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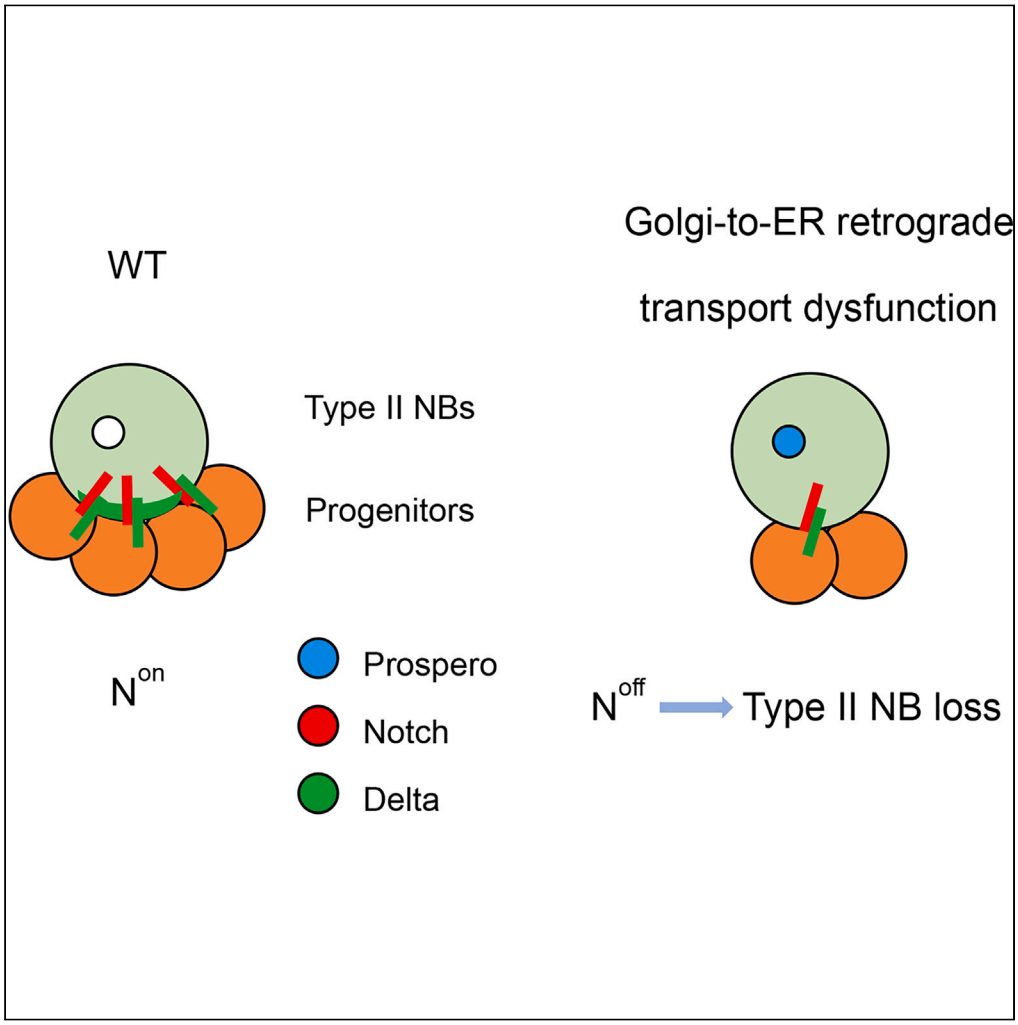

Huanhuan Zhang, Menglong Rui, Zhixin Ma, ..., Congfeng Gan, Wenting Gong, Su Wang

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Highlights

Blocking RT specifically affects type II NBs by reducing Notch activity

INPs provide Delta to NBs as niche

The Delta generated by type II NBs was asymmetrically distributed into INPs

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Golgi-to-ER retrograde transport prevents premature differentiation of *Drosophila* type II neuroblasts via Notch-signal-sending daughter cells

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SUMMARY

Stem cells are heterogeneous to generate diverse differentiated cell types required for organogenesis; however, the underlying mechanisms that differently maintain these heterogeneous stem cells are not well understood. In this study, we identify that Golgi-to-endoplasmic reticulum (ER) retrograde transport specifically maintains type II neuroblasts (NBs) through the Notch signaling. We reveal that intermediate neural progenitors (INPs), immediate daughter cells of type II NBs, provide Delta and function as the NB niche. The Delta used by INPs is mainly produced by NBs and asymmetrically distributed to INPs. Blocking retrograde transport leads to a decrease in INP number, which reduces Notch activity and results in the premature differentiation of type II NBs. Furthermore, the reduction of Delta could suppress tumor formation caused by type II NBs. Our results highlight the crosstalk between Golgi-to-ER retrograde transport, Notch signaling, stem cell niche, and fusion as an essential step in maintaining the self-renewal of type II NB lineage.

INTRODUCTION

During neurogenesis, neural stem cells are heterogeneous and regulated by distinct mechanisms. *Drosophila* larval neural stem cells, called neuroblasts (NBs), can be classified into two types based on their different cell identities, developmental potential, and proliferative capacity. Type I NBs give rise to ganglion mother cells (GMCs) through asymmetric divisions, and each GMC divides once more to generate two differentiated neurons and/or glial cells. In contrast, type II NBs produce intermediate neural progenitors (INPs) that divide asymmetrically to generate GMCs and further neural cells through terminal divisions.[1–3] Type II NBs lack the Asense (Ase) and Prospero (Pros) expression[4] but express the pointedP1 (PntP1 or pnt), which is required for specifying the identity of type II NBs.[5,6] In addition, two distinct NB lineages show different responsiveness toward Notch and Hippo signaling pathways. Upregulation of Notch activity induced by notch intracellular domain (NICD) overexpression or *numb* knockdown leads to the over-proliferation of type II NBs but only a lower overgrowth phenotype in type I NBs.[7,8] Inhibiting Hippo pathway by knocking down *Tao-1*, *Hippo*, or *Wts* significantly increases type I NB clone volume, while the type II NB clone volume remains unchanged under the same condition.[9] To gain a better understanding of the CNS in *Drosophila*, further research into the mechanisms that differentiate between type I and II NBs is needed.

The Notch signaling pathway is essential for maintaining the self-renewal of many types of stem cells from *Drosophila* to mammals. In mammals, the disruption of Dll1/Notch2 signaling pathway results in a decrease in the number of satellite cells, accompanied by the transfer of myogenic cells to myofibers.[10] In *Drosophila* CNS, loss of Notch or its downstream components, such as Spdo or Aph-1, abolishes pnt expression and activates ectopic Erm expression, consequently leading to premature loss of type II NBs.[11–14] In *Drosophila*, there are two Notch ligands: Delta and Serrate.[15] Although the mechanism of how Notch maintains stem cell identity is well studied, it is still unknown which ligand is required for the activation of Notch in type II NBs, and, more importantly, which cells express and provide such ligand.

Various behaviors of stem cells are regulated by cell intrinsic programs, as well as extrinsic cues that are provided by niche cells adjacent to the stem cell. Increasing evidence suggests that the niche is crucial for the behavior and function of neural stem cells/progenitors. In mammalian CNS, the vascular endothelial cells and astrocytes function as a microenvironment, secreting epidermal growth factor (EGF), Noggin, or Ephs that is required for neural stem cell (NSC) proliferation and self-renewal.[16] In *Drosophila* brain, the glial cells are regarded as the NB niche, secreting insulin-like peptides to promote NB reactivation and expressing extracellular factors required for NB proliferation.[17–26] In addition to glial cells, the newly generated daughter cells are also adjacent to NBs; it is tempting to determine whether these daughter cells are part of the NB niche. For type I NBs, the division orientation has been demonstrated to rely on the new-born GMCs, as an extrinsic cue, to

