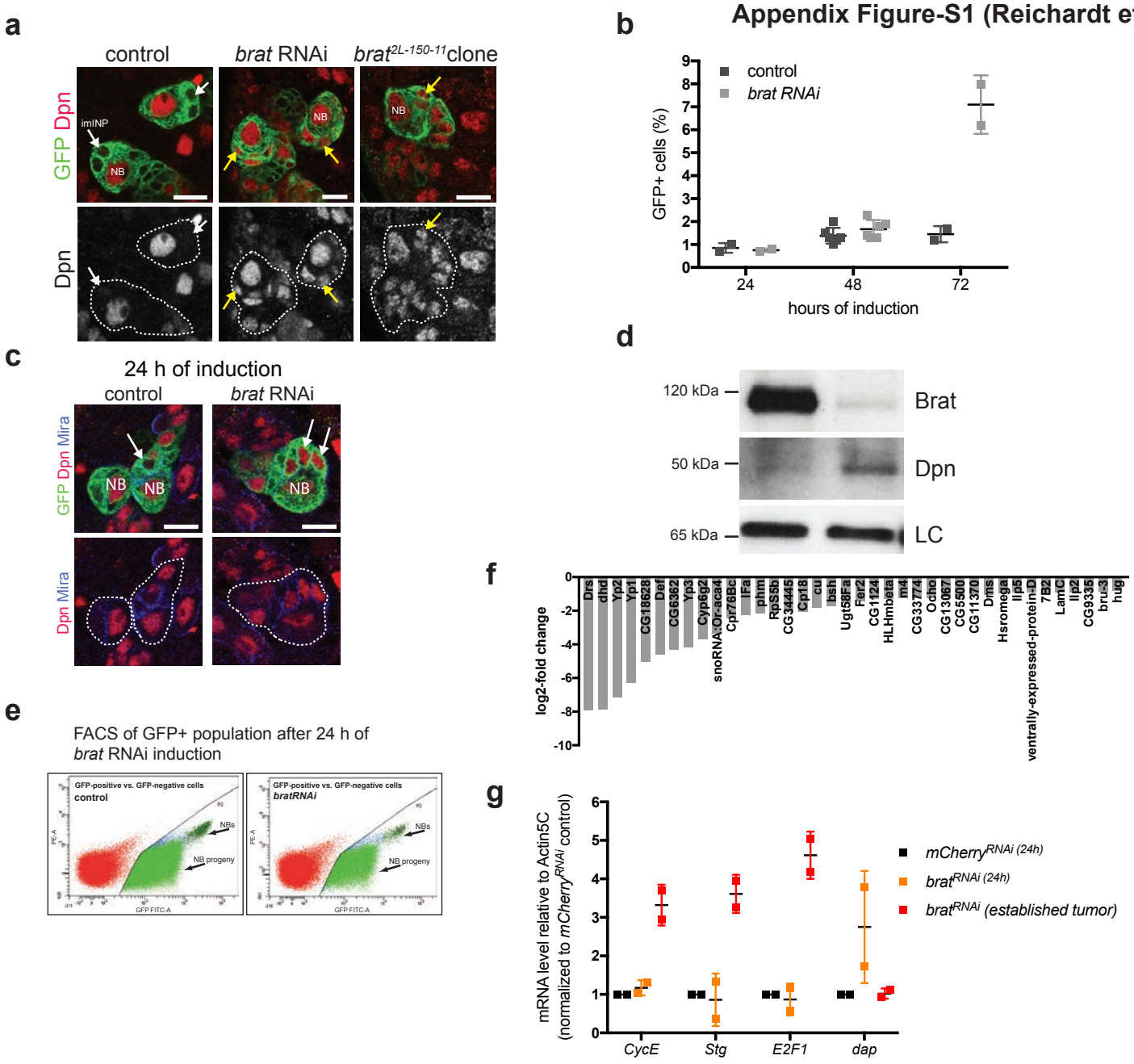
