Glia-derived temporal signals orchestrate neurogenesis in the *Drosophila* mushroom body

Mengying Yang[a,b,c,1], Honglei Wang[a,1], Changyan Chen[d], Shiping Zhang[a], Mengxiao Wang[a], Bhagyashree Senapati[e,2], Shuhua Li[a], Shuanglong Yi[a], Linfang Wang[a], Min Zhang[a], Shuai Yin[d], Yijing He[d], Lei Xue[f], Suewei Lin[e], and Margaret S. Ho[a,3]

[a]School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China; [b]Institute of Neuroscience, State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; [c]University of Chinese Academy of Sciences, Beijing 100049, China; [d]Department of Anatomy and Neurobiology, School of Medicine, Tongji University, Shanghai 200092, China; [e]Institute of Molecular Biology, Academia Sinica, Taipei, 115, Taiwan; and [f]Institute of Intervention Vessel, Shanghai 10th People's Hospital, Shanghai Key Laboratory of Signaling and Diseases Research, School of Life Science and Technology, Tongji University, Shanghai 200092, China

Edited by Claude Desplan, New York University, New York, NY, and approved April 26, 2021 (received for review September 27, 2020)

Intrinsic mechanisms such as temporal series of transcription factors orchestrate neurogenesis from a limited number of neural progenitors in the brain. Extrinsic regulations, however, remain largely unexplored. Here we describe a two-step glia-derived signal that regulates neurogenesis in the *Drosophila* mushroom body (MB). In a temporal manner, glial-specific ubiquitin ligase dSmurf activates non–cell-autonomous Hedgehog signaling propagation by targeting the receptor Patched to suppress and promote the exit of MB neuroblast (NB) proliferation, thereby specifying the correct α/β cell number without affecting differentiation. Independent of NB proliferation, dSmurf also stabilizes the expression of the cell-adhesion molecule Fasciclin II (FasII) via its WW domains and regulates FasII homophilic interaction between glia and MB axons to refine α/β-lobe integrity. Our findings provide insights into how extrinsic glia-to-neuron communication coordinates with NB proliferation capacity to regulate MB neurogenesis; glial proteostasis is likely a generalized mechanism in orchestrating neurogenesis.

glia | neurogenesis | mushroom body

Temporal patterning timely specifies neural progenies from a small pool of neural stem cells (NSCs) in the brain. Upon division in different modes, NSCs self-renew and produce a more differentiated daughter cell with distinct cell fate. To confer NSC temporal identity, a cascade of temporal transcription factors (tTFs) is sequentially expressed to specify neural cell fates within the lineage, whereas spatial cues intersect to assign regional identity among different lineages. As a consequence, a vast number of diverse cell types are generated as building blocks for the brain circuitry and functional connections underlying animal behaviors. Interestingly, execution of the tTF transcriptional program coordinates with the timing of NSC proliferation and modulates its proliferative capacity, ensuring neurogenesis proceeds at the expense of NSC competence and neuronal number (1, 2). To date, how these intrinsic factors coordinate with others, and the mechanism of temporal control on NSC proliferation, remain largely elusive.

Drosophila NSCs, termed neuroblasts (NBs), are excellent models to study the temporal mechanism for neurogenesis (3). Conserved sequential tTF series have been found in *Drosophila* NBs, with modes of division strikingly similar to mammals (4–8). Well-characterized embryonic NBs enter quiescence at the end of embryogenesis and are awakened by nutritional cues to enter a second phase of proliferation at the early larval stage, generating neuronal diversity in the adult central nervous system (9, 10). Among these postembryonic NBs, four NBs generating MB neurons—Kenyon cells (KCs)—do not undergo quiescence but continue to proliferate into the pupal stage until ecdysone signals for NB shrinking and apoptosis (6, 11, 12). Each of the four NBs generates three types of KCs (γ, α'/β', and α/β) in a sequential manner (13). Different from other types of NBs, opposing gradients of two IGF-II mRNA-binding proteins (Imp) and Syncrip (Syp) comprise the intrinsic temporal program for MB NBs (14). These distinct features imply that the behaviors of MB NBs are under tight regulation and that other temporal mechanisms might exist in regulating their division and proliferation (15).

In addition to intrinsic mechanisms, extrinsic signaling has been implicated in NSC regulation in terms of daughter cell fate commitment and the initiation of proliferation (9, 10, 16). Yet much less is known about whether the termination of NSC proliferation requires extrinsic factors. It has been shown that extrinsic activin and ecdysone signaling play key roles in regulating MB neurogenesis (6, 17, 18). Whereas the late-acting temporal factor, E93, integrates extrinsic hormonal cues to the intrinsic Imp and Syp program for terminating MB neurogenesis (19), signals activated by the glia-secreted activin ligand Myoglianin (Myo) regulates γ axon pruning (20, 21) and α'/β' neuron differentiation (17, 18). These findings imply pivotal roles for glia as extrinsic regulators, but whether they regulate the temporal patterning of MB neurogenesis, particularly the termination and exit of NSC proliferation, awaits further exploration.

Smad ubiquitin regulatory factor (Smurf) proteins (Smurf1 and Smurf2) are C2-WW-HECT ubiquitin E3 ligases that belong to the family of genes including the neural precursor cell–expressed

Significance

Brain function relies on tasks executed by different types of neurons generated via diverse mechanisms. Combining a series of genetic, cellular, and behavioral approaches, we identify a two-step glia-to-neuron signal that regulates how neurons are produced in the *Drosophila* mushroom body, a key learning center for animal behavior. This glial signal requires the action of an E3 ubiquitin ligase dSmurf that regulates the stability of two downstream effectors: the Hedgehog receptor Patched and the cell-adhesion molecule Fasciclin II. Our findings demonstrate the significance of glial cells in regulating neuron production, hence the brain function and animal behavior.

Author contributions: M.Y., H.W., S.Z., and M.S.H. conceived and designed the study; M.Y., H.W., C.C., S.Z., M.W., B.S., S.L., S.Y., L.W., M.Z., S.Y., and Y.H. performed the experiments; L.X. and S.L. analyzed the data; and M.Y., H.W., and M.S.H. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]M.Y. and H.W. contributed equally to this work.

[2]Present address: Centre for Neural Circuits and Behaviour, University of Oxford, Oxford OX1 3TA, United Kingdom.

[3]To whom correspondence may be addressed. Email: margareth@shanghaitech.edu.cn.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2020098118/-/DCSupplemental.

Published June 2, 2021.

developmentally down-regulated gene 4 (Nedd4) (22, 23). Whereas the C2 domain binds to phospholipids and mediates intracellular targeting to the plasma membrane, the WW domains recognize substrates via a proline-rich sequence (PPXY motif) (24, 25), and the carboxyl-terminal HECT domain mediates the catalytic transfer of ubiquitin. Smurf proteins and their *Drosophila* homolog dSmurf have been shown to regulate a variety of developmental processes such as ovary and adult germline stem-cell differentiation, wing disk development, and epithelial cell polarity by targeting the degradation of components in bone morphogenetic protein (BMP), Hedgehog (Hh), and Hippo pathways (26–31). Nonetheless, Smurf proteins' function in the nervous system has not been fully investigated. Here, we identify a dSmurf-mediated glial signal that regulates *Drosophila* MB neurogenesis. Glial dSmurf regulates the distribution of Hh receptor Patched (Ptc) on the membrane to convey the paracrine Hh signal for suppressing MB NB proliferation in a temporal manner. This glia-derived temporal signal is required for the exit of MB NB proliferation so that the α/β cell number is precisely controlled. Furthermore, glial dSmurf stabilizes the membrane expression of the cell-adhesion molecule Fasciclin II (FasII) via its WW domains and modulates homophilic FasII interaction between glia and axons to refine α/β lobe formation. These regulatory mechanisms demonstrate the significance of glia–neuron interaction, revealing a distinct route of glia-to-neuron communication controlling neurogenesis in an extrinsic manner.

Results

Glial-Specific dSmurf Misexpression Selectively Disrupts MB α/β-Lobe Formation. Previous literature has indicated that dSmurf is expressed in both neurons and glia (32). Using the anti-dSmurf antibodies (27), dSmurf expression was analyzed in fly brains expressing UAS-IVS-Syn21-GFP-p10 under the control of repo-Gal4 (*repo > synGFP*), which labels glial nuclei and membranes. Expressing the RNA interference (RNAi) transgene targeting dSmurf (*UAS-dSmurf-RNAi*, v24681) in glia abolished the dSmurf-positive immunofluorescent intensities in third-instar larvae, pupa at 48 h after puparium formation (APF), and 3-d-old adult animals (*SI Appendix*, Fig. S1). Western blot analysis also revealed a corresponding change in glial dSmurf protein levels upon expressing dSmurf with the Myc