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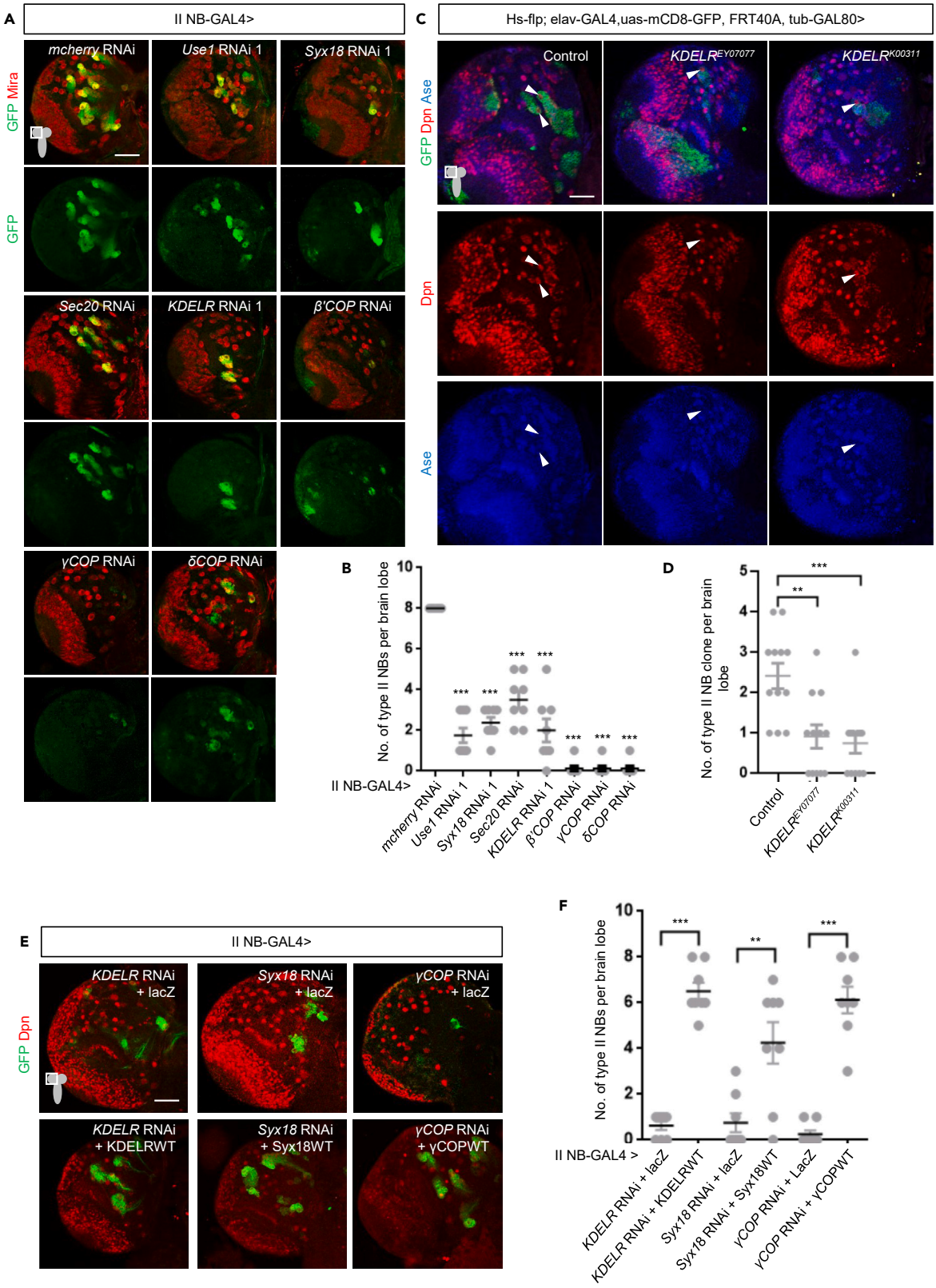
A
II NB-GAL4>
GFP Mira
GFP
mcherry RNAi
Use1 RNAi 1
Syx18 RNAi 1
Sec20 RNAi
KDELR RNAi 1
β'COP RNAi
γCOP RNAi
δCOP RNAi
C
Hs-flp; elav-GAL4,uas-mCD8-GFP, FRT40A, tub-GAL80>
GFP Dpn Ase
Dpn
Ase
Control
KDELR EY07077
KDELR K00311
B
No. of type II NBs per brain lobe
II NB-GAL4>
mcherry RNAi
Use1 RNAi 1
Syx18 RNAi 1
Sec20 RNAi
KDELR RNAi 1
β'COP RNAi
γCOP RNAi
δCOP RNAi
D
No. of type II NB clone per brain lobe
Control
KDELR EY07077
KDELR K00311
E
II NB-GAL4>
GFP Dpn
KDELR RNAi + lacZ
Syx18 RNAi + lacZ
γCOP RNAi + lacZ
KDELR RNAi + KDELRWT
Syx18 RNAi + Syx18WT
γCOP RNAi + γCOPWT
F
No. of type II NBs per brain lobe
II NB-GAL4 >
KDELR RNAi + lacZ
KDELR RNAi + KDELRWT
Syx18 RNAi + lacZ
Syx18 RNAi + Syx18WT
γCOP RNAi + LacZ
γCOP RNAi + γCOPWT

Figure 1. Golgi-to-ER RT is required for maintaining type II NB number

(A) Knocking down RT genes leads to type II NB loss. Third-instar larval brains expressing the indicated RNAi transgenes were driven by II NB-GAL4. Type II NB lineages were stained with GFP and Mira. Scale bars: 20 μm.

(B) Quantification of type II NB number in per brain lobe from genotypes in (A). N = 8. Mean ± SEM, ***p < 0.001.

(C) The phenotype of type II NB MARCM clones in *KDELR*^{EY07077} and *KDELR*^{K00311} mutants. Type II NB lineages were marked by GFP, Dpn, and Ase. White arrowheads: type II NBs. Scale bars: 20 μm.

(D) Quantification of type II NB MARCM clone number in per brain lobe from genotypes in (C). N = 12. Mean ± SEM, **p < 0.01; ***p < 0.001.

(E) Overexpression of wild-type KDELR, Syx18, and γCOP partially rescued type II NB loss. Type II NB lineages were labeled by GFP and Dpn. Scale bars: 20 μm.

(F) Quantification of type II NB number in per brain lobe from genotypes in (E). N = 8. Mean ± SEM, **p < 0.01; ***p < 0.001. p values were calculated using a one-way ANOVA compared to mCherry or control groups. Unpaired Student's t tests were used for comparisons between two groups. See also Figures S1 and S2.

stably maintain the axis polarity between cell cycles.[27] In the type II NB lineage, whether the newly generated INPs act as niche cells to regulate the behavior and function, especially the self-renewal and differentiation, of NBs needs to be answered.

Golgi-to-endoplasmic reticulum (ER) retrograde transport (RT) is an important biological process to recycle ER-resident proteins required for correct protein folding and synthesis.[28–30] The key molecules involved in RT are seven subunits (α, β, β′, γ, δ, ε, and ζCOP) that form the coatomer of the coat protein (COP) I vesicles, KDELR protein that binds to ER escape proteins, and solule N-ethylmaleimide sensitive factor attachment protein receptors (SNARE) proteins, including Syx18, Sec20, Use1, and Ykt6, that mediate vesicle fusion.[31–38] Previous studies have shown that disruption of the formation of COP I vesicles leads to damage of endomembrane system, impairment of secretion function, induction of ER stress (ERS), and ultimately cell death without ERS relief.[39–42] Furthermore, in *Drosophila KDELR* mutants, the soluble ER proteins, such as Boca and Wbl, failed to be recycled and detected in the lumen of salivary glands, leading to dysplastic salivary glands compared to the wild type.[43] During *Drosophila* male meiosis divisions, knockdown of COPI subunits leads to the failure of cytokinesis.[44] Although Golgi-to-ER RT plays critical roles during organogenesis, it still remains unclear whether RT functions in the CNS to regulate NBs in *Drosophila*.

Our study reveals that INPs function as the niche of type II NBs, inheriting Delta from NB to *trans*-activate Notch signaling. Blocking RT reduces the number of INPs, resulting in decreased Notch activity and ectopic expression of nuclear Pros, ultimately leading to premature differentiation of type II NBs.

RESULTS

Golgi-to-ER RT dysfunction specifically leads to the loss of type II NBs

To identify essential genes that specifically regulate *Drosophila* larval type II NBs, we used the GAL4/UAS system to perform a paired screen in type II and type I NBs. In our screen, individual type II NB lineage was labeled with UAS-mCD8-GFP driven by wor-GAL4, ase-GAL80 (referred to as II NB-GAL4).[45] In the control group, each brain lobe is stereotyped with eight type II NBs (Figures 1A and 1B). We found that knocking down genes involved in Golgi-to-ER RT, including *KDELR*, *Syx18*, *Sec20*, *Use1*, *Ykt6*, *βCOP*, *β′COP*, *γCOP*, *ζCOP*, and *δCOP*, significantly reduced the number of type II NBs (Figures 1A, 1B, S1A, and S1B). To further verify this result, we used another type II NB lineage-specific driver, PntP1-GAL4, to drive UAS-mCD8-GFP expression, and knocked down these genes again. Compared to control, similar loss of type II NBs was observed when RT was blocked (Figure S2A). While knocking down these genes using type I NB driver, ase-GAL4, we found no significant loss of type I NBs or any abnormal phenotype compared to the control group (Figure S2B). Therefore, these results suggest that blocking RT specifically affects the maintenance of type II, but not type I NBs.

To exclude the possibility of off-target effects of RNAi, we took advantage of the MARCM system,[46,47] which allows the generation of wild-type or mutant clones of both type I and type II NBs that express membrane-bound GFP in an otherwise wild-type, GFP-negative background, to observe the number of NB clones in the background of *KDELR* mutants. Two hours after larvae hatching (ALH), subsequent 37°C heat shock for 1.5 h drove the expression of the recombinant enzyme flippase, thereby inducing homologous recombination between the two FLP recombination target (FRT) sites. Two *KDELR* mutants, *KDELR*^{EY07077}and *KDELR*^{K00311}, in which the P element was inserted into the 5′UTR of the *KDELR* transcription unit, were used to generate mutant clones.[43] In the control group, the number of type II NB clones in each brain lobe was 2.42 ± 0.31, while the clone number was 0.92 ± 0.28 in *KDELR*^{EY07077} mutants and 0.75 ± 0.25 in *KDELR*^{K00311} mutants (Figures 1C and 1D). However, the number of type I NB clones was 8.29 ± 0.52 in *KDELR*^{EY07077} and 8.58 ± 0.8 in *KDELR*^{K00311} mutants, which was comparable to control (9 ± 1.15) (Figures S2C and S2D). These results further demonstrate that Golgi-to-ER RT is required for maintaining type II NBs specifically. For convenience, we focused our subsequent analysis on three key components in the RT process, including KDELR, Syx18, and γCOP, which are highly conserved from *Drosophila* to mammals.[43,48,49]

To further confirm that the loss of type II NBs was indeed caused by RT genes knockdown, we performed rescue experiments by overexpressing the wild-type form of coding sequence of KDELR, Syx18, and γCOP which partially rescued the loss of type II NBs induced by RT genes single knockdown (Figures 1E and 1F). These results further support that the loss of type II NBs is attributed to inhibiting RT.