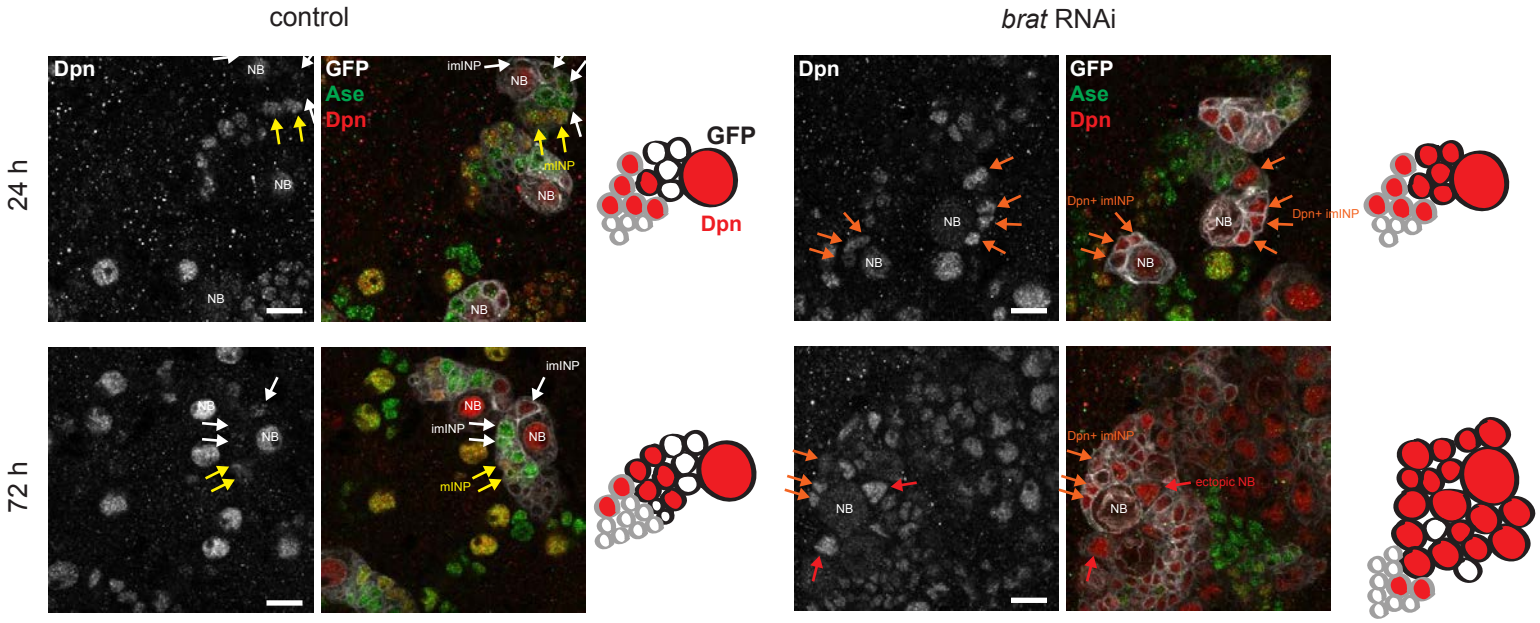
**Appendix – Table of contents**