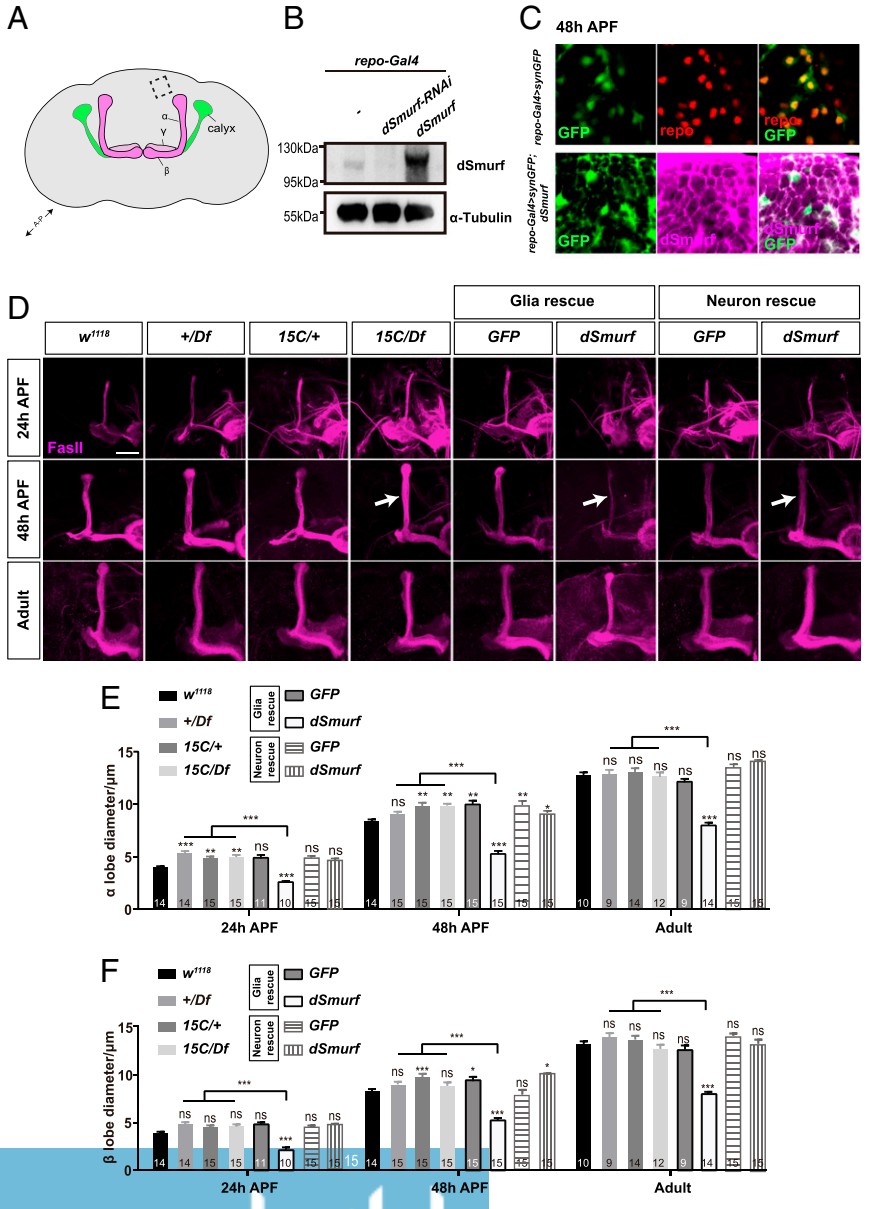

Fig. 1. dSmurf mutants exhibit α/β-lobe defects at 48 h APF. (*A*) Illustration of a symmetrical MB in a fly brain with α/β and γ lobes located anteriorly (magenta) and cell-body calyces located posteriorly (green). Dashed square in black indicates the area where images in *C* were acquired. (*B*) Western blot analysis on the protein extracts collected from adult fly heads with the indicated genotypes. Note that dSmurf protein levels increase and decrease when dSmurf or dSmurf-RNAi is expressed, respectively. α-Tubulin serves as an internal control. (*C*) Fly brains expressing glial GFP (*repo > synGFP*) at 48 h APF were dissected and immunostained with antibodies against Repo (red, *repo > synGFP*) or dSmurf (magenta, *repo > synGFP; dSmurf*). Overlay images are shown (magenta or red and green). Note that dSmurf expression is detected on the glial membrane. (*D–F*) Representative confocal images (*D*) and quantifications (*E* and *F*) of α/β lobe formation in heterozygous (*15C/+* and *+/Df*) and trans-heterozygous (*15C/Df*) mutant brains. Three different developmental stages: 24h APF, 48h APF, and 3-d-old adult were analyzed. α/β and γ lobes were immunostained with antibodies against FasII (magenta). Lobe diameters were quantified at the midst of lobe (total length/2). Note that α/β lobes are significantly thicker in dSmurf mutant brains (arrows). Re-introducing dSmurf expression in glia (Glia rescue) rescues and further suppresses the α/β lobe formation, whereas dSmurf expression in MB neurons (Neuron rescue) does not rescue the defects (arrows). Average 15 brains (30 α/β lobes) for each genotype were dissected and quantified. (Scale bar: 50 μm.) Data are shown as mean ± SEM. Only merged hemispherical α/β lobes are shown. *P* values of significance (indicated with asterisks; ns, no significance; $*P < 0.05$, $**P < 0.01$; and $***P < 0.001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above. Approximately 40 confocal Z-stack sections were taken with 0.5 μm each to thoroughly visualize MB lobes. Genotypes analyzed are detailed in the *SI Appendix*, Table S1 and simplified in the legends for all figures. All data in subsequent figures are present and analyzed as described in Fig. 1 unless stated otherwise.

epitope tag (*UAS-Myc-dSmurf*, henceforth abbreviated as *dSmurf*) (27) or dSmurf-RNAi (Fig. 1*A*). dSmurf-positive immunofluorescence was colocalized with glial membranous GFP in fly brains overexpressing dSmurf, suggesting that dSmurf is localized on the glial membrane (Fig. 1*C*). Under endogenous conditions, dSmurf-positive immunofluorescence also coincided with glial membranous GFP in the vicinity of MB calyces or in the entire brain (*SI Appendix*, Fig. S2). Taken together, these results validate the antibody specificity and demonstrate dSmurf expression in glia.

To investigate dSmurf function in glia, dSmurf or dSmurf-RNAi was selectively expressed using the pan-glial *repo-Gal4*. MB lobe formation was first analyzed using the anti-FasII antibody, a marker that stains α/β lobes strongly and γ lobes weakly (33). The location of MB lobes with respect to the whole brain is illustrated in Fig. 1*A*, and different developmental stages, including 24 h APF, 48 h APF, and 3-d-old adult, were analyzed. Interestingly, FasII-positive MB lobes analyzed over different developmental stages revealed relatively normal γ lobes across brains from all genotypes, whereas α/β lobes of repo > dSmurf flies were significantly distorted and became thinner in all developmental time points (24 h APF, 48 h APF, and 3-d-old adult, arrows in Fig. 1*D*, quantified in Fig. 1 *E* and *F*). Conversely, significantly thicker α/β lobes were found in repo > dSmurf-RNAi flies at all time points except the adult stage. Moreover, glial expression of two additional dSmurf-RNAi, v24680 or v107349, also caused an increase in the α/β lobe diameter at 48 h APF, confirming that dSmurf expression in glia is required for α/β lobe formation (Fig. 1 *H* and *I*), with their knockdown efficacies verified by Western blot analysis (Fig. 1*G*). Coexpression of dSmurf-RNAi and dSmurf or dSmurf expression in the $dSmurf^{15C}$ mutant heterozygotes rescued the α/β lobe defects (Fig. 1 *D*–*F* and *SI Appendix*, Fig. S3 *A* and *B*), suggesting that these phenotypes are specifically due to glial dSmurf. Notably, Trio-labeled α'/β' lobes remain normal across brains from all genotypes (*SI Appendix*, Fig. S3*C*). Taken together, these results indicate that glial dSmurf selectively regulates MB α/β lobe formation commencing at the beginning of the pupal stage; this glial regulation exhibits the greatest penetrance at 48 h APF.

dSmurf Mutants Exhibit MB α/β-Lobe Defects at 48 h APF. To further support a role for dSmurf in regulating MB lobe formation, dSmurf mutant brains ($dSmurf^{15C}/Df$) (27, 34) were analyzed for MB lobe formation. Consistently, FasII-positive MB α lobes were significantly thicker in the heterozygous and homozygous dSmurf mutant brains at 24 and 48 h APF, whereas β lobes of the same genotypes exhibited similar trends (arrows in Fig. 2*A*, quantified in Fig. 2 *B* and *C*). Similar to the glial dSmurf-RNAi, the mutant thick-lobe phenotype was most robust and penetrant at 48 h APF yet did not persist into the adult stage (Fig. 2 *A*–*C*). To test whether dSmurf is required in different cell types to mediate MB lobe formation, tissue-specific rescue experiments under the control of the MB α/β/γ driver mb247-Gal4 or repo-Gal4 were performed. Interestingly, whereas MB-specific expression of dSmurf (*mb247 > dSmurf*) did not rescue the mutant thick-lobe phenotype, glial-specific dSmurf expression (*repo > dSmurf*) rescued and further suppressed the lobe formation (Fig. 2 *A*–*C*). These results indicate that glial dSmurf expression is critical for MB α/β lobe formation, prompting us to analyze dSmurf function in glial cells.

dSmurf Expression in Neurons Is Not Required for MB Formation. Results from dSmurf mutant and tissue-specific expression analysis indicate that dSmurf functions in glial cells to regulate MB lobe formation. To further confirm, dSmurf or dSmurf-RNAi were also selectively expressed in neurons using a pan-neuronal driver elav-Gal4. Interestingly, FasII-positive α/β and γ lobes were largely normal when expressing dSmurf, dSmurf-RNAi, or both in neurons (*SI Appendix*, Fig. S4*A*). There are no significant differences in the diameter of α/β lobes between control and experimental brains (*SI Appendix*, Fig. S4 *D* and *E*). dSmurf protein levels were also affected upon expressing dSmurf transgenes using *elav*-Gal4 (*elav > dSmurf-RNAi* or *dSmurf*, *SI Appendix*, Fig. S4*C*). α/β neuron-specific expression of dSmurf, another dSmurf overexpression transgene Flag-$dSmurf^{877}$, or a catalytic inactive form of dSmurf, Flag-$dSmurf^{C1029A}$, did not cause any α/β lobe defects (*SI Appendix*, Fig. S4 *B*, *F*, and *G*). In addition, α'/β' lobe formation and MB NB proliferation remain unaffected upon α'/β' neuron-specific expression of dSmurf or dSmurf-RNAi (*SI Appendix*, Fig. S4 *H*–*K*). Notably, a previous study has shown that abolished dSmurf expression in neurons does not alter synaptic bouton numbers at the *Drosophila* larval neuromuscular junction (35). Taken together, these results suggest that neuronal dSmurf does not play a significant role in MB and synapse development.

Glial dSmurf Selectively Regulates MB α/β Cell Number. Our results indicate a concurrent change in the diameter of α/β lobes upon altering glial dSmurf expression. Thus, the morphological difference of α/β lobes is unlikely due to a misguidance defect, since misguided axons of one lobe will reroute to the other, leading to the thickening and thinning of α/β lobes at the same time. Based on these observations, we next sought to determine if the disruptive α/β lobe formation is due to a change in the overall cell number, such that an irregular number of axons are bundled together, reflecting a change in the diameter.