Knocking down RT genes leads to low Notch activity and ectopic Pros expression in type II NBs

We further explored the direct cause of type II NB loss induced by blocking RT. By counting the number of type II NBs at the end of the first-instar larvae, we found that type II NB number was around four when the RT gene was knocked down (Figures S3A and S3B), which was

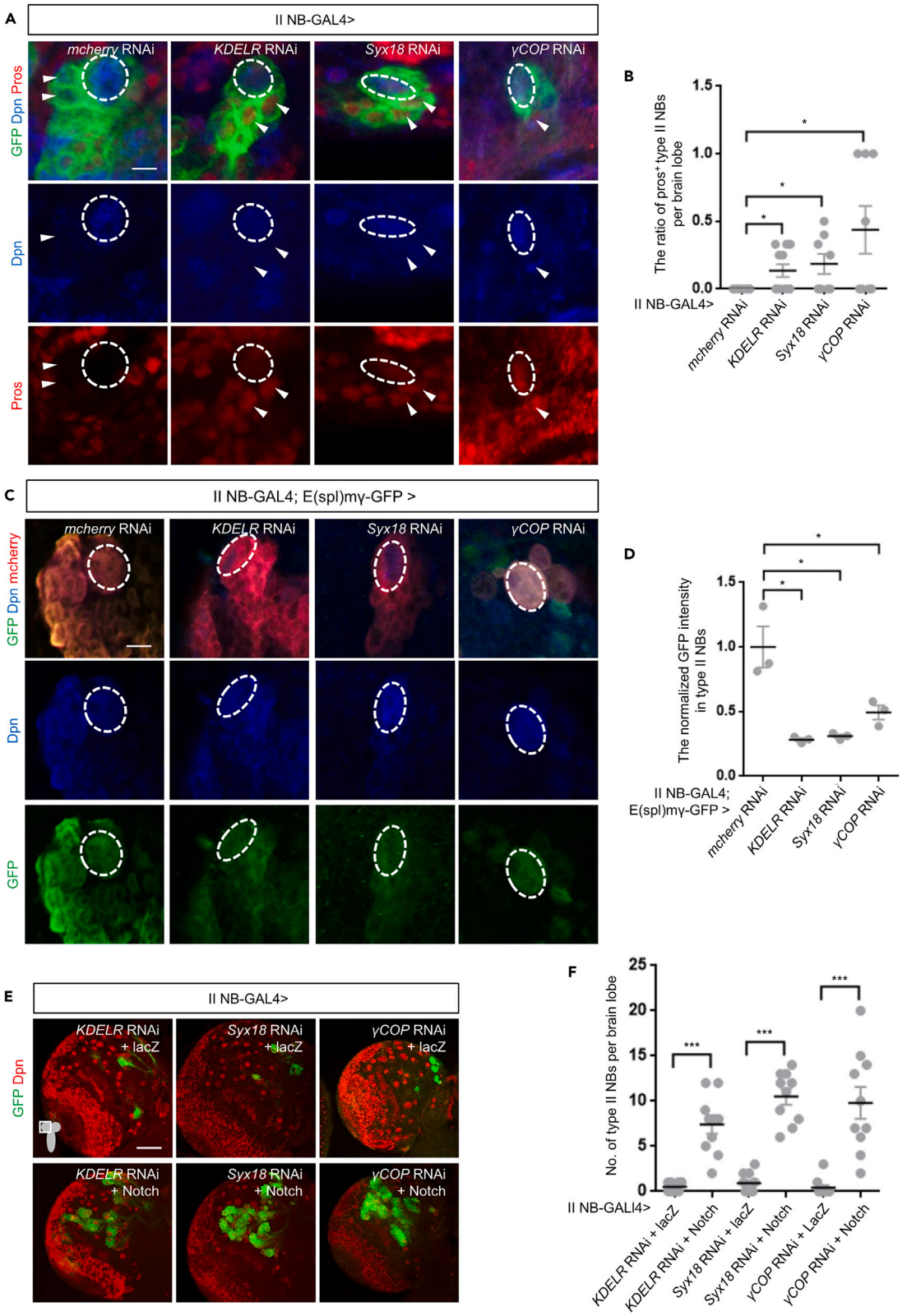
A
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
GFP Dpn Pros
Dpn
Pros
B
The ratio of pros+ type II NBs per brain lobe
1.5
1.0
0.5
0.0
*
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
C
II NB-GAL4; E(spl)mγ-GFP >
GFP Dpn mcherry
GFP
D
The normalized GFP intensity in type II NBs
II NB-GAL4; E(spl)mγ-GFP >
E
II NB-GAL4>
KDELR RNAi + lacZ
Syx18 RNAi + lacZ
γCOP RNAi + lacZ
KDELR RNAi + Notch
Syx18 RNAi + Notch
γCOP RNAi + Notch
F
No. of type II NBs per brain lobe
25
20
15
10
5
0
***
II NB-GALI4>
KDELR RNAi + lacZ
KDELR RNAi + Notch
Syx18 RNAi + lacZ
Syx18 RNAi + Notch
γCOP RNAi + LacZ
γCOP RNAi + Notch

Figure 2. Knockdown of the RT gene results in ectopic expression of nuclear Pros and a decrease in Notch activity in type II NBs

(A) Nuclear Pros is detected in type II NBs (outlined by dashed line) and INPs (white arrowheads) after knocking down *KDELR*, *Syx18*, and *γCOP*. Type II NB lineages were labeled by GFP and Dpn. Scale bars: 2 μm.

(B) The Ratio of Pros ectopically expressed in type II NBs from genotypes in (A). N = 8, 11, 8, and 8, respectively.

Mean ± SEM, *p < 0.05.

(C) The expression pattern of E (spl)mγ-GFP in type II NBs (outlined by dashed line) after knocking down *KDELR*, *Syx18*, and *γCOP*. GFP represents the activity of Notch. Type II NB lineages were marked by mCherry and Dpn. Scale bars: 2 μm.

(D) Quantification of normalized GFP intensity in type II NBs from genotypes in (C). N = 23, 18, 26, and 25, respectively. Mean ± SEM, *p < 0.05.

(E) Overexpression of Notch successfully rescued type II NB loss. Type II NB lineages were marked with GFP and Dpn. Scale bars: 20 μm.

(F) Quantification of type II NB number in per brain lobe from genotypes in (E). N = 10. Mean ± SEM, ***p < 0.001. p values were calculated using a one-way ANOVA compared to mCherry groups. Unpaired Student's t tests were used for comparisons between two groups. See also Figure S3.

significantly higher than that in the third-instar larvae, indicating that the number of type II NBs was decreased gradually, rather than not being formed during embryonic stage. Therefore, it seems that RT is not required for the origin but for the maintenance of type II NBs.

We next explored whether blocking RT leads to apoptosis of type II NBs. With the apoptotic marker Dcp1, we found that, after blocking RT, the apoptosis level showed no significant change compared to the control (Figure S3C), indicating that the loss of type II NBs may not be due to apoptosis. More importantly, we detected whether the loss of type II NBs could be rescued by overexpressing the baculovirus anti-apoptotic molecule P35. Consistently, overexpression of P35 did not rescue the loss of type II NBs caused by blocking RT (Figure S3D). Taken together, these data suggest that type II NB loss induced by RT block is independent of apoptosis.

Next, we continued to investigate if blocking RT would lead to premature differentiation of type II NBs by staining the differentiation-promoting factor Pros. In wild-type type II NBs, the level of nuclear Pros is almost undetectable (Figures 2A and 2B). However, when RT was blocked in type II NBs, the ratio of type II NBs with ectopic nuclear Pros was significantly increased (Figures 2A and 2B). Therefore, type II NBs were lost in the absence of RT due to the ectopic expression of nuclear Pros.

Next, we wondered by which means RT genes knockdown caused the ectopic expression of nuclear Pros. Given that the NB loss phenotype only appeared in type II NBs, we focused on genes or signaling pathways that specifically maintain type II NBs. Previous studies have shown that loss of pnt results in ectopic nuclear Pros expression in type II NBs.[5] Therefore, we examined whether the expression of Pnt was affected by RT genes knockdown. We used pnt-lacZ, an enhancer trap line, to characterize pnt expression, and found that the signal of lacZ in type II NBs was comparable to control after knocking down RT genes (Figure S4A), indicating that the expression level of Pnt was not affected by blocking RT. We next overexpressed Pnt in RT genes knockdown background and found that Pnt overexpression could not rescue the loss of type II NBs (Figure S4B). Therefore, these data suggest that type II NB loss induced by blocking RT was not due to the absence of Pnt.

Previous studies have reported that decreased Notch signaling leads to ectopic expression of nuclear Pros in type II NBs.[11] We next wonder whether blocking RT would lead to a reduction in Notch activity. Using the Notch activity indicator E(spl)mγ-GFP, in which the full length of GFP fragment was ligated into the coding sequence of *E(spl)mγ*,[7,11] we found that, after blocking RT, the GFP fluorescence intensity was significantly reduced in type II NBs (Figures 2C and 2D), indicating that the Notch activity was downregulated. We next tested whether overexpressing Notch can rescue type II NB loss. Remarkably, Notch overexpression completely rescued the loss of type II NBs (Figures 2E and 2F). Thus, we conclude that blocking RT reduces Notch activity, which leads to ectopic expression of nuclear Pros, consequently the premature differentiation, and ultimately the loss of type II NBs.

Blocking RT leads to decreased Delta protein at interface between NB and INPs

Next, we want to investigate how blocking RT reduced Notch activity in type II NBs. To analyze the mechanism of this regulation, we first performed immunofluorescence staining with an antibody against Notch extracellular domain (NECD) and found that NECD in type II NBs remains unchanged after blocking RT (Figure S4C). Thus, the total Notch protein level appears to be normal after blocking RT. Then, we proposed that the level of Notch ligands was reduced after blocking RT. To test this hypothesis, we detected the expression pattern of Notch ligands. We took advantage of Serrate::GFP protein trap line and observed that Serrate was expressed mainly in glial cells, enveloping the whole type II NB lineage (Figures S5A and S5B). We also used Delta::GFP protein trap line and found that Delta was distributed in the cytoplasm and enriched on the interface between type II NB and INPs (Figures S5A and S5B). The expression pattern of Delta and Serrate is in line with previous findings.[50]

Previous data have demonstrated that Delta is more important for maintaining Notch activity in NBs than Serrate;[50] thus, we focused on Delta for our further research. Type II NBs need to receive Delta provided by neighboring cells to activate Notch. Therefore, we used Delta::GFP to test whether blocking RT will lead to the decreased Delta protein at the interface between type II NBs and neighboring cells. We found that, in knockdown of RT genes, the total Delta protein was decreased in the whole type II NB lineage, and in particular the interface between type II NB and INPs (Figures 3A and 3B). Therefore, our findings indicate that type II NBs receive less Delta protein from INPs after blocking RT, which may diminish Notch activity and ultimately the loss of type II NBs.