**Appendix Figure S1 (related to Figure 1).**

(**a**) Close-up images of type II NB lineages marked with membrane-bound GFP (green) and stained for Dpn (red). imINPs of control lineages do not show Dpn expression (white arrows). In lineages expressing *brat* RNAi or in *brat*$^{2L\text{-}150\text{-}11}$ mutant lineages NB daughter cells kept Dpn expression (yellow arrows). (**b**) Percentage of GFP-positive cells within a FACS population of control versus *brat* RNAi 3rd instar larval brains at indicated time points. Note that after 24 h and 48 h the amount of GFP+ cells is about the same in control and *brat* RNAi larval brains, whereas after 72 h the amount of GFP-positive cells increased 7 fold. (**c**) Close-up images of control and *brat* RNAi type II NB lineages marked by membrane-bound GFP (*wor-Gal4, ase-Gal80*) and stained for Dpn (red) and Mira (blue). Note that after 24 h of *brat* RNAi induction Dpn is not repressed in imINPs. (**d**) Western Blot of FACS-sorted GFP-positive cells from control and *brat* RNAi larval brains 24 h after induction reveals reduced Brat and increased Dpn protein levels. LC, loading control anti-Lamin. (**e**) FACS plots (y-axis: size; x-axis: GFP intensity) reveal similar populations of control and *brat* RNAi after 24 h of induction. (**f**) Plot showing log2-fold change of the expression of downregulated genes upon *brat* RNAi (one independent experiment). (**g**) qPCR analysis of cell-cycle related genes expression in FACS-sorted control and *brat* RNAi type II lineages (induced with *wor-Gal4, ase-Gal80*) after 24 h of induction or from established *brat* tumor cells (no tub>Gal80TS) (two independent experiments).