To examine the α/β cell number, a binary system was established with α/β neuron–specific LexA (*GMR70F05-LexA*) driving the expression of a membrane-bound GFP (*13xLexAop2-myrGFP*) and repo-Gal4 driving dSmurf expression (*GMR70F05-LexA > GFP; repo > dSmurf* or *dSmurf-RNAi*). Interestingly, the α/β cell number quantified by counting the posterior GFP-positive α/β cell bodies (green, Fig. 3*A*) decreased dramatically upon glial dSmurf misexpression, and coexpression of dSmurf and dSmurf-RNAi in glia rescued such reduction (Fig. 3 *B* and *C*). Expressing dSmurf RNAi v24681 or v107349 in glia also exhibited a decrease in α/β cell number (Fig. 3*D*). These results indicate that glial dSmurf is required for a correct output of α/β cell number.

To confirm that glial dSmurf only modulates the α/β cell number, a different binary system was established with the α'/β' neuron–specific LexA (*GMR41C07-LexA > GFP; repo > dSmurf* or *dSmurf-RNAi*). Quantifications of the posterior GFP-positive α'/β' cell bodies indicated no significant difference in the α'/β' cell number upon expressing dSmurf or dSmurf-RNAi in glia (*SI Appendix*, Fig. S3 *D* and *E*). Furthermore, γ cell number marked by the anti-EcRB1 antibodies in the third-instar larval brains remain unaffected by glial dSmurf (*SI Appendix*, Fig. S3 *F* and *G*). These results suggest that glial dSmurf unlikely regulates the sequential differentiation of γ, α'/β', and α/β neurons during development; rather, glial dSmurf specifies a correct output of α/β cell number, hence the proper formation of α/β lobes.

Glial dSmurf Regulates MB NB Proliferation. Given that glial dSmurf regulates the α/β cell number, we next analyzed whether it regulates the ability of MB NBs to divide and generate KCs. We proposed that MB NBs divide and proliferate correctly until α/β cells are differentiated at the pupal stage. To test this hypothesis, MB NB proliferation was first analyzed at 48 h APF using antibodies against phospho-Histone 3 (pH3). The anti-pH3 antibodies mark the four MB NBs while *mb247-LexA > GFP* labels the cell bodies and calyces (Fig. 3*E*). Analysis of the pH3-positive cell number (present as multiple spots within the puncta) indicated a reduction in the proliferative potential of MB NBs when glial dSmurf or dSmurf-RNAi was expressed, and reintroducing dSmurf expression rescued this decrease (Fig. 3 *F* and *G*). Similarly, glial expression of dSmurf RNAi v24680, v107349, or $dSmurf^{C1029A}$ caused a reduction in the pH3-positive cell number (Fig. 3*H*). dSmurf

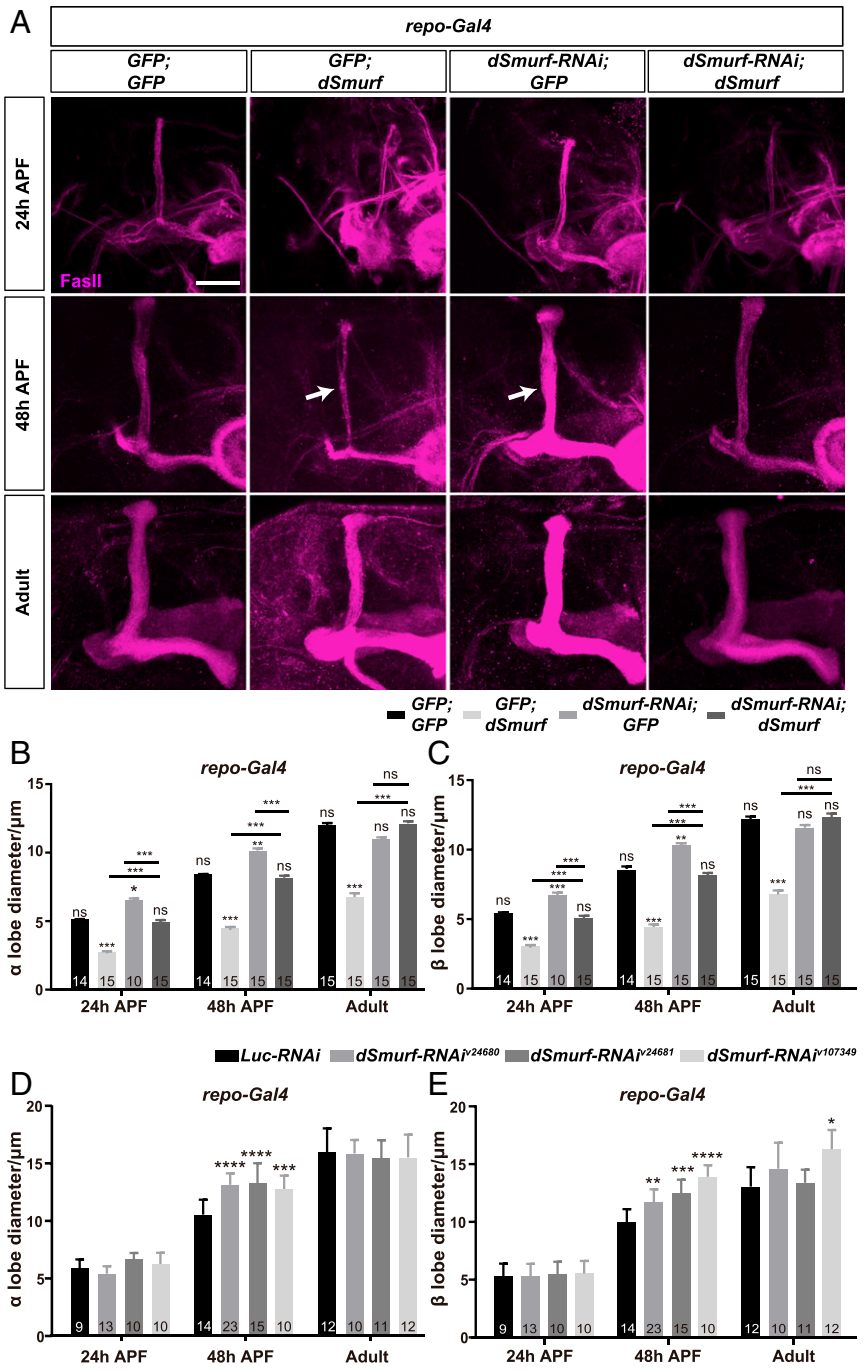

Fig. 2. Glial-specific dSmurf misexpression selectively disrupts α/β lobe formation. (*A*–*C*) Representative confocal images (*A*) and quantifications (*B* and *C*) of MB α/β lobe formation in fly brains overexpressing or downregulating glial dSmurf. (*D* and *E*) Quantifications of MB α/β lobe formation in fly brains expressing three independent dSmurf RNAi in glia. The genotypes analyzed above included: control (*GFP*; *GFP*), overexpression (*GFP*; *dSmurf*), downregulation (*dSmurf-RNAi*; *GFP*), rescue (*dSmurf-RNAi*; *dSmurf*), and two other independent RNAi lines (*dSmurf-RNAi*^{v24680} and *dSmurf-RNAi*^{v107349}). dSmurf-RNAi refers to the v24681 line throughout the text and figures in this study, whereas two other independent dSmurf-RNAi lines are referred by the number v24680 and v107349. FasII-positive α/β and γ lobes were analyzed at 24h APF, 48h APF, and 3-d-old adult. Note that α/β lobes are significantly thinner and thicker when glial dSmurf expression is upregulated and downregulated, respectively (arrows in *A*). Co-expression of dSmurf and dSmurf-RNAi rescues the defective α/β lobe diameter. Downregulation of dSmurf expression using either of the two additional RNAi lines causes similar thick-lobe phenotype to dSmurf-RNAi and mutant animals at 48h APF. Average 15 brains (30 α/β lobes) for each genotype were dissected and quantified. (Scale bar: 50 μm.) P values of significance (indicated with asterisks; ns, no significance; $*P < 0.05$, $**P < 0.01$; and $***P < 0.001$; and $****P < 0.0001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above.

mutant brains also exhibited a reduced number of pH3-positive cells. Glial dSmurf expression rescued and further suppressed the reduction but not MB neuron dSmurf expression (Fig. 3*I*). Taken together, these results suggest that glial dSmurf is required for MB NB proliferation.

To further analyze MB NB proliferation, antibodies against bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) were used to label the incorporated BrdU during cell proliferation. Consistent with the pH3-staining results, a striking reduction in the number of BrdU-positive cells was detected when expressing dSmurf or dSmurf-RNAi in glia (Fig. 3 *F* and *J*). Coexpression of dSmurf and dSmurf-RNAi also rescued this decrease. As assessed by pH3 staining, NB proliferation remain unaffected by glial dSmurf in earlier stages such as late third-instar larvae and 0 h APF (Fig. 3*K*). In toto, these results suggest that glial dSmurf regulates MB NB proliferation in a temporal manner to precisely control the α/β cell number.