Delta expressed in type II NBs regulates Notch activity

As we observed that Delta decreases at the interface and the Notch ligand is provided by NB surrounding cells, we speculated that the Delta required for the activation of NB Notch is produced in INPs. Therefore, we took advantage of multiple INP-specific drivers, 9D10-GAL4 and 9D11-GAL4,[51,52] to knockdown *Delta* in INPs to verify whether it will affect the number of type II NBs. Surprisingly, we found that knocking

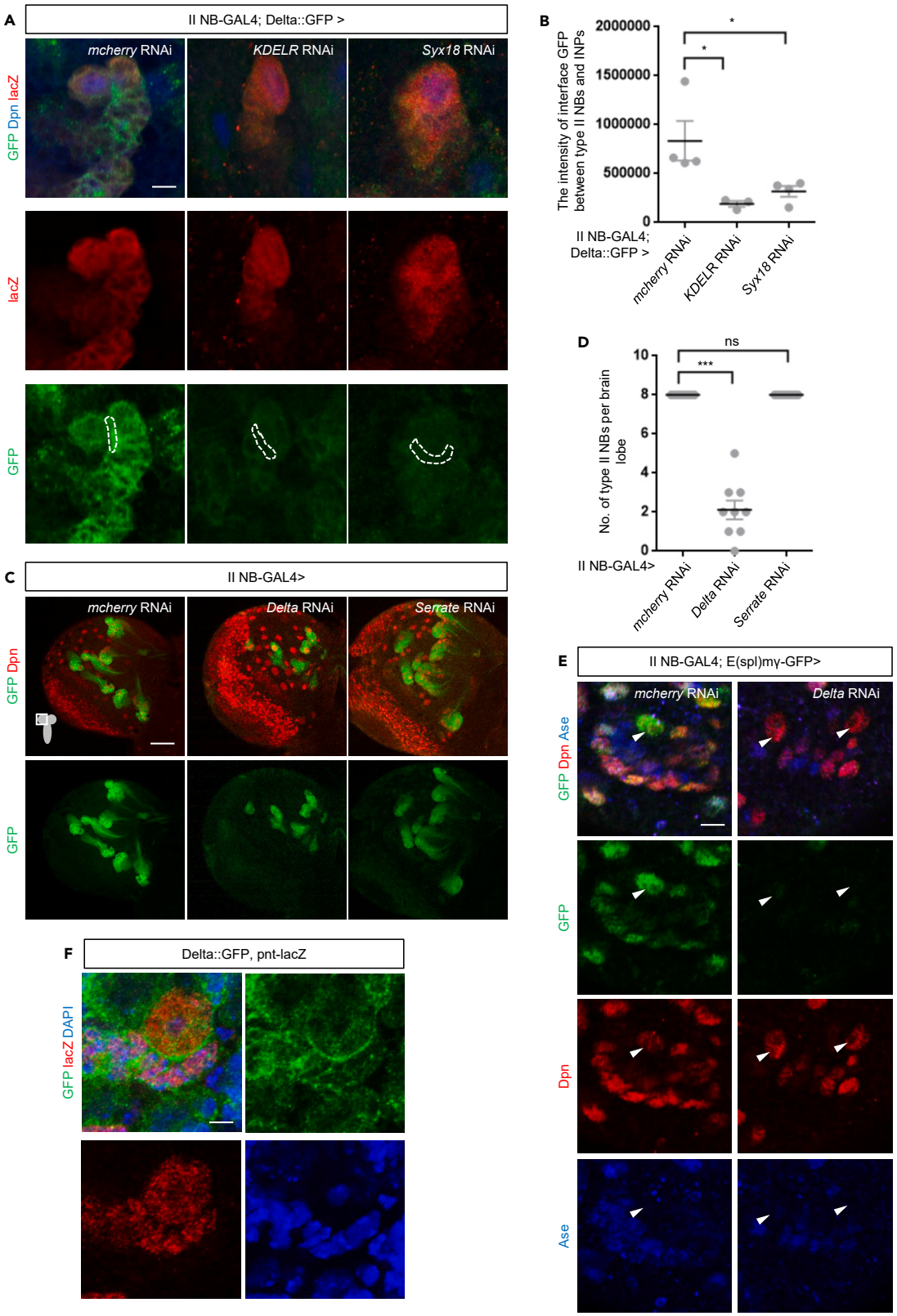
A
II NB-GAL4; Delta::GFP >
mcherry RNAi
KDELR RNAi
Syx18 RNAi
GFP Dpn lacZ
lacZ
GFP
B
The intensity of interface GFP between type II NBs and INPs
2000000
1500000
1000000
500000
0
*
*
II NB-GAL4; Delta::GFP >
mcherry RNAi
KDELR RNAi
Syx18 RNAi
C
II NB-GAL4>
mcherry RNAi
Delta RNAi
Serrate RNAi
GFP Dpn
GFP
D
No. of type II NBs per brain lobe
10
8
6
4
2
0
ns
***
II NB-GAL4>
mcherry RNAi
Delta RNAi
Serrate RNAi
E
II NB-GAL4; E(spl)mγ-GFP>
mcherry RNAi
Delta RNAi
GFP Dpn Ase
GFP
Dpn
Ase
F
Delta::GFP, pnt-lacZ
GFP lacZ DAPI

Figure 3. The decreased Delta protein at interface induced by blocking RT and the loss of Delta in NBs result in type II NB loss

(A) The expression pattern of Delta::GFP at interface between type II NB and INPs (outlined by dashed line) after *KDELR* and *Syx18* knockdown. Type II NB lineages were marked by lacZ and Dpn. Scale bars: 2 μm.

(B) The quantification of GFP intensity at the interface between type II NB and INPs from genotypes in (A). N = 4, 3, and 4, respectively. Mean ± SEM, *p < 0.05.

(C) Loss of *Delta* leads to type II NB loss, while type II NB was normal after knocking down *Serrate*. Type II NB lineages were stained with GFP and Dpn. Scale bars: 20 μm.

(D) Quantification of type II NB number in per brain lobe from genotypes in (C). N = 8. Mean ± SEM, ns (non-significant), p > 0.05; ***p < 0.001.

(E) Loss of *Delta* results in the activity of Notch was lost in type II NBs (white arrowheads). GFP represents the Notch activity. Type II NB lineages were stained with Dpn and Ase. Scale bars: 2 μm.

(F) The distribution of Delta::GFP in mitosis type II NBs. Type II NB lineages were labeled by pnt-lacZ. Scale bars: 2 μm. p values were calculated using a one-way ANOVA compared to mCherry groups. See also Figures S4 and S5.

down *Delta* in INPs did not change the number of type II NBs at all (Figures S5C and S5D). Thus, although Delta is expressed in INPs, the expressed Delta may not be necessary for maintaining type II NBs.

As the knockdown of *Delta* in INPs does not affect the number of type II NBs, we continued to investigate which cell produces Delta that maintains type II NBs. We used Delta-lacZ, in which P element carrying lacZ was inserted at the 5′ untranslated region (UTR) of the *Delta* genomic locus,[53,54] to identify the Delta expression pattern. In type II NB lineage, Delta expression is highest in the NB and becomes gradually lower in daughter cells (Figure S5E). Thus, we tried to detect whether knocking down *Delta* in type II NBs would lead to type II NB loss. Interestingly, knocking down *Delta* in type II NBs significantly reduced type II NB number (Figures 3C and 3D), while knocking down *Serrate* in type II NBs or glial cells had no effect on type II NB number (Figures 3C, 3D, and S5F). These results suggest that the Delta produced by type II NBs, rather than INPs, is necessary for maintaining type II NBs.

In order to investigate how the Delta produced by type II NBs affects type II NB number, we proposed that the Delta produced by type II NBs is used to activate Notch of themselves, and knocking down *Delta* would reduce Notch activity in type II NBs. Using E(spl)mγ-GFP, we found the Notch activity was significantly decreased in type II NBs after knocking down *Delta* by the type II NB driver (Figure 3E). Therefore, the Delta produced by type II NBs is used to activate Notch in type II NBs.

Our results promoted us to investigate how the Delta produced by type II NB activated Notch in the same cells. Previous study has shown that, when a type I NB divides, part of the Delta is inherited by the GMC, which serves as the Delta-sending cell and activates the Notch in type I NBs.[55] Similarly, we hypothesized that the Delta produced by type II NBs would also be asymmetrically distributed to the INPs during division, and then the Delta in the INPs activates Notch in type II NBs. To verify this hypothesis, by observing Delta::GFP, we found that, during the metaphase, GFP was mainly enriched at the basal cortex of NBs adjacent to the INPs (Figure 3F). Therefore, the Delta produced by type II NBs, as the main source of Delta in type II NB lineages, will be asymmetrically distributed to the INPs and then *trans*-activate Notch to maintain the self-renewal of type II NBs.

Knocking down RT genes decreases NB proliferation and INP number

Next, we aimed to investigate how blocking RT of type II NBs decreased Delta protein level at the interface. Because Golgi-to-ER RT is important for the recycle of ER-resident proteins that participate in the proper folding of polypeptides,[56–58] therefore, RT dysfunction leads to the accumulation of misfolded proteins and ERS.[59,60] To confirm the potential role of ERS in type II NB maintenance, we first overexpressed the dominant negative form of Bip (Bip^{DN}), which has no ATP binding/hydrolysis activities, or Psn to effectively induce ERS.[61–63] However, type II NB number remained eight in each brain lobe with overexpression of Bip^{DN} or Psn (Figures S6A and S6B). In addition, blocking each of the three downstream branches of ERS, inositol-requiring enzyme-1 (IRE), pancreatic eIF-2α kinase (PERK), and activating transcription factor 6 (ATF6) pathways, type II NB number remained unchanged (Figures S6C and S6D). Finally, we tried to alleviate ERS by reducing protein accumulation to rescue type II NB loss. We found that neither promoting autophagy by overexpressing atg8[64] nor knocking down Deubiquitinases (DUBs)[65,66] could rescue the loss of type II NBs (data not shown). Altogether, these results exclude the possibility that the loss of type II NBs is caused by aggravated ERS.