Pictures and plots are representative of three independent experiments if not otherwise indicated. Error bars represent standard deviation. Scale bars, 10 µm.

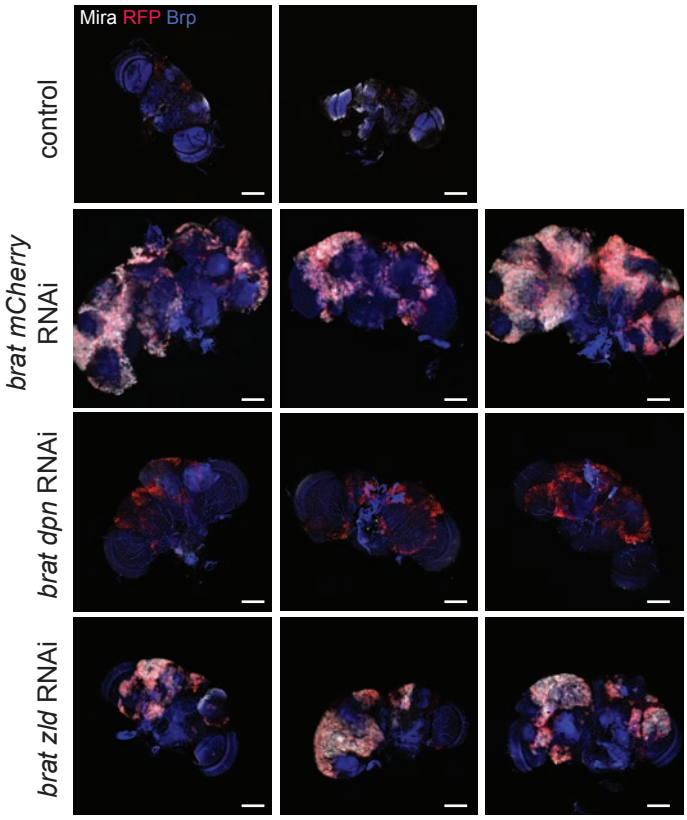



**Appendix Figure S2 (related to Figure 1).**

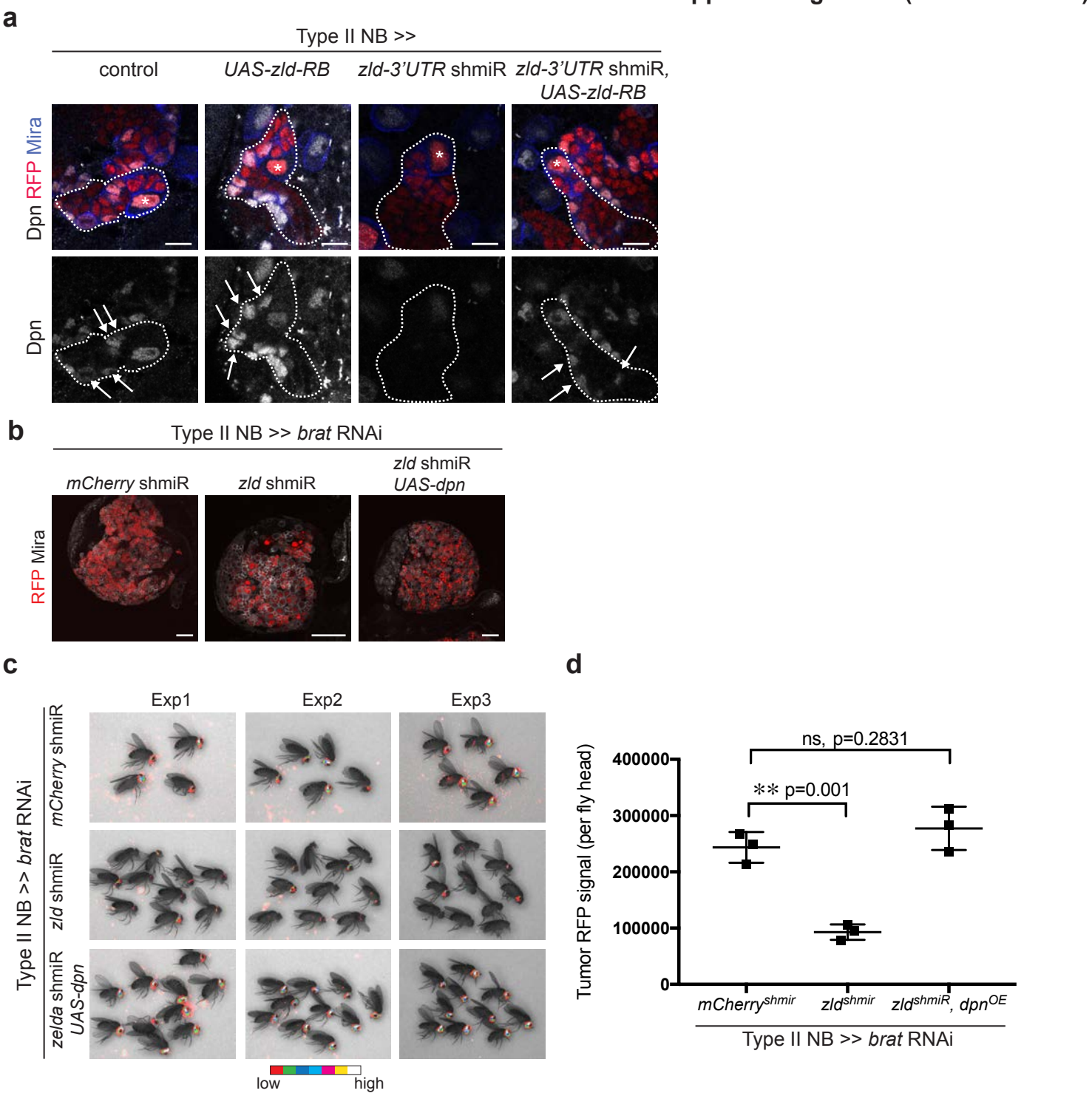
Close-up images of type II NB lineages 24h or 72h after control or *brat* RNAi, marked with membrane-bound GFP (grey) and stained for Dpn (red (merge picture) or grey (individual channel)) and Ase (green). imINPs of control lineages do not show Dpn expression (white arrows). In lineages expressing *brat* RNAi NB immediate daughter cells remain Dpn-positive (orange arrows) and further develop into ectopic NB (red arrows) after 72h of RNAi.

