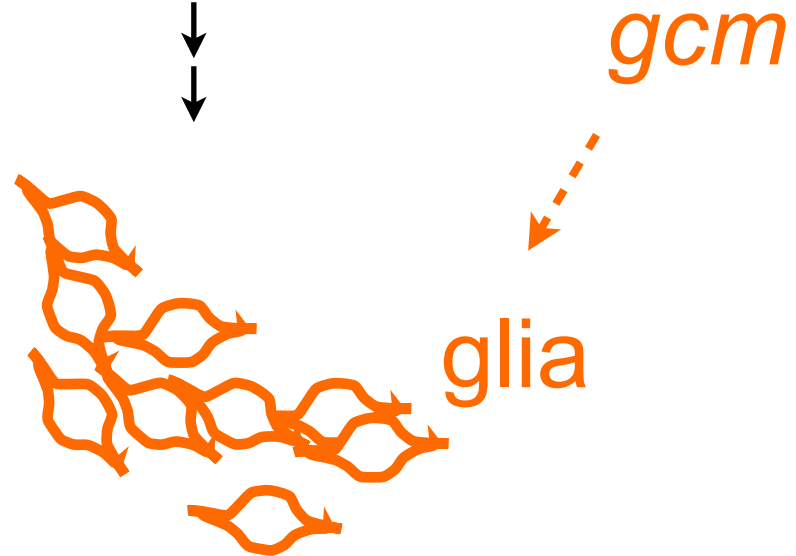
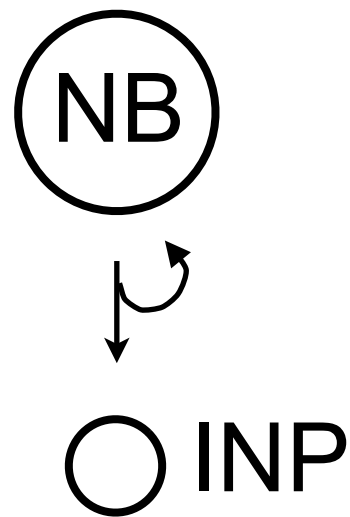


**A***gcm*

neurons

**B**

neurons

**C**

glia

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Table 1

	DM1	DM2	DM3	DM4	DM5	DM5 lateral	DM6
Mean $\pm$ s.d. (n $\geq$ 9 clones)	10 $\pm$ 2	20 $\pm$ 2	15 $\pm$ 2	2 $\pm$ 2	14 $\pm$ 4	20 $\pm$ 5	0.3 $\pm$ 0.5 (0-1 glia)

Table 2

		65-71 h L2	65-71 h L3	75-80 h L3	85 h L3	96 h L3
1	percentage of DM1-3 clones with glia (n $\geq$ 10 clones)	0 %	0 %	20 %	100 %	100 %
2	percentage of INP1-3 clones with glia (n $\geq$ 11 clones)	0 %	50 %	72 %	100 %	100 %
3	number of glia in DM1-3 clones (n $\geq$ 10 clones)	0 glia	0 glia	0.2 $\pm$ 0.4 (0-1 glia)	6 $\pm$ 4 glia	14 $\pm$ 5 glia
4	number of cells in INP1-3 clones (n $\geq$ 11 clones)	5 $\pm$ 1 cells	5 $\pm$ 1 cells	5 $\pm$ 1 cells	6 $\pm$ 2 cells	9 $\pm$ 2 cells
5	number of glia in INP1-3 clones (n $\geq$ 11 clones)	0 glia	1 $\pm$ 1 glia	1 $\pm$ 1 glia	3 $\pm$ 1 glia	3 $\pm$ 1 glia

## Supplemental Figure legends

**Fig. S1. INP-derived glia express the glial marker *gcm-lacZ*, but not the neuronal marker *Elav*.** (A-D') Example of a type II lineage MARCM clone (green; white outline, identified as DM1) colabeled with *gcm-lacZ* (magenta) and anti-*Elav* (cyan). (A-D) Clone-derived cells that express *gcm-lacZ* (magenta arrows) do not express *Elav* and are found far away from the neuroblast (orange outline) and its immediate, *Elav*-negative progeny (asterisks). (A'-D') The presence of *Elav*-negative cells (arrows) up to 10 cell diameters away from the neuroblast (orange outline copied from A-D) are indicative of type II lineages and correspond to intermediate progenitor cells and ganglion mother cells. (E-H) In a control type I neuroblast clone, *Elav*-negative cells (asterisks) are only found in close contact with the neuroblast (orange outline; out of focus, immediately above and touching the cells shown). Scale bar, 20  $\mu$ m.

**Fig. S2. Glia in the DM region first appear during early L3.** (A-C) Brains of L2 and L3 larvae during the L2-L3 transition around 70 hours AEL. (A) While *gcm* mRNA is already detectable in L2 larvae by in situ hybridization, *gcm-lacZ*<sup>A87</sup> expression in the DM1-3 region is not visible from L2 (B) and appears during L3 (C). Those cells already coexpress *Repo* (C', maximum projection of the central 71  $\mu$ m excluding large, *Repo*-expressing surface glia overlying the glia shown). A brain containing both a DM1 and a DM3 MARCM clone, shown magnified in (C''), was chosen to indicate the DM1-3 region. The area of the clone is outlined in (C-C') to show the position of DM1-3 in relation to the newly formed glia. Note that none of

the glia shown are part of the MARCM clones, as at that developmental time only IPC clones contain glia (see Table 2). Scale bars, 50  $\mu\text{m}$ .

**Fig. S3. INP-derived glia surround the developing central complex primordium.**

(A-C) Confocal sections of *erm-Gal4* G-trace (green) and Repo (magenta) co-labeled brains at the level of the fan-shaped body primordium at 10 hours (A-A"), 20 hours (B-B"), and 40 hours (C-C") APF. Single confocal sections in A, B; projection of 5  $\mu\text{m}$  confocal sections in C shows glia around the fan-shaped body primordium. Scale bar, 25  $\mu\text{m}$ .

**Fig. S4. Membrane labeling of INP-derived glia and neurons at the level of the fan-shaped body primordium at 20 hours APF.** (A-D) Single optical sections

through the central complex primordium reveals membrane structures of *erm-Gal4* neuronal and glial progeny (*erm-Gal4, UAS-flp, act>\*>Gal4, UAS-mCD8GFP*; A,B). All cell bodies (*UAS-nLacZ*; A,D) at this focal level are glial (Repo; A,C). The membrane structure, while containing axonal processes in addition to glial membranes (white in A,B), resembles that of ensheathing glia, as opposed to astrocyte-like glia, as reported in Awasaki et al. (2008). Scale bar, 20  $\mu\text{m}$ .