In addition to the decrease of Delta protein in each INP, the reduced number of INPs could also lead to the decrease of total Delta that activates Notch. Since RT is involved in biosynthesis and promote cell proliferation, we assumed that blocking RT might impair the proliferation of type II NBs, resulting in a reduced number of INPs. With immunofluorescence staining of PH3, our results showed that, after blocking RT, the proliferation rate of type II NBs was significantly reduced (Figures 4A and 4B). We also quantified the number of INPs of each type II NB lineage in defective RT brains and found that INP number was significantly reduced after blocking RT (Figures 4C and 4D). Therefore, blocking RT indeed reduces the proliferation of type II NBs and in turn leads to a decrease in the number of INPs, which might be the cause of decreased level of Delta around type II NBs.

Because blocking RT leads to a decrease in the number of INPs, we hypothesized that this event could be the reason for type II NB loss. We reduced the proliferation of type II NBs by knocking down CycE, a well-known G1/S regulator. At the first-instar larval stage, the type II NB number of *CycE* knockdown (6.63 ± 0.63) was slightly lower than that of the control (8 ± 0) (Figures 4E and 4F), while the NB-adjacent INP number in each type II NB lineage of *CycE* knockdown (1 ± 0.29) was dramatically decreased compared to the control (3.73 ± 0.36) (Figures 4G and 4H). At the third-instar larval stage, the number of type II NBs decreased significantly (1.25 ± 0.36) compared with the control (8 ± 0) (Figures 4F and S6E). These results suggest that the loss of INPs precedes type II NB loss, indicating the causality between INP number and type II NB maintenance.

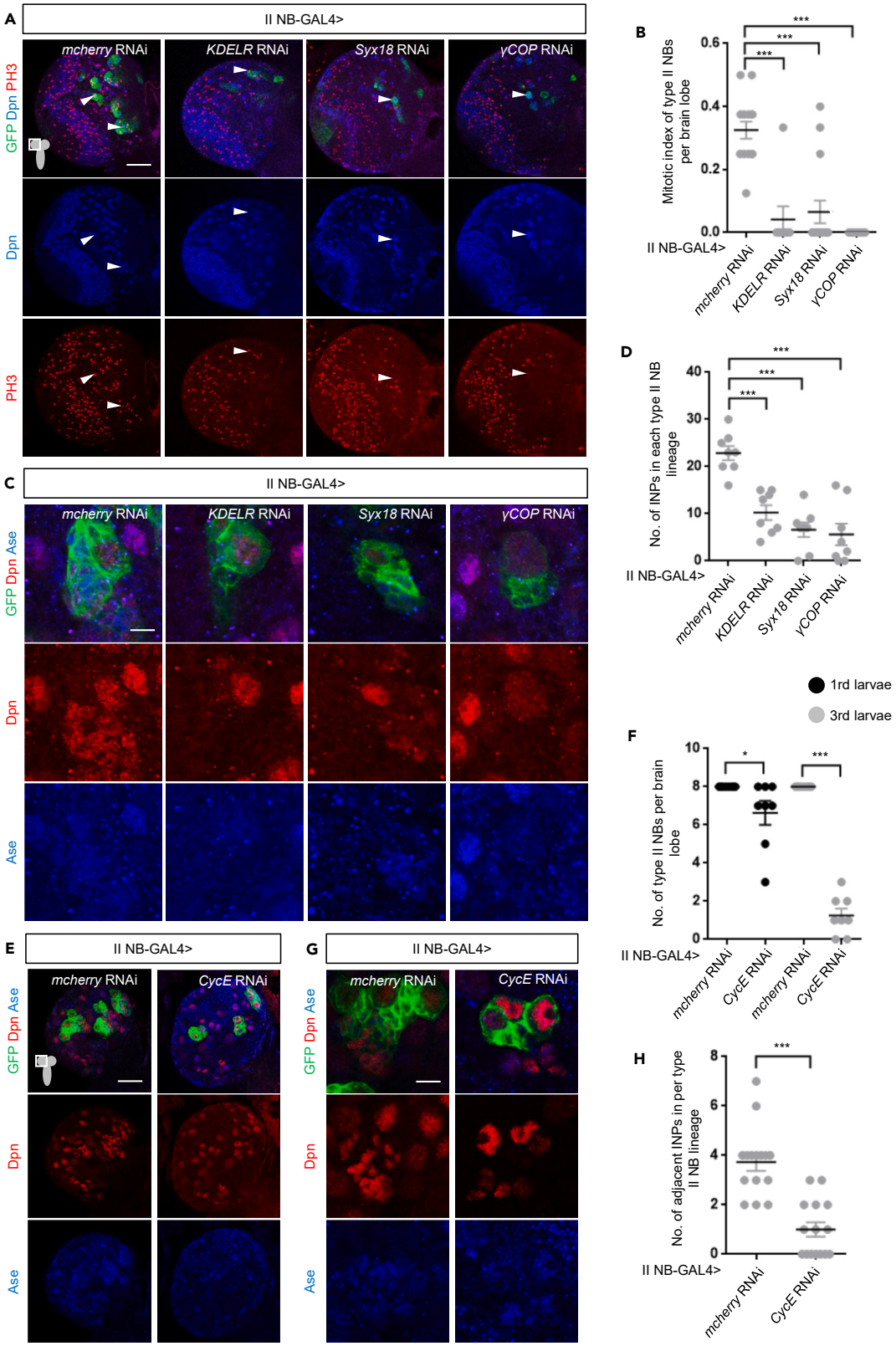
A
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
GFP Dpn PH3
Dpn
PH3
B
Mitotic index of type II NBs per brain lobe
0.6
0.4
0.2
0.0
***
***
***
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
C
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
GFP Dpn Ase
Dpn
Ase
D
No. of INPs in each type II NB lineage
40
30
20
10
0
***
***
***
II NB-GAL4>
mcherry RNAi
KDELR RNAi
Syx18 RNAi
γCOP RNAi
1rd larvae
3rd larvae
E
II NB-GAL4>
mcherry RNAi
CycE RNAi
GFP Dpn Ase
Dpn
Ase
F
No. of type II NBs per brain lobe
10
8
6
4
2
0
*
***
II NB-GAL4>
mcherry RNAi
CycE RNAi
mcherry RNAi
CycE RNAi
G
II NB-GAL4>
mcherry RNAi
CycE RNAi
GFP Dpn Ase
Dpn
Ase
H
No. of adjacent INPs in per type II NB lineage
8
6
4
2
0
***
II NB-GAL4>
mcherry RNAi
CycE RNAi

Figure 4. Knocking down the RT gene leads to a decrease in the number of INP, which could be a reason for type II NB loss

(A) Knocking down *KDELR*, *Syx18*, and *γCOP* decreases the proliferation of type II NBs (white arrowheads). PH3 labels the proliferating NBs. Type II NB lineages were stained with GFP and Dpn. Scale bars: 20 μm.

(B) The proliferation index of type II NBs in per brain lobe from genotypes in (A). N = 16, 8, 16, and 10, respectively. Mean ± SEM, ***p < 0.001.

(C) Loss of *KDELR*, *Syx18*, and *γCOP* in type II NBs reduces the number of INPs. Type II NB lineages were stained with GFP, Dpn, and Ase. Scale bars: 2 μm.

(D) Quantification of INP number in per type II NB lineage from genotypes in (C). N = 8. Mean ± SEM, ***p < 0.001.

(E) Loss of *CycE* leads to a slightly loss of type II NBs at first instar larvae. Type II NB lineages were stained with GFP, Dpn, and Ase. Scale bars: 50 μm.

(F) Quantification of type II NB number in per brain lobe from genotypes in (E) at different development stage. N = 8. Mean ± SEM, *p < 0.05; ***p < 0.001.

(G) Knocking down *CycE* results in a significant loss of INPs at first instar larvae. Type II NB lineages were stained with GFP, Dpn, and Ase. Scale bars: 2 μm.

(H) Quantification of INP number adjacent to type II NBs from genotypes in (G) at first-instar larvae. N = 15. Mean ± SEM, ***p < 0.001. p values were calculated using a one-way ANOVA compared to mCherry groups. Unpaired Student's t tests were used for comparisons between two groups. See also Figure S6.

INPs function as the niche for type II NB maintenance

As INPs are directly adjacent to type II NBs and inherit the Delta from NBs, which is used to activate Notch in NBs, INPs can be regarded as the niche of type II NBs and might be involved in type II NB self-renewal. To verify this hypothesis, we eliminated INPs by overexpressing pro-apoptotic genes *rpr* and *hid* with two INPs-specific drivers. Compared to the control, we found that INP number was significantly decreased after overexpressing pro-apoptotic genes (Figures S7A and S7B). To further confirm the apoptosis of INPs, we also stained the apoptosis marker, Dcp1, in INPs and found that the number of $Dcp1^+$ INPs was increased (Figures S7C and S7D). We next tested whether the elimination of INPs would lead to type II NB loss. As expected, the elimination of INPs leads to the decrease in the number of type II NBs (Figures 5A and 5B). We next overexpressed P35 in INPs to test whether the loss of type II NBs would be rescued. We found that the number of type II NBs was partially rescued (Figures S7E and S7F). Taken together, these data indicate INPs act as the niche to maintain type II NBs.

Next, to determine whether Delta is the only essential ligand that is provided by INPs for maintaining type II NB self-renewal, we tried to overexpress Delta to rescue the type II NB loss induced by INP elimination. Remarkably, the number of type II NBs increased from 2 ± 0.27 in Rpr and Hid overexpression to 6.12 ± 0.23 after overexpressing Delta in INPs (Figures 5C and 5D). These results, together with INPs-inherited Delta from type II NBs, support that INPs form the niche of type II NBs by providing Delta to activate Notch in adjacent NBs.