Pictures are representative of three independent experiments.

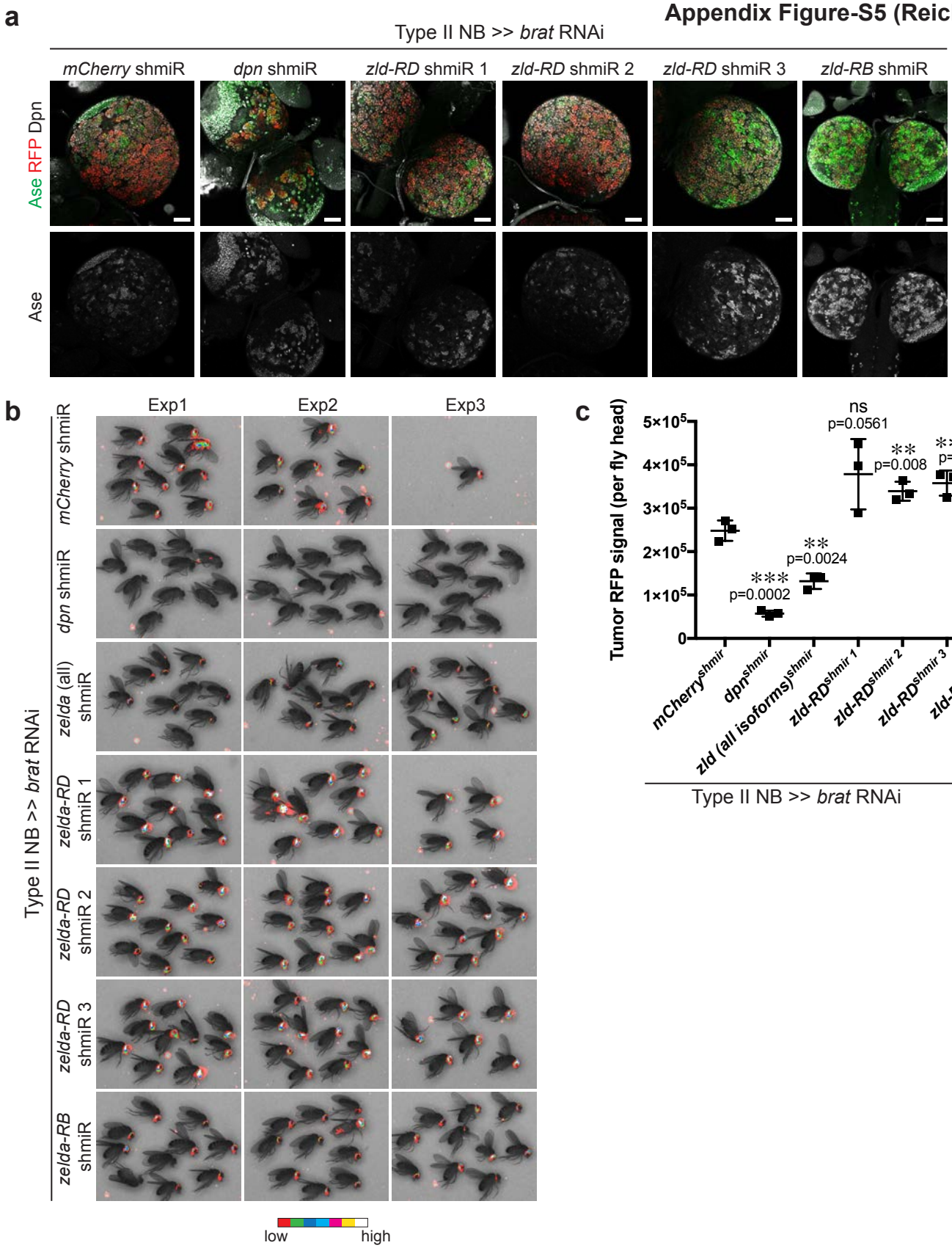
Scale bars, 10 µm.