Glial dSmurf Regulates MB NB Proliferation via Hh Signaling. To further elucidate the mechanism of glial dSmurf regulating MB NB proliferation, we investigated whether the Hh signaling pathway with components targeted by dSmurf is involved in MB NB proliferation. *Drosophila* Hh activation is mediated by interaction between the membrane receptor Ptc and extracellular Hh ligands, thus relieving Ptc-mediated inhibition on the signal transducer Smoothened (Smo) (28, 29, 31). Smo then activates downstream Hh signaling via a Cubitus interruptus–mediated transcriptional program. Interestingly, down-regulating Ptc expression in glia

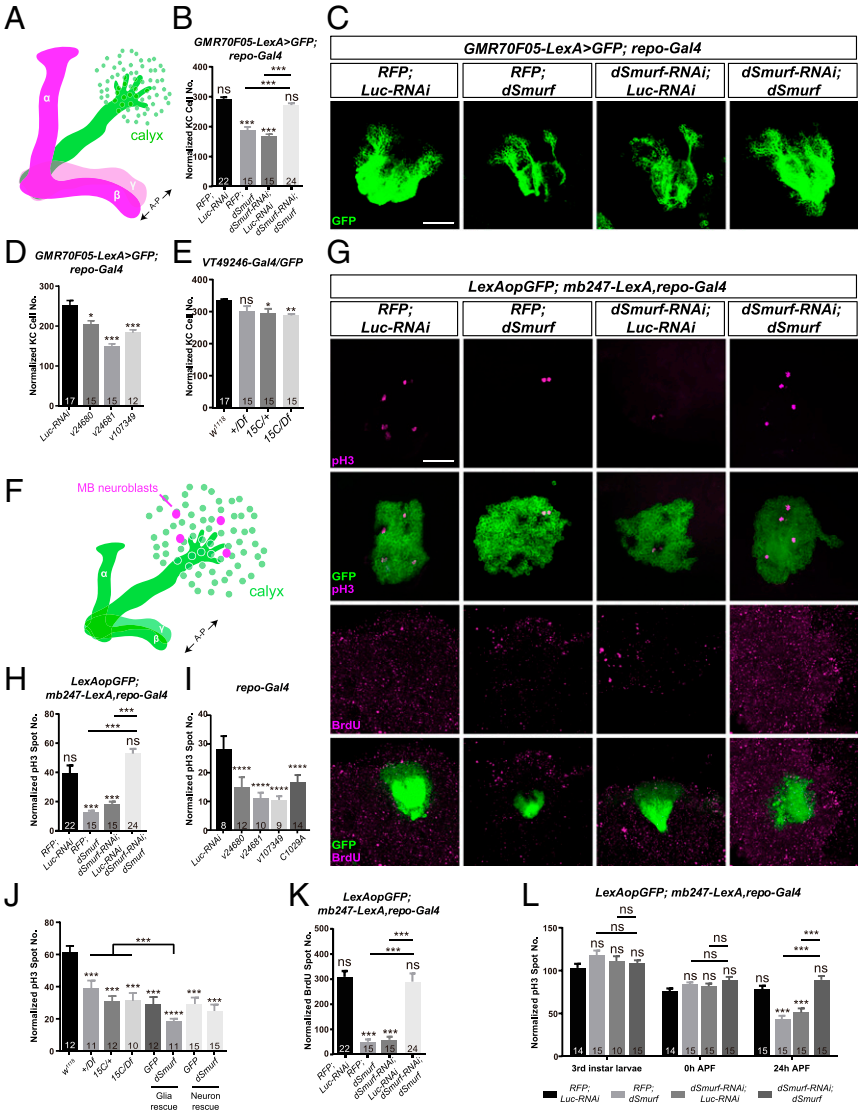

Fig. 3. Glial dSmurf regulates MB NB proliferation and α/β cell number (*A*) Illustration of hemispherical MB α/β and γ lobes (magenta) and α/β cell bodies (green). (*B* and *C*) Representative confocal images (*B*) and quantifications (*C*) of α/β cell number in adult brains carrying the genetic binary system to label α/β cell bodies (green) and manipulate glial dSmurf expression simultaneously (*GMR70F05-LexA > GFP; repo-Gal4*). Based on this system, genotypes analyzed included control (*RFP; Luc-RNAi*), overexpression (*RFP; dSmurf*), down-regulation (*dSmurf-RNAi; Luc-RNAi*), and rescue (*dSmurf-RNAi; dSmurf*). (*D* and *E*) Quantifications of α/β cell number in adult brains expressing different dSmurf RNAi lines (*D*) and mutants (*E*). Note that α/β cell number is significantly reduced in the absence of glial dSmurf. (*F*) Illustration of the four hemispherical MB neuroblasts (magenta) with respect to the location of lobes and calyx (green). (*G*) Representative confocal images of MB calyces in 48h APF brains carrying the genetic binary system to label α/β and γ cell bodies (green) and manipulate glial dSmurf expression simultaneously (*mb247-LexA > GFP, repo-Gal4*). Same genotypes were analyzed as in *C* using different binary system. Proliferative MB neuroblasts are marked by antibodies against pH3 or BrdU (magenta). Overlay images are shown. (*H–K*) The pH3- or BrdU-positive cell numbers were quantified by counting the number of pH3- or BrdU-positive spots with the indicated genotypes: RNAi (*H*, *I*, and *K*) and mutant (*J*) animals. Note that MB neuroblast proliferation is suppressed in the absence of glial dSmurf. (*L*) Quantifications of the pH3-positive cell number in three developmental stages: third instar larvae, 0h APF, and 24h APF. Note that glial *dSmurf* does not impact on MB neuroblast proliferation in earlier stage and only becomes effective at 24h APF. Average 15 brains (30 α/β lobes or posterior calyces) for each genotype were dissected and quantified. (Scale bar: 50 μm.) *P* values of significance (indicated with asterisks; ns, no significance; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above.

(*repo > ptc-RNAi)*, like dSmurf overexpression, caused a reduction in the pH3-positive cell number (Fig. 4 *B*, *C*, *E*, and *F*), whereas Ptc overexpression in glia led to an increase in the pH3-positive cell number (Fig. 4 *A* and *D*). Coexpression of Ptc and dSmurf or Ptc-RNAi and dSmurf-RNAi (or v107349) rescued the defect in MB NB proliferation caused by glial dSmurf misexpression. In line with the results on NB proliferation, the lobe diameter and the number of α/β cells were also rescued when expressing Ptc or Ptc-RNAi in the presence of dSmurf or dSmurf-RNAi (or v107349), respectively (*SI Appendix*, Fig. S5 *A–I*). These results suggest that Ptc is a substrate and acts downstream of dSmurf in mediating MB NB proliferation. Ptc protein levels analyzed by immunostaining with the anti-Ptc antibodies were dramatically reduced in the presence of dSmurf, and some of the Ptc-positive aggregates colocalized with lysosomes marked by the lysosomal-associated membrane protein 1 (Lamp1), supporting the notion that dSmurf regulates Ptc internalization and stability (*SI Appendix*, Fig. S5*J*). In addition, coexpression of Hh RNAi and dSmurf-RNAi (or v107349) rescued the NB proliferation defect caused by either RNAi (*SI Appendix*, Fig. S5 *K–N*). Taken together, these results suggest that glial dSmurf regulates MB NB proliferation, α/β cell number, and lobe formation via Hh signaling.

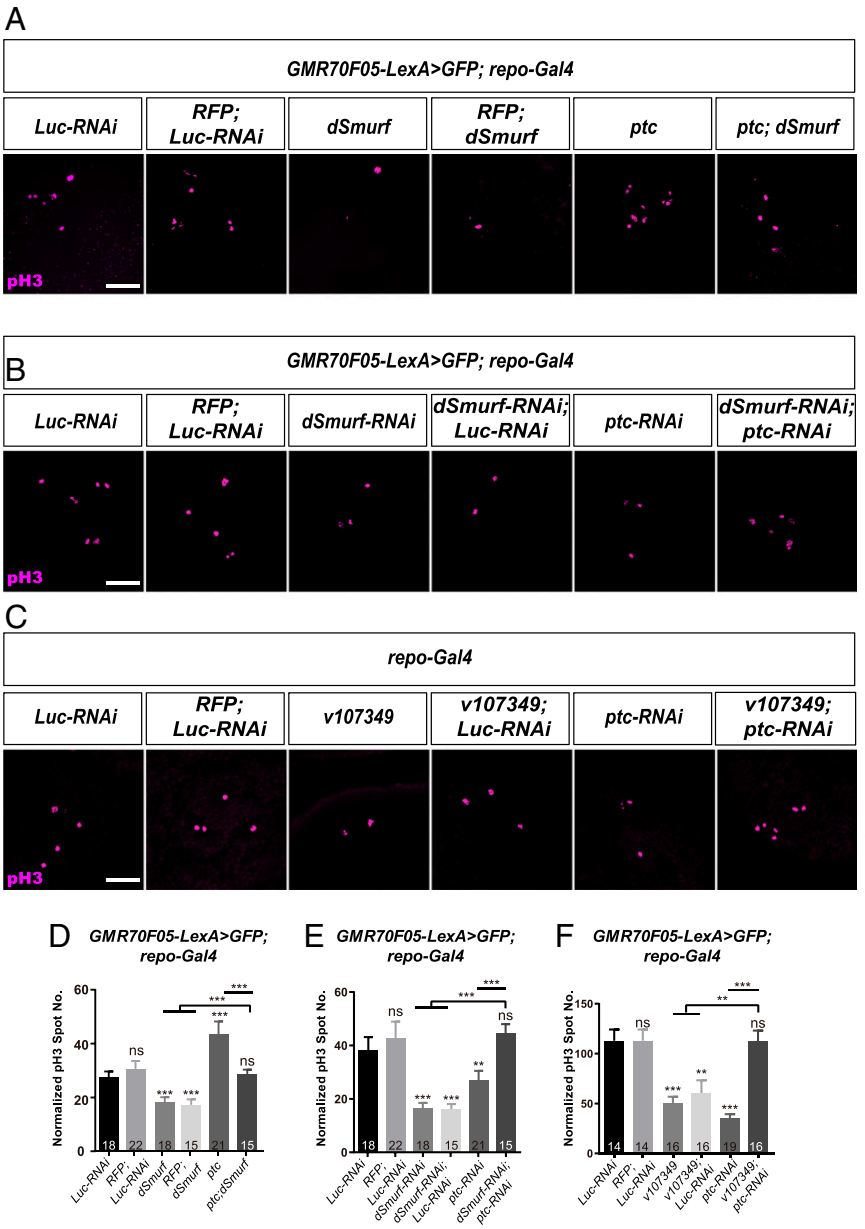

Fig. 4. Glial dSmurf regulates MB NB proliferation via Hh signaling (*A*–*F*) Representative confocal images (*A*–*C*) and quantifications (*E* and *F*) of NB proliferation in MB calyces of 48-h APF brains with altered dSmurf and Ptc expression. The same genetic binary system was used as in Fig. 3*C*. The genotypes analyzed included control (*Luc-RNAi* or *RFP; Luc-RNAi*), overexpression (*dSmurf* or *RFP; dSmurf*), down-regulation (*dSmurf-RNAi; Luc-RNAi*), *ptc*, *ptc-RNAi*, *v107349*, and genetic interaction (*ptc; dSmurf*, *dSmurf-RNAi; ptc-RNAi*, *v107349; ptc-RNAi*). Controls of one or two Upstream Activation Sequence (UAS) elements are shown for comparison to one or two UAS experimental transgenes in the same ratio. Proliferative MB NBs in the dissected brains were detected by immunostaining with α-pH3 antibodies (magenta). The pH3-positive cell numbers were quantified by counting the number of pH3-positive spots. Note that coexpression of Ptc and dSmurf or Ptc-RNAi and dSmurf-RNAi (or v108349) rescues the reduction in pH3-positive cell numbers. An average of 15 brains (30 posterior calyces) for each genotype were dissected and quantified. (Scale bar: 50 μm.) *P* values of significance (indicated with asterisks; ns, no significance; $^{**}P < 0.01$ and $^{***}P < 0.001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above.