Restore of INP number can rescue type II NB loss due to RT block

Blocking RT leads to the decrease in the proliferation rate of type II NBs, which in turn results in a reduction in the number of INPs and the decline of Delta protein in INPs. Next, we sought to restore the decreased INP number to rescue type II NB loss. We overexpressed CycE in type II NBs to promote cell cycle progression, which partially rescued type II NB loss (Figures 6A and 6B). Thus, promoting cell cycle can rescue type II NB loss. We further explored the genetic relationship between Notch and CycE. In the wing disc, *CycE* is a downstream target gene of Notch to control proliferation.[67,68] Consistently, we found that CycE overexpression failed to rescue the loss of type II NBs caused by *Notch* knockdown (Figures 6C and 6D). It seems plausible that CycE serves as an independent regulatory role in cell proliferation rather than self-renewal. However, overexpression of CycE could rescue type II NB loss caused by RT block. Taken together, these results further support that RT is only required for the Notch ligand assembling in the Notch-sending INPs, rather than Notch and its downstream components in the Notch-receiving NBs.

Since our results showed that blocking RT could effectively inhibit the excessive proliferation of type II NB caused by Notch overexpression via alleviating the Delta provided by adjacent INPs, we next explored whether knocking down *Delta* can also inhibit tumor growth. We found that tumor development induced by defective *numb* or *brat* can be effectively inhibited by *Delta* knockdown (Figure 6E). Thus, we conclude that cutting off the self-renewal maintaining signals from niche maybe a promising method to treat tumor clinically.

In summary, our research shows that in wild-type type II NBs, Golgi-to-ER RT is required for normal biosynthesis and proliferation, through which Delta produced by type II NBs is distributed to INPs to *trans*-activate Notch in type II NBs. However, when blocking RT, it leads to a decrease in biosynthesis, which in turn leads to a decrease in proliferation and the number of INPs, all of which reduces Delta localized in INPs to activate Notch in type II NBs and ectopic expression of Pros, eventually leading to premature differentiation of type II NBs (Figure 6F).

DISCUSSION

In this study, we demonstrate that Golgi-to-ER RT is essential for the maintenance of type II NBs. In wild-type type II NBs, the Notch ligand Delta is distributed to the basal side and segregated to INPs during division. The Delta localized in NB-adjacent INPs activates Notch signaling in NBs. Blocking RT will disrupt biosynthesis and proliferation, leading to a decreased number of INPs, reducing the Delta protein, and consequently decrease the Notch activity. This disruption ultimately results in ectopic nuclear Pros expression and premature differentiation of type II NBs. This mechanism can be adopted to suppress the tumor development initiated from type II NBs.

In the CNS, the Notch pathway is often involved in the maintenance of quiescence and self-renewal of NSCs. For instance, in the telencephalon of adult zebrafish, quiescent NSCs express the Notch3 to remain in a quiescent state. In the absence of Notch3, the ratio of activated NSC is significantly increased.[69,70] However, once NSCs are activated, they still require Notch3 to maintain their stemness, and loss of Notch3 leads to a progressive decrease in the number of activated NSCs accompanied by an increase in neuron number.[71] Similarly, in the subventricular zone of adult mammals, Notch signaling is highly active in quiescent NSCs and has a pivotal role in maintaining their quiescent state. Dysfunction in the Notch pathway will reactivate quiescent NSCs.[72–76] In mammalian embryonic brain, Notch pathway is essential

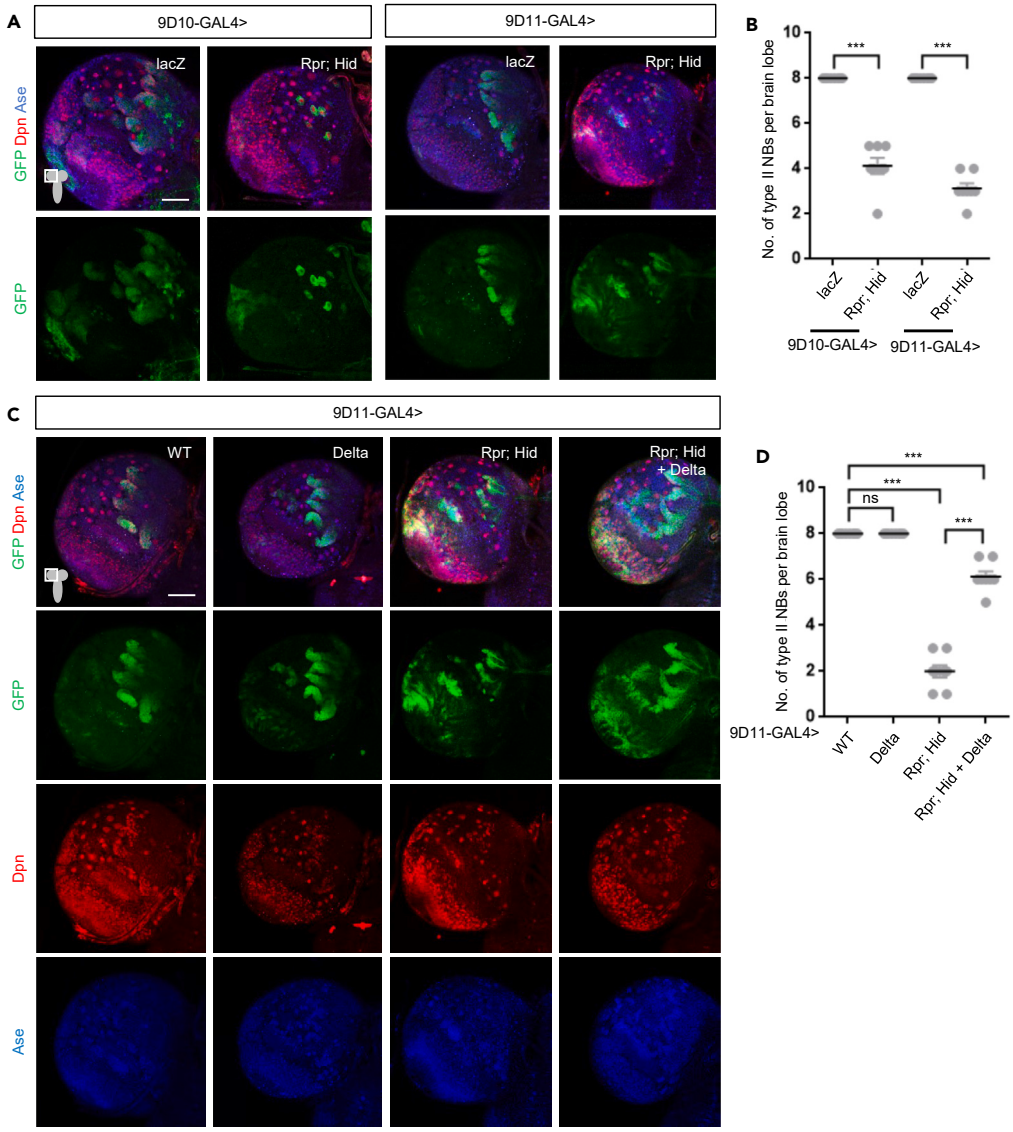

Figure 5. Delta overexpression can rescue type II NB loss induced by INP elimination

(A) Overexpression of Rpr and Hid in immINPs (9D10-GAL4) or mINPs (9D11-GAL4) leads to type II NB loss. Type II NB lineages were labeled by GFP, Dpn, and Ase. Scale bars: 20 μm.

(B) Quantification of type II NB number in per brain lobe from phenotypes in (A). N = 8. Mean ± SEM, ***p < 0.001.

(C) Overexpression of Delta partially rescued type II NB loss induced by the elimination of INPs. INP lineages were stained with GFP, Dpn, and Ase. Scale bars: 20 μm.

(D) Quantification of type II NB number in per brain lobe from genotypes in (C). N = 8. Mean ± SEM, ns (non-significant), p > 0.05; ***p < 0.001. p values were calculated using a one-way ANOVA compared to WT groups. Unpaired Student's t tests were used for comparisons between two groups. See also Figures S6 and S7.

for the self-renewal of NSCs, as the loss of Notch leads to the premature differentiation of most NSCs into neuron and finally depletion.[77–79] Likewise, in *Drosophila* type I NBs, loss of Notch delays entry into quiescence, while overexpression of Notch leads to an early entry into quiescence.[55] In this study, we found that reduced Notch activity induced by blocking RT selectively affects the self-renewal of type II NBs. Therefore, Notch pathway has different roles in two types of NBs, which is essential for entering quiescence in type I NBs and for maintaining the self-renewal in type II NBs. In conclusion, Notch plays critical roles in regulating quiescence and self-renewal of NSCs, which is determined by the cell state, developmental stage, or cell type of NSCs.