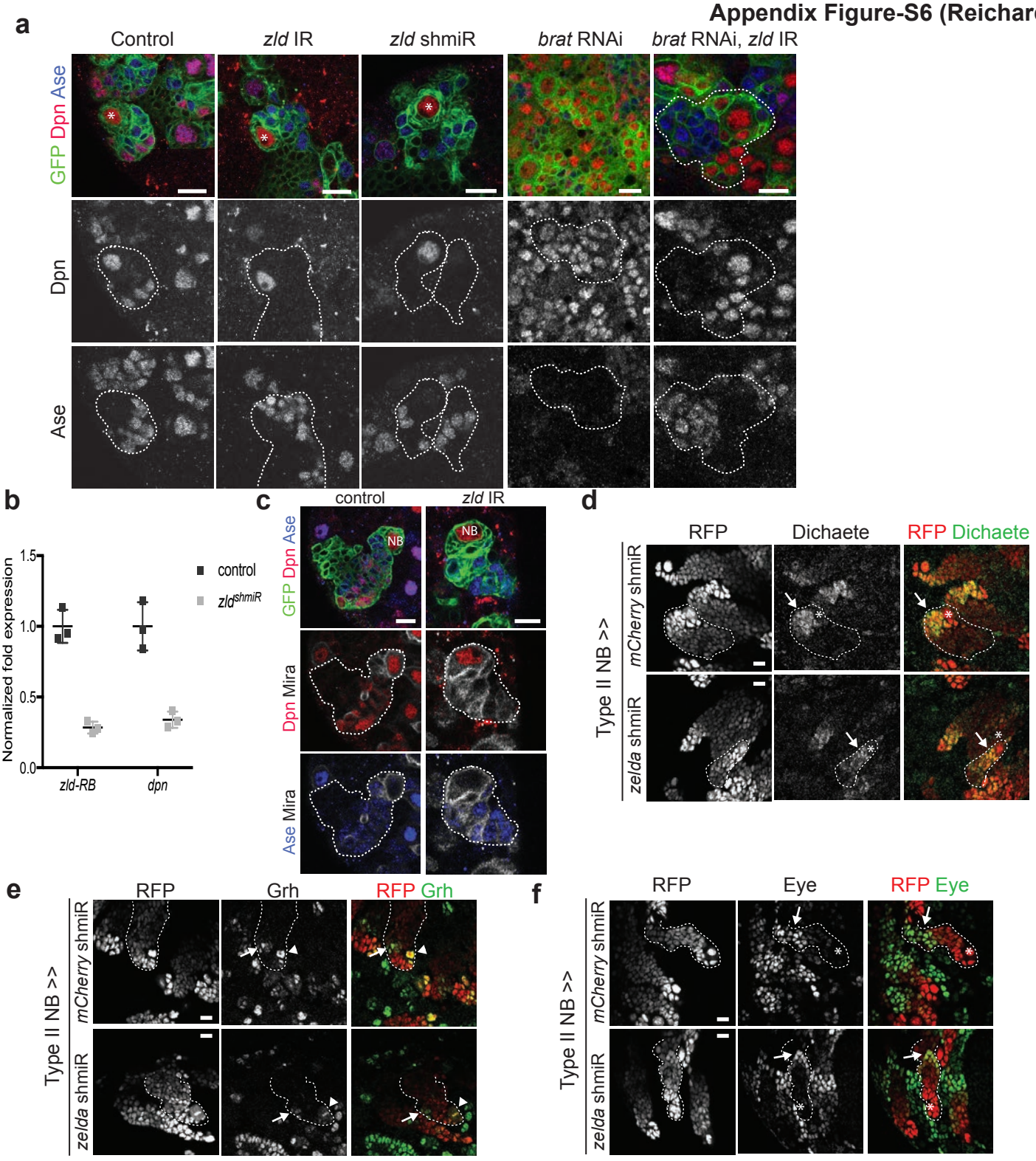

**Appendix Figure S3 (related to Figure 2).**
Images of adult brains of control, *brat* RNAi *mCherry* shmir, *brat* RNAi *dpn* RNAi and *brat* RNAi *zld* shmir (all isoforms) induced by *wor-Gal4, ase-Gal80*. Type II NB lineages are marked with stinger::RFP (red). Brains are stained for Bruchpilot (Brp, blue) and Mira (white). *brat* RNAi adult brains are overgrown by Mira-positive NB-like cells, whereas upon *brat zld* or *brat dpn* double knockdowns tumors are reduced in size.
Pictures are representative of two (control) to three (other conditions) independent experiments.
Scale bars, 120 µm.




**Appendix Figure S4 (related to Figure 2).**
(**a**) Close-up images of larval brains expressing nuclear RFP together with *zld-RB*, *zld* shmiR (targeting the 3'UTR of *zld-RB*) or both together from type II NB driver (*wor-Gal4, ase-Gal80*) and stained for Dpn (grey) and Mira (blue). Unlike the three other conditions, *zld* shmiR showed no Dpn+ INP progenies. (**b**) Overview images of larval brains expressing *brat* RNAi and nuclear RFP together with *mCherry* shmiR (negative control) or *zld* shmiR alone or together with *dpn* overexpression from type II NB driver (*wor-Gal4, ase-Gal80*) and stained for Miranda (grey). Scalebars, 50 µm. (**c, d**) Tumor burden analysis of adults with *brat* RNAi and nuclear RFP together with *mCherry* shmiR (negative control), *zld* shmiR alone, or *zld* shmiR together with *UAS-dpn* expressed from type II NB driver (*wor-Gal4, ase-Gal80*) (three independent experiments). *zld* shmiR show significantly less tumor burden compared to the two other conditions.
Pictures and plots are representative of three independent experiments. Error bars represent standard deviation. Statistical analyses were done using unpaired T-test. *p<0.05 **p <0.01 ****p<0.0001. Scale bars, 10µm.