dSmurf Activates Glial Hh Signaling. Based on our results, we hypothesized that dSmurf regulates Ptc turnover, hence the activation of glial Hh signaling. To test this hypothesis, the enhancer-trap reporter *ptc-LacZ*, a historical tool for monitoring the levels of Hh signaling (36), was used. As shown in Fig. 1*C*, GFP-positive cells from brains expressing *repo > synGFP* are Repo-positive, indicating their glial origin. In the regime here, these GFP-positive glial cells near the MB calyces (illustrated in Fig. 5*A*) were assessed via immunostainings with the anti-β-galactosidase (β-gal) antibodies, identifying cells positive for GFP and β-gal, thus allowing quantifications of Hh-signaling activity in glia. Interestingly, Smo or Ptc-RNAi expression, both representing conditions of Hh activation, caused elevated β-gal intensities in glial cells (dashed circles in magenta, Fig. 5 *A* and *D*, β-gal intensities per glial nucleus). These results indicate that changes in *ptc-LacZ* levels nicely recapitulate the activity of Hh signaling. Importantly, dSmurf overexpression caused elevated β-gal intensities in glial cells (Fig. 5 *A* and *D*), suggesting that increased dSmurf levels activate glial Hh signaling. Functionally, Hh activation by Smo or Ptc-RNAi expression in glia, like dSmurf overexpression, caused a dramatic decrease in the pH3-positive cell number, reinforcing the notion that dSmurf suppresses MB NB proliferation via glial Hh activation (Fig. 5*C*).

Glial dSmurf Activates Neuronal Hh Signaling to Suppress NB Proliferation. Given that non-cell–autonomous propagation of Hh signaling has been reported (31), we proposed that neuronal Hh signaling is activated by the dSmurf-activated glial Hh signaling, thereby suppressing MB NB proliferation. To test this hypothesis, the *ptc-LacZ* reporter was used to monitor neuronal Hh signaling in α/β cells while altering glial dSmurf expression (*GMR70F05-LexA > GFP; repo > dSmurf* or *dSmurf-RNAi)*. GFP-positive α/β cells (illustrated in Fig. 5*B*) in the MB calyces were colabeled with Elav and β-gal, identifying GFP- and β-gal–positive α/β cells for quantifications of Hh-signaling activity. Already shown to activate glial Hh signaling, Smo- or Ptc-RNAi expression in glia also caused a significant increase of β-gal intensities in α/β cells (Fig. 5 *B* and *E*, intensities in arbitrary unit per GFP- and Elav-positive α/β cell).

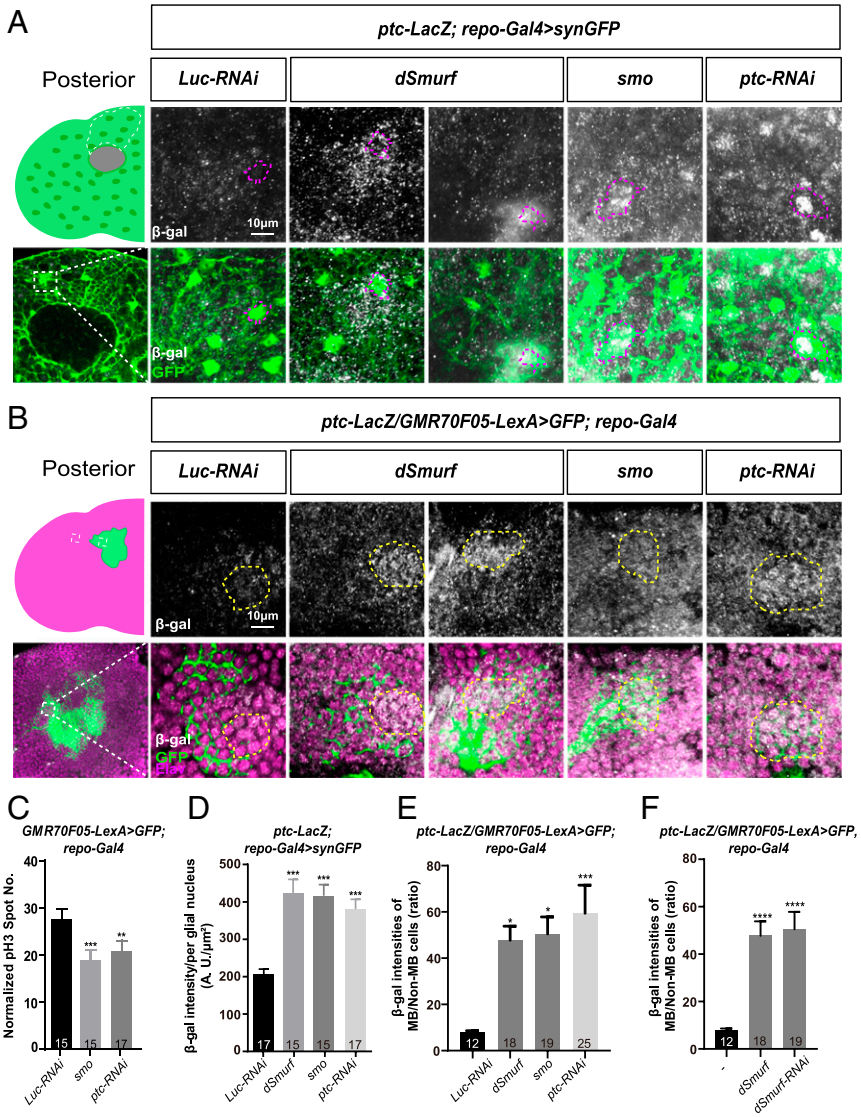

Fig. 5. Glial dSmurf activates glial and neuronal Hh signaling (*A–F*) Representative confocal images (*A* and *B*) and quantifications (*C–F*) of *ptc-LacZ* levels in adult brains with the indicated genotypes: control (*Luc-RNAi*), overexpression (*dSmurf*), Hh activation (*smo* and *ptc-RNAi*). *ptc-LacZ* expression levels in dissected brains were detected by immunostaining with antibodies against β-gal (gray). Glial nuclei and membranes adjacent to the posterior MB calyces (*A*) are marked by GFP, whereas α/β cells in the MB calyces (*B*) are marked by GFP and Elav (magenta). For clarity, single confocal section images are shown. β-gal intensities in glia or neurons were carefully quantified through each section, making sure that each cell object remained to be seen. Overlay images are shown (gray, green, and magenta). White dashed lines encircle the areas of quantifications and normalization in the schematic diagrams in *A* and *B*. Dashed circles in *A* (magenta) or *B* (yellow) enclose the respective areas of a single glial nucleus or a group of α/β-cell nuclei for quantifying β-gal intensities. Note that the average β-gal intensities in arbitrary unit per glial cell (AU/μm ^{2}) or normalized to non-MB regions (ratio, α/β cell) are significantly elevated when dSmurf, dSmurf-RNAi, Smo, or Ptc-RNAi is expressed (*D–F*). Glial Smo or Ptc-RNAi expression suppresses NB proliferation (*C*). An average of 15 brains (30 posterior MB calyces) for each genotype were dissected and quantified. Approximately 20 confocal Z-stack sections were taken with 0.4 to 0.5 μm each to thoroughly visualize β-gal–positive glia and α/β neurons. (Scale bar: 50 μm [10 μm in *A* and *B*].) *P* values of significance (indicated with asterisks; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above.

These results demonstrate the non-cell–autonomous nature of Hh signaling, as neuronal Hh signaling is also up-regulated in conditions of glial Hh activation. Similarly, dSmurf overexpression or dSmurf-RNAi expression at 48 h APF caused elevated β-gal intensities in α/β cells (Fig. 5 *B*, *E*, and *F*). Elevated β-gal intensities in α/β cells upon glial dSmurf misexpression recapitulates neuronal Hh activation, as Smo- or Ptc-RNAi expression in α/β cells (*C739* > *smo* or *ptc-RNAi*) caused a similar increase (*SI Appendix*, Fig. S6 *A* and *B*) and suppressed MB NB proliferation (*SI Appendix*, Fig. S6*C*). Taken together, these results suggest that dSmurf activates non-cell–autonomous Hh-signaling propagation from the glia to the neurons. Neuronal Hh activation then suppresses MB NB proliferation.

It has been shown that the transcription factor foxo (forkhead box class O) exhibits nuclear localization upon the exit of MB NB proliferation in response to a decrease in insulin/PI3K signaling (37). Interestingly, Hh activation upon Smo overexpression in α/β cells caused nuclear localization of foxo-GFP (*SI Appendix*, Fig. S6*D*). Furthermore, MB NBs upon glial dSmurf expression were colabeled with the NB markers Deadpan (Dpn) and Miranda (Mira) (38–40). Interestingly, fewer Dpn-positive MB NBs were detected with shrinkage in size marked by Mira staining, indicating that some MB NBs die early and that their fates are prematurely terminated (*SI Appendix*, Fig. S6 *E–G*). Taken together, these results further demonstrate that glial dSmurf triggers paracrine Hh-signaling propagation and that neuronal Hh activation causes precocious termination of MB NB proliferation.