The niche refers to the microenvironment, in which stem cells are located, and has a regulatory effect on stem cells. Generally, niche-forming cells are either cells independent of the stem cell lineage such as somatic cap cells or hub cells, which form the female or male *Drosophila* germline stem cell niche, respectively,[80] or highly differentiated daughter cells of stem cells, like Paneth cells acting as the niche of mammalian

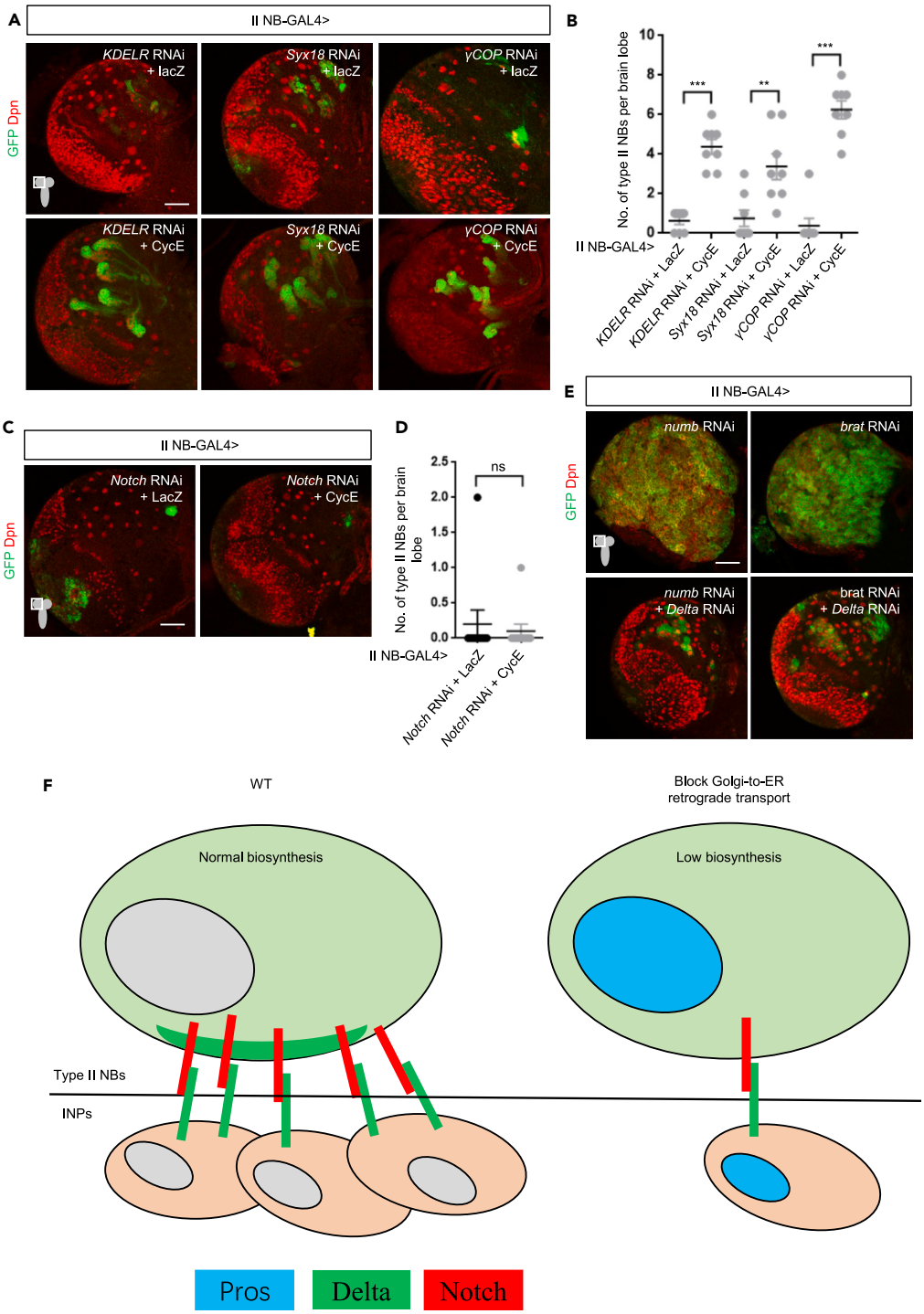

Figure 6. Overexpression of CycE can rescue type II NB loss caused by knocking down RT genes, not by *Notch* knockdown

(A) Overexpression of CycE partially rescued the loss of type II NBs. Type II NB lineages were labeled by GFP and Dpn. Scale bars: 20 μm.

(B) Quantification of type II NB number in per brain lobe from genotypes in (A). N = 8. Mean ± SEM, **p < 0.01; ***p < 0.001.

(C) Overexpression of CycE failed to rescue type II NB loss induced by knocking down *Notch*. Type II NB lineages were labeled by GFP and Dpn. Scale bars: 20 μm.

(D) Quantification of type II NB number in per brain lobe from genotypes in (C). N = 10. Mean ± SEM, ns (non-significant), p > 0.05.

(E) Knockdown of *Delta* inhibits the over-proliferation of type II NBs induced by *numb* or *brat* knockdown. Type II NB lineages were labeled by GFP and Dpn. Scale bars: 20 μm. Unpaired Student's t tests were used for comparisons between two groups.

(F) Working model. In wild-type II NBs, RT maintains normal biosynthesis and proliferation. The newly generated INPs inherit Delta from type II NBs and then transactivate Notch in NBs. After blocking RT, the proliferation of type II NBs decreases, and the number of INPs providing Delta decreased, ultimately leading to a decrease in Notch activity, Pros entering the nucleus, and pre-differentiation of type II NBs.

intestinal stem cells.[81] In the *Drosophila* CNS, previous studies have shown that highly differentiated glial cells form the NB niche, which regulates the quiescence, reactivation, survival, and proliferation of NBs.[23,24,26,82,83] Therefore, the newly born daughter cells are not regarded as part of stem cell niche. However, recent studies have shown that the *trans*-amplifying cells generated by ISCs regulate the proliferation of ISCs through the Wnt pathway.[84,85] In mammalian CNS, the intermediate progentior cells (IPCs) provide Notch ligands to activate Notch in NSCs.[86–88] In this study, we found that the newly generated INPs, as the NBs niche, provide Delta to *trans*-activate Notch and regulated the self-renewal of type II NBs. Together, these studies support the conception that the immediate daughter cells might function as the stem cell niche.

The activation of the Notch pathway requires adjacent cells to provide ligands. In this study, we have found that INPs provide Delta for maintaining the self-renewal of type II NBs, resembling the role of mammalian IPCs in producing Delta to activate NSCs in the CNS.[86] However, the regulatory mechanisms of these two Notch activations are still different. In the *Drosophila* type II NB lineage, the NB expresses the highest level of Delta, which is distributed to INPs for activating Notch in type II NBs. Furthermore, knocking down *Delta* in type II NBs leads to a decrease in Notch activity, indicating that type II NBs produce Delta that is required for the activation of their own Notch signaling, resembling how Notch is activated to maintain the NSC quiescence in mammals.[89] Therefore, our research deepens the understanding of the regulation of NB self-renewal through Notch signaling pathway.

The Golgi-to-ER RT is a critical process in maintaining ER homeostasis and protein synthesis and secretion. Therefore, blocking this process is highly correlated with serious cellular defects and neurodegeneration diseases. For instance, in *KDELR* mutant mice, cells are sensitive to ERS, which finally develop into dilated cardiomyopathy.[90] Protein misfolding or accumulation resulting from RT blocking is closely related to the development of many neurological diseases, such as Parkinson's disease and Alzheimer's disease.[34,91] In this study, we blocked RT in *Drosophila* type II NBs, which led to type II NB loss. However, type II NB loss was not due to ERS induction. Instead, blocking RT resulted in reduced biosynthesis and cell proliferation of type II NBs, eventually leading to attenuated Notch activity, and premature differentiation of type II NBs, which provides a new mechanism for future research on the consequences related to RT block. It is worth noting that we identified several genes related to protein modification or epigenetics that were also specifically required for the maintenance of type II NBs in our screen (data not shown), which possibly share the same mechanism of RT block, suggesting that regulating INP number may be a general mechanism on maintaining type II NBs.

In our study, we observed that some retained type II NBs were labeled by mGFP (mCD8-GFP) after eliminating INPs, which is theoretically impossible to appear in the original type II NBs (Figure S6F). We speculate that the original type II NB is lost due to INP elimination, which creates room for an INP to occupy the NB position, receive Delta from other INPs, and eventually become the mGFP-labeled new NB. However, further evidence is required to confirm this hypothesis.

In this study, we revealed that Golgi-to-ER RT selectively maintains type II NBs by regulating Notch activity. Notch is highly expressed in various tumors, such as breast cancer, gliomas, malignant melanoma, and small cell lung cancer.[92–95] Our results demonstrated that blocking RT could effectively inhibit Notch-overexpressing tumors by reducing the number of INPs in *Drosophila* brain. Thus, targeting the cells that provide Notch ligands to tumor cells could potentially be a promising treatment to suppress tumors, but further research is needed in the future to fully explore this avenue.