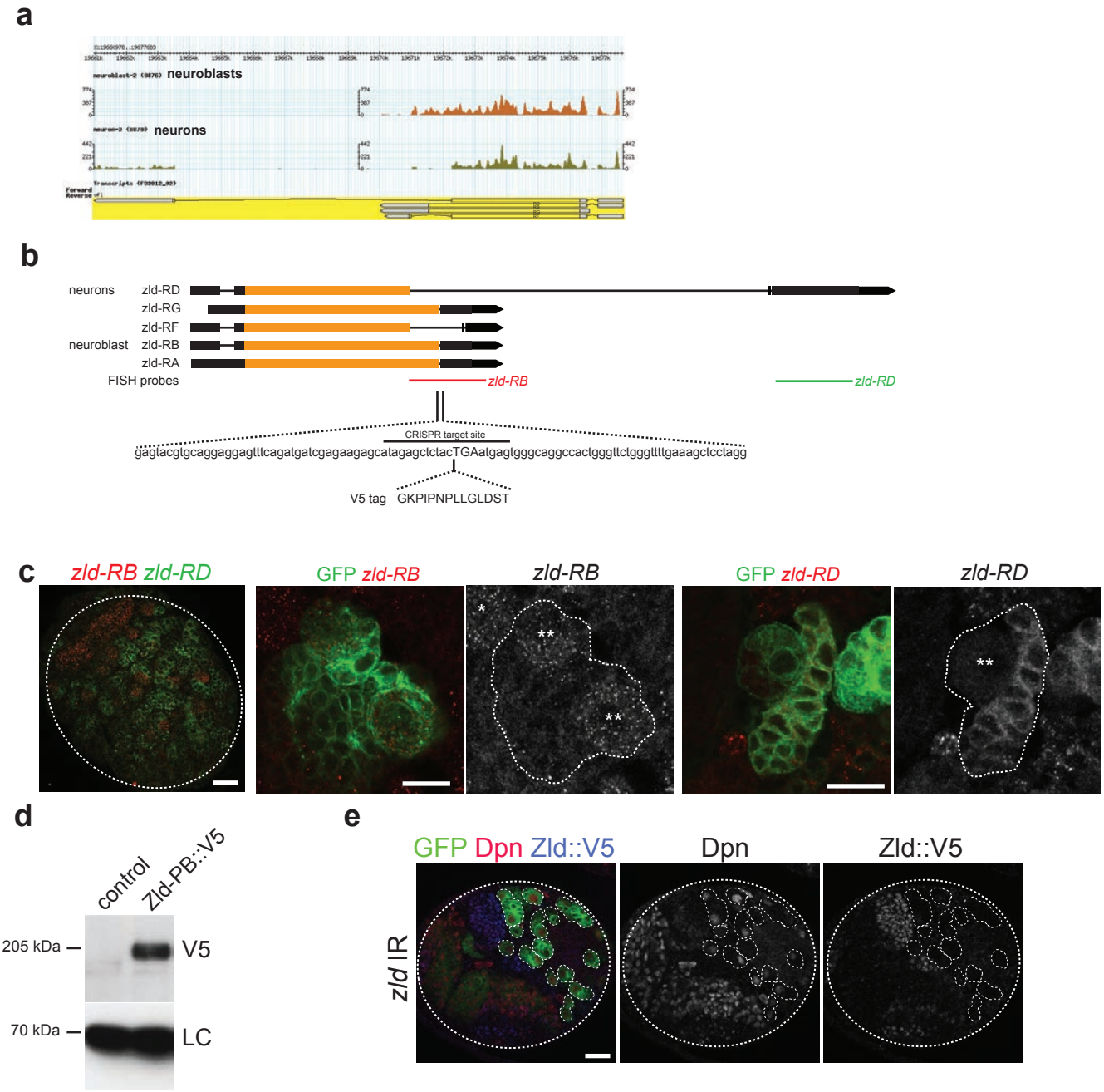

**Appendix Figure S5 (related to Figure 2).**
(**a**) Overview images of larval brains expressing *brat* RNAi and nuclear RFP together with *mCherry* shmiR (negative control), *dpn* shmiR (positive control), *zld-RD* specific shmiRs or *zld-RB* specific shmiR from type II NB driver (*wor-Gal4, ase-Gal80*) and stained for Dpn (grey) and Ase (green). Unlike *mCherry* and *zld-RD* shmiRs, *zld-RB* shmiR brat-induced tumor is smaller and contained a majority of Ase+ tumor (RFP+) cells. (**b, c**) Tumor burden analysis of adults with *brat* RNAi and nuclear RFP together with *mCherry* shmiR (negative control), *dpn* shmiR (positive control), *zld* shmiR (all isoforms), *zld-RD* specific shmiRs or *zld-RB* specific shmiR expressed from type II NB driver (*wor-Gal4, ase-Gal80*). *dpn*, *zld* (all isoforms) and *zld-RB* shmiRs show significantly less tumor burden compared to *mCherry* shmiR control, while *zld-RD* shmiRs show similar or significantly higher tumor burden.
Pictures are representative of three independent experiments. Error bars represent standard deviation. Statistical analyses were done comparing mCherry with the other shmiRs using T-test. *p<0.05 **p <0.01 ***p<0.001. Scale bars, 50µm.