Glial FasII Rescues dSmurf-Mediated Lobe Defects Independently of NB Proliferation. Whereas a reduction in NB proliferation and α/β cell number is consistently detected upon altering glial dSmurf expression, the α/β-lobe formation is not as sensitive to the manipulated changes, implying that other glial dSmurf-mediated mechanisms might exist to complement Hh signaling in regulating α/β-lobe formation. Based on our observations, α/β lobes with altered glial dSmurf expression consistently exhibited higher levels of FasII staining (Fig. 1). Furthermore, flip-out clonal analysis of α/β neurons revealed abnormal axons when glial dSmurf was misexpressed (Fig. 6*A*). These results prompted us to investigate the possibility that FasII, a cell-adhesion molecule involved in axon fasciculation (33, 41), participates in dSmurf-mediated MB α/β-lobe formation. Different FasII isoforms have been predicted, including the neuronal FasII with or without the intracellular PEST motif (FasIIA and FasIIA-PEST) and the glial GPI-anchored FasII (FasIIB), a variant lacking the intracellular domain (Fig. 6*B*).

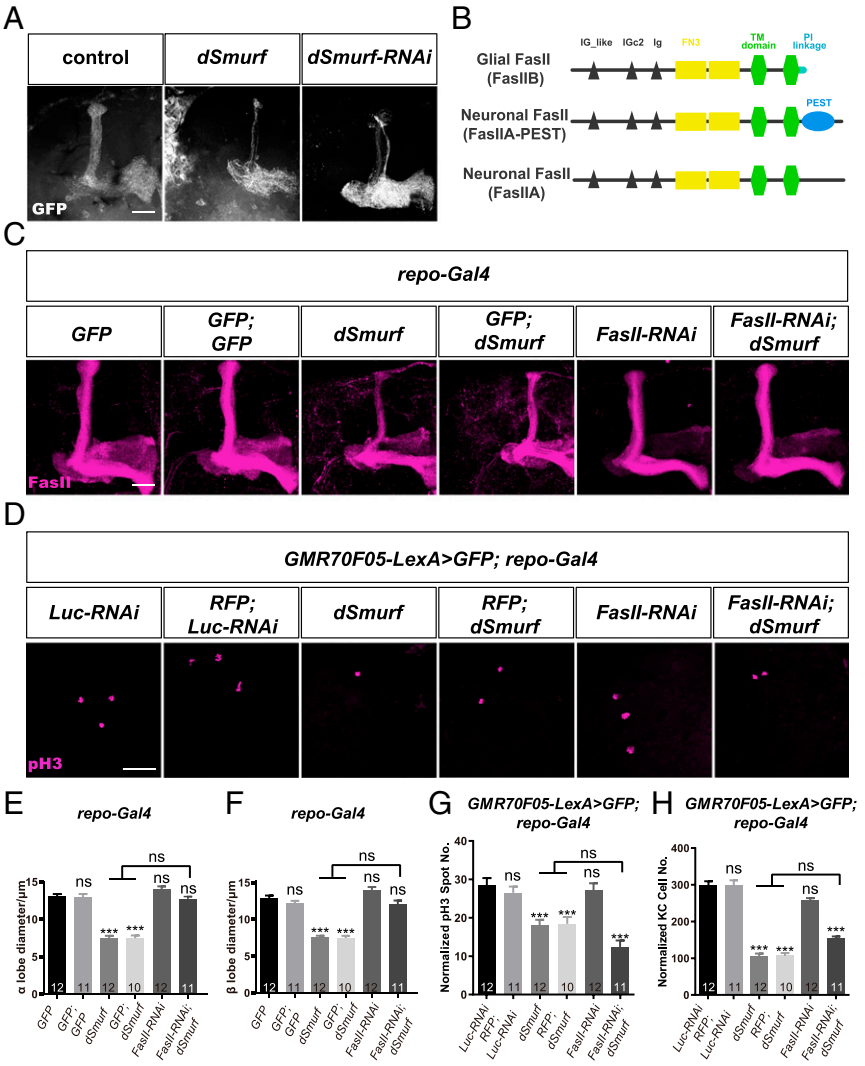

Fig. 6. Glial FasII rescues dSmurf-mediated lobe defects independently of NB proliferation. (*A*) Flip-out MB neuron clones of adult brains were induced by heat shock in the presence or absence of glial dSmurf. Note the disruptive and defasciculated α axons when misexpressing glial dSmurf (intensities are adjusted so that axons can be clearly seen). (*B*) Illustration of predicted FasII isoforms including the glial FasII (FasIIB) lacking the intracellular domain and neuronal FasII (FasIIA and FasIIA-PEST) with or without the intracellular PEST motif (blue oval). (*C–H*) Representative confocal images (*C* and *D*) and quantifications (*E–H*) of α/β lobes and MB NB proliferation in adult (*C*, *E*, and *F*) and 48-h APF (*D*, *G*, and *H*) brains expressing different combinations of dSmurf and FasII. The same binary system as in Fig. 3*C* was used for *D*, *G*, and *H*. Controls of one or two UAS elements are shown for comparison to one or two UAS experimental transgenes in the same ratio. Genotypes are detailed in *SI Appendix*, Table S1. Brains were dissected and immunostained with α-FasII (*C*, magenta) or α-pH3 (*D*, magenta). Both anterior α/β and γ lobes (*C*, adult brains) and posterior pH3-positive cells (*D*, 48-h APF brains) are shown. The α/β-lobe diameter in the middle of the lobe (*E* and *F*), the pH3-positive cell number (*G*), and the α/β cell number (*H*) were quantified. Note that knock-down of glial FasII rescues the dSmurf-induced defective α/β-lobe diameter but not the reduction in pH3-positive-cell number, nor the α/β cell number. An average of 10 brains (20 α/β lobes or posterior MB calyces) for each genotype were dissected and quantified. (Scale bar: 50 μm.) *P* values of significance (indicated with asterisks; ns, no significance; ***$P < 0.001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test among three groups or above.

To investigate whether glial FasII is involved in dSmurf-mediated α/β-lobe formation, we first took advantage of an RNAi targeting all three forms of FasII (*FasII-RNAi*) to selectively down-regulate glial FasII expression. Whereas FasII-RNAi expression alone did not induce any change in α/β lobes, reducing glial FasII expression rescued the dSmurf-induced α/β-lobe defects (Fig. 6 *C*, *E*, and *F*). Surprisingly, coexpressing FasII-RNAi did not rescue the dSmurf-mediated reduction in MB NB proliferation (Fig. 6 *D* and *G*) nor the α/β cell number (Fig. 6*H*). FasII-RNAi expression alone did not affect NB proliferation nor the α/β cell number. These results suggest that glial FasII functions downstream of dSmurf in regulating α/β-lobe formation, a process independent of MB NB proliferation. Given that a decrease in glial FasII expression rescues the dSmurf-mediated α/β-lobe defects, we propose that dSmurf stabilizes glial FasII expression on the membrane.

dSmurf Stabilizes Glial FasII. To further elucidate the mechanism of glial FasII in dSmurf-mediated α/β-lobe formation, we tested whether dSmurf forms a complex with glial FasII. Plasmids expressing glial FasII engineered with a Myc epitope in six tandem repeats (6×Myc-FasIIB) and dSmurf engineered with a Flag epitope in three tandem repeats (3×Flag-dSmurf) were constructed and expressed in S2 cells. Coimmunoprecipitation (Co-IP) analysis revealed that dSmurf was pulled down together with FasIIB in the eluate, indicating that both proteins are in the same complex (Fig. 7*A*). To determine which dSmurf region is important for complexing with FasIIB, plasmids of dSmurf lacking different domains (dSmurfΔC2, dSmurfΔWW, and dSmurfΔHECT) were constructed and coexpressed with FasIIB in S2 cells. Interestingly, while low levels of dSmurfΔHECT expression hindered further analysis, expression levels of dSmurfΔC2 and dSmurfΔWW were relatively higher, enabling the determination of protein-complex formation (Fig. 7*C*). Co-IP analysis revealed that dSmurf or dSmurfΔC2 were in the same eluate with FasIIB but not dSmurfΔWW, suggesting that WW domains are required for dSmurf regulation on glial FasII.

To further support our in vivo results that showed that dSmurf stabilizes glial FasII expression, FasIIB stability was analyzed in the presence of different dSmurf variants in S2 cells treated with cycloheximide at various time points. Despite the fact that it is hard to predict the half-life of FasIIB examined over the course of 8 h, the protein was apparently stabilized in the presence of dSmurf yet further destabilized by dSmurfΔWW (Fig. 7 *D* and *E*). Furthermore, FasIIB ubiquitination was significantly reduced in the presence of dSmurf (*SI Appendix*, Fig. S7 *A* and *B*). These results indicate that, unlike a typical substrate, glial FasII is stabilized by dSmurf on the membrane, and the dSmurf WW domain is critical for glial FasII stability; glial FasII expression on the cell surface potentially regulates glia–MB axon association, thus affecting axon fasciculation.