Limitations of the study

This study focused on Golgi-to-ER RT, which maintains *Drosophila* type II NBs by regulating Notch activity. We observed that, during the division of type II NBs, Delta is asymmetrically distributed to INPs. In the future, it is necessary to explore the mechanisms that mediate Delta distribution. Besides, we found that Golgi-to-ER RT maintains the self-renewal of *Drosophila* type II NBs. Therefore, using other types of mammal stem cells to explore the conservative regulatory mechanism of Golgi-to-ER RT in self-renewal is very meaningful.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, H.Z. and S.W.; methodology, H.Z. and S.W.; software, H.Z.; formal analysis, H.Z., Z.M., S.G., S.Z., Q.Z., C.G., and W.G.; writing – original draft, H.Z. and S.W.; Writing – review & editing, H.Z., M.R., and S.W.; funding acquisition, S.W. and M.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Asense	This paper	N/A
Chicken polyclonal anti-GFP	Thermo Fisher Scientific	Cat# A10262; RRID: AB_2534023
Rabbit polyclonal anti-GFP	Torrey Pines Biologies	Cat# TP401; RRID: AB_10013661
Mouse monoclonal anti-GFP	Santa Cruz	Cat# sc-9996; RRID: AB_627695
Rat monoclonal anti-Miranda	Abcam	Cat# ab197788 RRID: AB_2936368
Rabbit monoclonal anti-mcherry	Abcam	Cat#ab213511; RRID: AB_2814891
Rat monoclonal anti-Dpn	Abcam	Cat# ab195173; RRID: AB_2687586
Chicken polyclonal anti-lacZ	Abcam	Cat# ab9361; RRID: AB_307210
Mouse monoclonal anti-lacZ	Developmental Studies Hybridoma Bank (DSHB)	Cat# 40-1a; RRID: AB_528100
Mouse monoclonal anti-pros	DSHB	Cat# MR1A; RRID: AB_528440
Mouse monoclonal anti-NECD	DSHB	Cat# C458.2H; RRID: AB_528408
Mouse monoclonal anti-Delta	DSHB	Cat# C594.9B; RRID: AB_528194
Rabbit polyclonal anti-PH3	Cell Signaling Technology (CST)	Cat# 9701; RRID: AB_331535
Rabbit polyclonal anti-Dcp1	CST	Cat# 9578; RRID: AB_2721060
Donkey anti-rabbit Alexa Fluor 555	Thermo Fisher Scientific	Cat#A31572; RRID: AB_162543
Donkey anti-rabbit Alexa Fluor 488	Thermo Fisher Scientific	Cat# A21206; RRID: AB_2535792
Donkey anti-rabbit Alexa Fluor 647	Thermo Fisher Scientific	Cat# A31573; RRID: AB_2536183
Goat anti-rat Alexa Fluor 555	Thermo Fisher Scientific	Cat# A21434; RRID: AB_2535855
Goat anti-rat Alexa Fluor 633	Thermo Fisher Scientific	Cat# A21094; RRID: AB_2535749
Goat anti-mouse Alexa Fluor 555	Thermo Fisher Scientific	Cat# A21437; RRID: AB_2535858
Goat anti-chicken Alexa Fluor 555	Thermo Fisher Scientific	Cat# A21424; RRID: AB_141780
Goat anti-chicken Alexa Fluor 488	Thermo Fisher Scientific	Cat# A11039; RRID: AB_2534096
Experimental models: Organisms/strains		
D. melanogaster: w; +; pnt-lacZ	Zhouhua Li	N/A
D. melanogaster: w; +; E(spl) mγ-GFP	Yan Song	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: w; +; Delta-lacZ	Shaozong Zhai	N/A
D. melanogaster: UAS-Dicer2; wor-GAL4, ase-GAL80, UAS-mCD8-GFP; + (II NB-GAL4)	This paper	N/A
D. melanogaster: w; ase-GAL4; UAS-Dicer2	This paper	N/A
D. melanogaster: w; UAS-Dicer2; PntP1-GAL4, UAS-mCD8-GFP	This paper	N/A
D. melanogaster: w; 9D10-GAL4, UAS-mCD8-GFP; +	This paper	N/A
D. melanogaster: w; UAS-mCD8-GFP; UAS-Dicer2, 9D11-GAL4	This paper	N/A
D. melanogaster: w; UAS-Delta; 9D11-GAL4, UAS-mCD8-GFP	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mcherry; UAS RNAi *KDELR*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mcherry; UAS- RNAi *Syx18*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mcherry; UAS- RNAi *γCOP*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mCD8-GFP; UAS-RNAi *KDELR*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mCD8-GFP; UAS-RNAi *Syx18*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80, UAS-mCD8-GFP; UAS-RNAi *γCOP*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; UAS-lacZ; UAS-RNAi *KDELR*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; UAS-lacZ; UAS-RNAi *Syx18*, UAS-Dicer2	This paper	N/A
D. melanogaster: w; wor-GAL4, ase-GAL80; Delta::GFP	This paper	N/A
D. melanogaster: w; UAS-γCOP; +	This paper	N/A
D. melanogaster: w; +; UAS-KDELR	This paper	N/A
D. melanogaster: UAS-Rpr; +; UAS-Hid	This paper	N/A
D. melanogaster: w; UAS-RNAi *Delta*; UAS-RNAi *numb*	This paper	N/A
D. melanogaster: w; UAS-RNAi *Delta*; UAS-RNAi *brat*	This paper	N/A
D. melanogaster: w; +; UAS-RNAi *Sec20*	TsingHua Fly Center (THU)	THU1448
D. melanogaster: w; UAS-RNAi *Ykt6*; +	THU	THU5021
D. melanogaster: w; UAS-RNAi *KDELR*; +	THU	THO4777.N
D. melanogaster: w; +; UAS-RNAi1 *β'COP*	THU	THU3447
D. melanogaster: w; +; UAS-RNAi *βCOP*	THU	THU4854
D. melanogaster: w; +; UAS-RNAi *δCOP*	THU	THU3459
D. melanogaster: w; +; UAS-RNAi *γCOP*	THU	THU3484
D. melanogaster: w; +; UAS-RNAi *εCOP*	THU	THU3495
D. melanogaster: w; +; UAS-RNAi *Delta*	THU	THU1537
D. melanogaster: w; +; UAS-RNAi *numb*	THU	THU1674
D. melanogaster: w; +; UAS-RNAi *brat*	THU	THU3660
D. melanogaster: w; +; UAS-RNAi *Serrate*	THU	THU3681

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: UAS-RNAi1 *Syx18* TRiP.JF02263}attP2	Bloomington Drosophila Stock Center (BDSC)	BDSC: 26721; FBst0026721
D. melanogaster: UAS- RNAi4 *KDELR* TRiP.HMC05779}attP40	BDSC	BDSC: 64906; FBst0064906
D. melanogaster: hs-flp, elav-GAL4, UAS-mCD8-GFP; +; +	BDSC	BDSC: 5146; FBst0005146
D. melanogaster: w; FRT40A, tub-GAL80; +	BDSC	BDSC: 5192; FBst0005192
D. melanogaster: w; +; UAS-PntP1	BDSC	BDSC: 869; FBst0000869
D. melanogaster: w; +; UAS-CycE	BDSC	BDSC: 4781; FBst0004781
D. melanogaster: w; +; Delta::GFP	BDSC	BDSC: 59819; FBst0059819
D. melanogaster: w; UAS-Notch^{FL}; +	BDSC	BDSC: 52309; FBst0052309
D. melanogaster: w; UAS-Bip^{DN}; +	BDSC	BDSC: 5841; FBst0005841
D. melanogaster: w; UAS-P35; +	BDSC	BDSC: 5072
D. melanogaster: w; UAS-RNAi1 *Use1*; +	Vienna Drosophila Resource Center (VDRC)	v100019; FBst0471893
D. melanogaster: w; +; UAS-RNAi2 *Use1*	VDRC	v42549; FBst0464643
D. melanogaster: W; UAS-RNAi2 *Syx18*; +	VDRC	v105113; FBst0476941
D. melanogaster: W; +; UAS-RNAi2 *KDELR*	VDRC	v9235; FBst0471424
D. melanogaster: w; UAS-*KDELR* RNAi3; +	VDRC	v9236; FBst0471425
D. melanogaster: w; UAS-Delta; +	Core Facility of Drosophila Resource and Technology	BCF198
D. melanogaster: w; FRT40A, *KDELR*^{K00311}; +	Fly ORF	F111125; FBst0313949
D. melanogaster: w; FRT40A, *KDELR*^{EY07077}; +	Fly ORF	F114572; FBst0317280
D. melanogaster: w; +; UAS-Syx18	Fly ORF	F003037; FBst0502014
Oligonucleotides		
KDELR forward primer: tgaatagggaattgggaattcATGCAGTGGCT CAAAATGAAGC	This paper	N/A
KDELR reverse primer: cgcagatctgttaacgaattcTTACAGCTCCTC GTGCACGTT	This paper	N/A
Software and algorithms		
ImageJ	https://imagej.net/	RRID:SCR_003070
GraphPad Prism (version 6.0.0)	http://www.graphpad.com/	RRID:SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Su Wang (wangsu@seu.edu.cn).

Materials availability

Fly lines generated in this study will be made available from the lead contact upon request.

Antibodies generated in this study will be made available from the lead contact upon request.

Data and code availability

- The datasets and images generated during this study are available from the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fly strains and genetics

Drosophila melanogaster fly stocks were mainly obtained from BDSC, THU or VDRC. Pnt-lacZ (on 3rd), E(spl)mγ-GFP (on 3rd), Delta-lacZ (on 3rd), 9D10-GAL4 (on 2rd), 9D11-GAL4 (on 3rd) were obtained from other labs. A full list of the fly lines used in this study can be found in Table S1. Flies were reared at 25°C on standard medium. For RNAi knockdown, animals were raised at 29°C after larval hatching to boost the efficiency.

METHOD DETAILS

Immunohistochemistry and confocal microscopy

Larval brains were dissected in 1X PBS buffer, fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature and then washed three times in PBS with 0.3% Trition X-100 (PBST). Brains were blocked in 2% BSA for 1 hour at room temperature and then antibodies were added and incubated overnight at 4°C. The primary antibodies were listed in key resources table. Images were recorded on a Zeiss LSM700 confocal microscope.

MARCM clonal analysis

To successfully generate MARCM clones, we used the well-established methods:[47] early larvae were heated shock at 37°C for 1.5 hours, and at 10-16 hours after the first heat shock for second heat shock. And then, larvae were further aged at third larvae at 25°C for dissection and immunostaining.

Hs-flp, elav-GAL4, UAS-mCD8-GFP; FRT40A, tub-GAL80 flies were crossed with FRT40A as control. FRT40A, *KDELR*^{K00311}and FRT40A, *KDELR*^{EY0707} were used as experimental groups, respectively.

Plasmid construction and generation of transgenic line

To generate UAS-KDELR fly line, the cDNA of KDELR was cloned into the pUAST-attB vector. The transgenic plasmids were verified by DNA sequencing before transformation. The success plasmids were injected and integrated into the third chromosome of flies. The primers used in this experiment were listed in key resources table.

Generation of ase antibody

To generate effective anti-Asense antibodies, two rabbits were injected with the synthetic peptide CLSDESMIDAIDWWEAHAPKSNGACTNLSV corresponding to a fragment in the C-terminal domain of Ase protein.[96] With the assistance of MERR YBIO, we successfully obtained Rabbit anti-Ase antibody.

Statistical method of pros into nuclear

The ratio of the number of type II NBs with ectopic expression of Pros ($GFP^+Dpn^+Pros^+$) in each brain lobe to the total number of type II NBs ($GFP^+Dpn^+Pros^-$) was calculated to test whether type II NBs have undergone premature differentiation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software) software and data are presented as the mean ± standard error of mean (SEM). The total number of animals quantified, p values, and significance levels are indicated in each Figure legend.