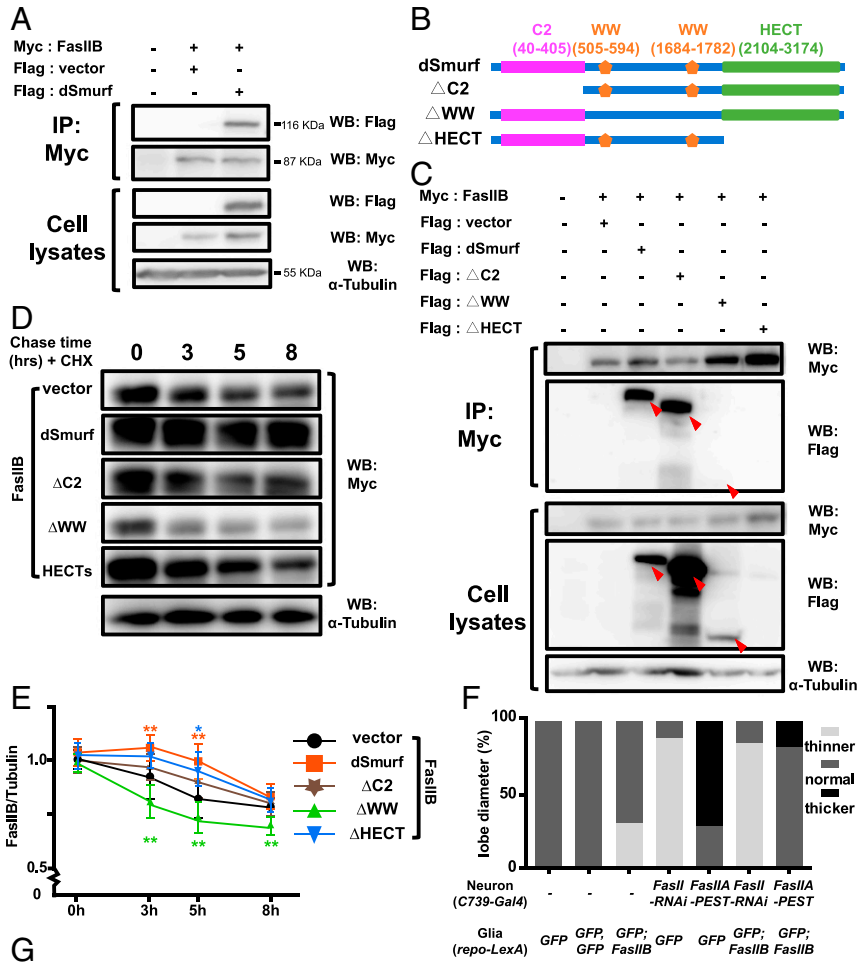

Fig. 7. Homophilic FasII interactions between glia and MB axons regulate axon integrity (*A*) Representative Western blot images for dSmurf-FasIIB complex formations. Note that dSmurf proteins are in the same eluates with FasIIB, indicating that both proteins are in the same complex. (*B*) Schematic diagram of dSmurf protein variants with deletions in different domains. (*C*) Truncated dSmurf proteins (red arrowheads) are in the same complex with FasIIB except dSmurfΔWW, indicating that WW domains are required for the complex formation. (*D* and *E*) Representative Western blot images (*D*) and quantifications (*E*) of pulse-chase analysis on FasIIB stability. Note that FasIIB is significantly stabilized in the presence of dSmurf but not dSmurfΔWW. (*F* and *G*) Representative confocal images (*G*) and quantifications (*F*) of α/β lobes in adult brains carrying the binary system expressing FasIIB in glia and FasIIA-PEST in α/β neurons. FasII-RNAi is used as a control for comparative expression in α/β neurons. Controls of one or two UAS elements are shown for comparison to one or two UAS experimental transgenes in the same ratio. Genotypes are detailed in *SI Appendix*, Table S1. More than 30 brains were analyzed for each genotype except for FasII-RNAi (17 brains). For quantifications, α/β lobes are categorized as thinner, normal, and thicker groups. Note that coexpression of FasIIA-PEST, but not FasII-RNAi, and FasIIB in neurons and glia, respectively, results in a higher percentage of normal lobe morphology in brains analyzed. (Scale bar: 50 μm.)

Homophilic FasII Interaction Regulates α/β-Lobe Integrity. Due to the adhesive nature of FasII, it is conceivable that glial FasII interacts with neuronal FasII via homophilic interaction, thus regulating α/β-lobe integrity and axon fasciculation. Interestingly, increased glial FasII expression (*repo > 13xLexAop2-FasIIB*), as verified by both immunostaining and Western blot analysis in *SI Appendix*, Fig. S6 *C* and *D*, induced lobe deficits similarly to glial dSmurf overexpression but with a lower penetrance due to the efficiency of the LexA system (*SI Appendix*, Fig. S6 *E* and *F*, 30% exhibiting thinner lobes). On the other hand, expressing the neuronal FasII in α/β neurons (*C739>FasIIA-PEST*) also caused significant distortion of α/β lobes (*SI Appendix*, Fig. S7 *G* and *H*, 72% exhibiting thicker lobes). These results are consistent with previous findings and indicate that neuronal FasII plays a crucial role in regulating α/β-lobe formation (42). Like glial FasII, neuronal FasII-mediated α/β-lobe formation is independent of MB NB proliferation and does not affect the KC number (*SI Appendix*, Fig. S7 *I* and *J*).

To further test whether homophilic FasII interaction mediates α/β-lobe formation, the binary system that manipulates gene expression in both α/β neurons and glia was used (*C739-Gal4; repo-LexA > 13xLexAop-FasIIB*). Strikingly, whereas expressing either glial FasII or neuronal FasII induced severe lobe defects, coexpression of both FasII isoforms rescued the α/β-lobe defects, and more than 73% of the lobes examined were normal (Fig. 7 *F* and *G*). As a control, coexpression of FasII-RNAi (in α/β cells) and glial FasII only slightly rescued the lobe defects, with 17% of the lobes examined being normal (Fig. 7 *F* and *G*). These results indicate that homophilic FasII interaction between glia and α/β neurons mediates α/β-lobe formation. An increased distribution of FasII on the glial membrane (as stabilized by dSmurf) causes α/β-lobe defects, possibly via increased homophilic FasII interaction with MB axons, hence the decrease in homophilic FasII interaction among MB axons and subsequent dysregulation in axon fasciculation.

Glial dSmurf Misexpression Causes MB-Associated Behavior Deficits. Previous reports have implied a role for MBs in locomotion, learning and memory, and anti-seizure paralysis (43–45). In particular, MB α/β lobes are critical for long-term memory formation (46) and seizure susceptibility in a *Drosophila* model of human epilepsy (47). To further investigate whether glial dSmurf-mediated neuronal diversity links to any functional consequences, three behavioral

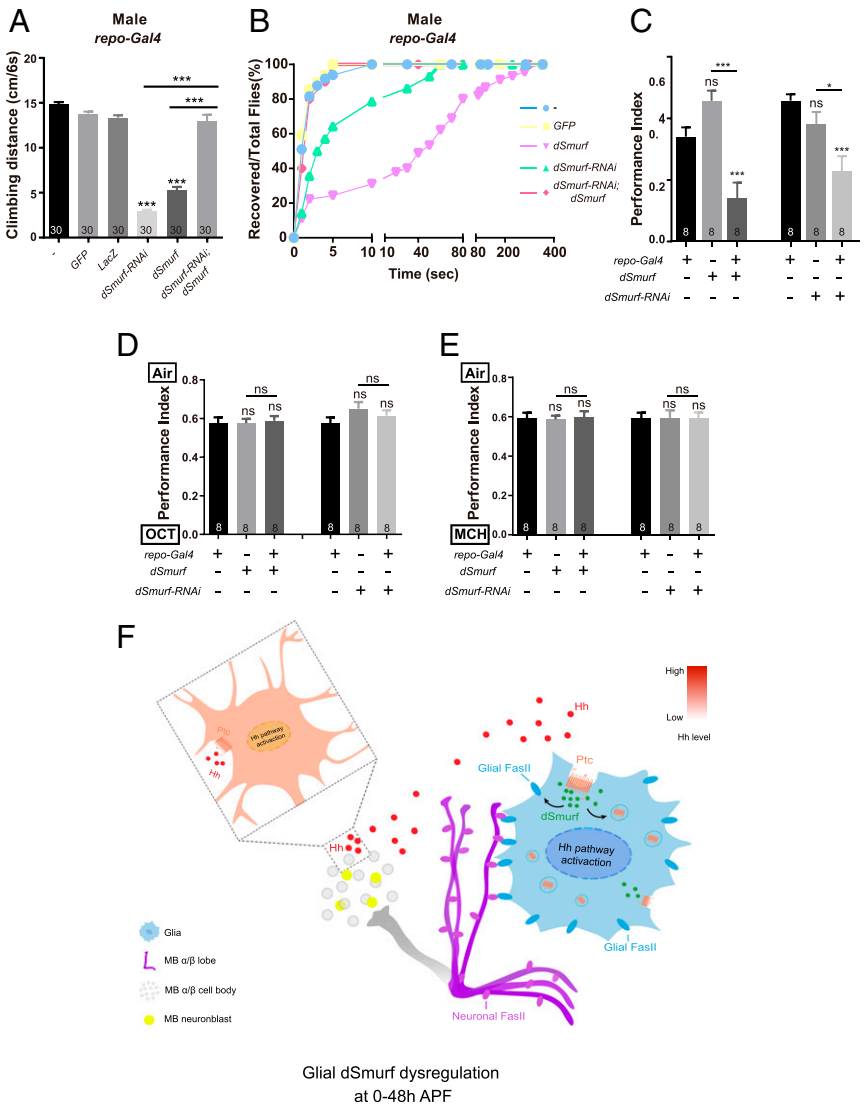

Fig. 8. Glia confer temporal signals in regulating MB neuronal diversity and function (*A*–*C*) Glial dSmurf misexpression causes climbing deficits (*A*), anti-seizure paralysis (*B*), and impairs 24-h sugar memory (*C*). *P* values of significance (indicated with asterisks; ns, no significance; $*P < 0.05$; and $***P < 0.001$) are calculated by one-way ANOVA with Bonferroni multiple comparison test ($n = 10$, at least three independent replicates, *A* and *B*) and one-way ANOVA with Tukey's test ($n = 8$, *C*). (*D* and *E*) Odor preference is not affected by glial dSmurf expression in the training for 24-h sugar memory. (*F*) Upon dSmurf dysregulation in the pupal stage, glial Hh signaling is activated due to an enhanced rate of Ptc internalization and turnover, leading to the lack of their distribution on the glial membrane for sensing and activating Hh signaling. Activated glial Hh signaling propagates non-cell–autonomously via reallocating extracellular Hh ligands to interact with adjacent neuronal Ptc receptors. Activated neuronal Hh signaling suppresses MB NB proliferation, leading to a decrease in the α/β cell number and aberrant lobe formation. Moreover, glial dSmurf stabilizes FasII expression on the membrane, which mediates homophilic interaction with neuronal FasII, hence regulating the interaction between the glial membrane and MB axons. Both routes lead to selective changes in the α/β-lobe diameter and cell number.

paradigms including locomotor activity, anti-seizure paralysis, and learning and memory were tested. Our results indicated that adult flies misexpressing dSmurf exhibited severe locomotor and seizure-like behavioral deficits (Fig. 8 *A* and *B*). In addition, long-term memory was also impaired under a 24-h sugar memory protocol (Fig. 8*C*), while odor preference remained unaffected (Fig. 8 *D* and *E*). These results suggest a strong correlation between glial dSmurf-mediated neuronal diversity and MB function and underscore the physiological significance of extrinsic glia-to-neuron communication in neuronal function.

Discussion

Understanding how neuronal diversity is achieved in the brain helps understand how the neuronal network interacts and orchestrates behavior. In the present study, we identify a two-step glia-derived temporal signal utilizing a ubiquitin enzyme to regulate MB neurogenesis Different from ligand secretion or phagocytosis, perturbation of glial protein homeostasis by the ubiquitin enzyme not only evokes non-cell–autonomous Hh-signaling propagation to suppress MB NB proliferation but also modulates glia–MB axon adhesion. Both processes contribute to a precise output of α/β neurons. Together, these mechanistic insights shed light on how glia crosstalk with neurons and define a form of glial signaling in regulating neurogenesis and neuronal function (Fig. 8*F*).

Glial dSmurf Is a Temporal Signal Regulating MB Neurogenesis. Glial dSmurf controls MB neurogenesis by selectively targeting NB proliferation in the pupal stage for a precise output of α/β neurons; its misexpression causes suppressed NB proliferation leading to altered α/β-lobe diameter and cell number, while α'/β'- and γ-neuron development remain unaffected. Given that MB NBs proliferate to generate three types of KCs in a sequential manner, our results suggest that this glial signal is specific, temporal dependent, and does not impact the sequential differentiation of different cell types. Interestingly, dSmurf expression drops to the lowest level at the end of the larval stage and resurges when the pupal stage begins (*SI Appendix*, Fig. S9). In addition, a physiological surge in the dSmurf level occurs at the beginning of the pupal stage when MB NBs begin to generate α/β neurons (modENCODE development RNA-seq, Flybase), suggesting that dSmurf activity is under precise regulation at this time window to modulate NB proliferation and the α/β cell number. Too little or too much dSmurf, as exemplified by dSmurf-RNAi or dSmurf expression in this specific time window, would drastically impact MB NB proliferation, leading to a premature stop of α/β-neuron differentiation. Our findings indicate that glia act as goalkeepers during normal physiological conditions and control the exit of NB proliferation to regulate MB neuronal diversity.

Glial cells have been shown to provide crucial extrinsic cues for NSC proliferation in different contexts (48). For instance, extracellular

factors such as Dally-like, a heparan sulfate proteoglycan, and Glass bottom boat, a BMP homolog, are important for regulation of NB proliferation by *Drosophila* surface glia. On the other hand, Pvr receptor tyrosine kinase signaling activates DE-cadherin in cortex glia to maintain the niche for NB proliferation (7, 8). Furthermore, *Drosophila* glia secrete the glycoprotein anachronism (49) or the insulin/insulin-like peptide Dilp6 (in response to nutrition signals) to control the exit of NBs from quiescence into the larval proliferative phase. This glial control of postembryonic neurogenesis does not impact MB NBs—the few that continue to proliferate into the larval stage. Our findings identify a second phase of glial signaling controlling MB NB proliferation. This glial signal is potentially independent of the metabolic ecdysone signaling, as glial dSmurf misexpression does not affect EcRB1 levels (*SI Appendix*, Fig. S3). Unlike in the first phase, in which glia contribute to earlier MB γ-neuron remodeling via a secretory mechanism, glia signal through an internal ubiquitination enzyme and control substrate availability for their diverse function, further contributing to the temporal patterning of MB neurogenesis.

Hh-Signaling Propagation from Glia to Neurons. Similar to dSmurf-mediated Ptc degradation in other developmental contexts, our genetic evidence supports a model in which dSmurf regulates Ptc distribution on the glial membrane. Ptc overexpression rescues dSmurf-induced lobe defects, the reduced cell number, and NB proliferation. Vice versa, Ptc down-regulation also rescues the defects caused by dSmurf-RNAi. Importantly, Ptc protein levels are dramatically reduced, and Ptc-positive aggregates are detected colocalizing with Lamp1-positive lysosomes in the presence of dSmurf. These findings support the model that, upon internalization, a lack of Ptc receptors on the glial membrane leads to Smo-mediated Hh activation in glia as well as nearby MB neurons, as more Hh ligands are now available for binding to the neuronal Ptc receptors. Neuronal Hh activation then suppresses MB NB proliferation, hence the reduced cell number and aberrant lobe formation (Fig. 8*F*). Interestingly, Smo also is a dSmurf substrate in wing-disk development (29). Our genetic interaction analysis suggests that Smo also rescues dSmurf-induced defects in MB NB proliferation and lobe formation (*SI Appendix*, Fig. S8). Due to the opposite roles of Ptc and Smo in Hh signaling, it is possible that down-regulating dSmurf expression also causes Smo stabilization on the membrane, which counteracts the effect of dSmurf-mediated Ptc stabilization on Smo inhibition, leading to similar effects when down- or up-regulating dSmurf.

Hh signaling has been shown to play pivotal roles in glial diversity and MB γ-neuron remodeling (50–52). Our results reveal an additional role for Hh signaling on MB NB proliferation. As excess Hh signaling causes premature NB cell cycle exit and hypoproliferation in *Drosophila* postembryonic neuroblasts (53), it is likely that glial dSmurf triggers the non-cell–autonomous Hh signaling, leading to up-regulated neuronal Hh signaling that suppresses proliferation and promotes MB NB cell cycle exit. Interestingly, Hh has been shown to act downstream of Castor (Cas) and upstream of Grainyhead (Grh), both tTFs at the end of the temporal series required for postembryonic NB cell cycle exit (53). Furthermore, the late-acting temporal factor, E93, integrates extrinsic hormonal cues to the intrinsic Imp and Syp programs for terminating MB neurogenesis (19). Although the mechanism remains unclear at this point, it is intriguing to speculate that MB intrinsic factors play a role in integrating the glial dSmurf–Hh signal to regulate MB NB proliferation and cell-cycle exit. Our results suggest that silencing the expression of Cas or Syp partially rescues the Hh-mediated suppression of MB NB proliferation and premature cell-cycle exit, whereas Grh, Imp, and E93 do not seem to play an important role in the process (*SI Appendix*, Figs. S10–S12). In addition, glial dSmurf-mediated Hh activation regulates MB neurogenesis independently from the other extrinsic activin signaling, as silencing the expression of the ligand Myo in glia only partially rescues the dSmurf-mediated suppression on MB NB proliferation and NB size but not NB number (*SI Appendix*, Fig. S13). On the other hand, silencing the expression of the activin receptor Baboon in neurons does not rescue the Hh-mediated suppression on MB NB proliferation (*SI Appendix*, Fig. S14). Taken together, our findings demonstrate a route of extrinsic glial signaling evoked by protein homeostasis, which acts as the source of neuronal Hh signaling to regulate neurogenesis, possibly via interacting with the intrinsic tTFs.

Homophilic FasII Interaction between Glia and MB Axons. In addition to regulation of MB NB proliferation, glial dSmurf also confers regulatory signals on MB axon fasciculation. We identify the glial GPI-anchored cell-adhesion molecule FasII as a downstream dSmurf player. Independent of the ubiquitination mechanism, dSmurf stabilizes glial FasII via its WW domains. Not only does glial FasII overexpression induce similar lobe defects as dSmurf overexpression, reducing its expression rescues the dSmurf-induced lobe defects. Interestingly, α/β-lobe integrity is rescued when simultaneously expressing neuronal and glial FasII, indicating that a proportional increase in neuronal FasII expression restores proper homophilic interaction between MB axons, hence rescuing the lobe integrity. It is conceivable that dSmurf stabilizes glial FasII on the membrane, resulting in increased interaction of the neuronal FasII to regulate adhesion between glial membranes and MB axons, thus modulating the dynamics of fasciculation among axon bundles. These findings provide another layer of complexity in the glial mechanism regulating MB neurogenesis via cell–cell adhesion; the glial mechanism underlying cell adhesion and axon guidance has been reported (54, 55).

Membrane Proteins As E3 Ligase Targets. Our findings identify a distinct route of glia-to-neuron communication utilizing an E3 ligase to modulate membrane protein distribution and availability. E3 ligases have been shown to regulate NB growth and MB development via intrinsic mechanisms (56, 57). Here, we identify an E3 ligase dSmurf acting extrinsically from glial cells. By modulating the distribution of two different membrane proteins, Ptc and FasII, dSmurf relays extrinsic signals to regulate MB neuronal diversity via two means: a ubiquitination-dependent mechanism in modulating Ptc turnover for Hh-mediated suppression on NB proliferation and a stabilizing paradigm for FasII-dependent axon integrity. Our findings expand the pool of dSmurf targets and identify mechanisms in dSmurf-mediated signaling: how dSmurf stabilizes a downstream target instead of mediating its degradation. Thus, diversifying actions downstream from these targets execute layers of regulation in a coordinated fashion to regulate MB neuron identity and progeny diversity. Future investigation will be needed to elucidate whether different types of glia-derived signals correlate with certain aspects of neuronal function and how these signals integrate with intrinsic mechanisms to regulate neurogenesis underlying circuits and behavior.

Materials and Methods

All animal experiments were conducted according to proper regulation and guideline under ShanghaiTech. Details of all fly strains and experimental crosses used are included in *SI Appendix*, Table S1. Methods including molecular cloning, immunohistochemistry, confocal microscopy and statistical analysis, and behavioral analysis are described in the *SI Appendix*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank the Bloomington Stock Center, Tsinghua Fly Center, Vienna Drosophila Resource Center, the Core Facility of *Drosophila* Resource and Technology, the Shanghai Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences, Developmental Studies Hybridoma Bank, Dahua Chen, Jin Jiang, Yun Zhao, Haiwei Pi, Yuu Kimata, Laixin Xia, Xiaohang Yang, Wanzhong Ge, Yongmei Xi, Yi Lu, and Yanshan Fang for providing fly stocks and antibodies. We also thank the Molecular Imaging Core Facility, the

Molecular and Cell Biology Core Facility, and the Multi-Omics Core Facility at the School of Life Science and Technology, ShanghaiTech University for providing technical support. We thank Tzumin Lee, Cheng-Ting Chien, Hongyan Wang, Yun Zhao, Bing Ye, Rob Jackson, and the Ho laboratory members for their discussion and comments. This work was supported by grants from ShanghaiTech and National Natural Science Foundation of China (31871039).

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