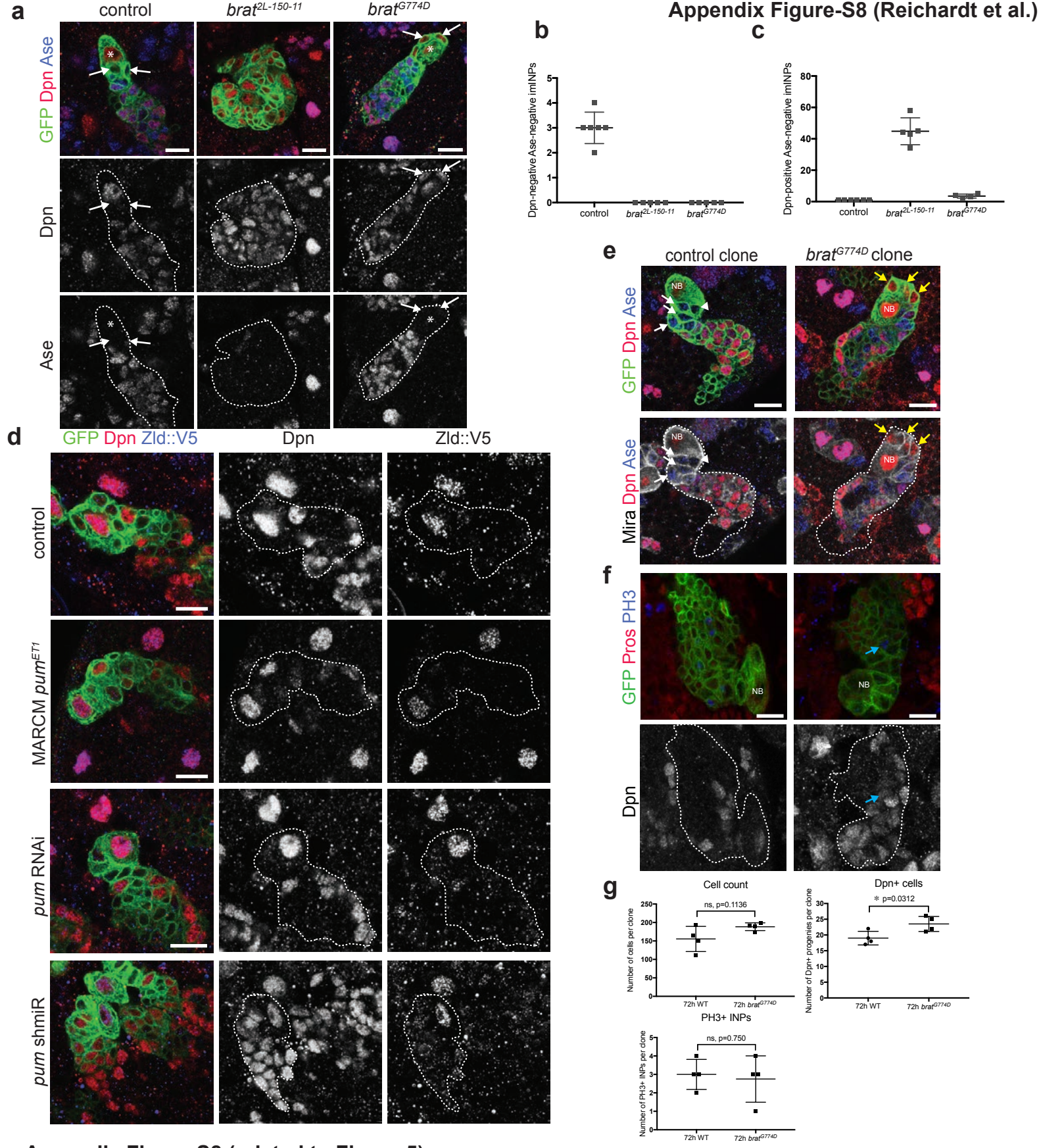

**Appendix Figure S6 (related to Figure 3).**

**(a)** Close-up images of type II NB lineages marked with membrane-bound GFP of control, *zld* IR, *zld* shmiR, *brat* RNAi or *brat* RNAi, *zld* IR (induced with *wor-Gal4, ase-Gal80*) stained for Dpn (red) and Ase (blue). Note that NB lineages of *zld* IR and *zld* shmiR contain several Ase-positive imINPs but no mINPs positive for Dpn and Ase. Asterisks indicate type II NB. **(b)** qPCR analysis of *zld* and *dpn* expression levels in larval brains of control versus *zld* shmiR. *zld* knockdown was induced by *insc-Gal4* driver. **(c)** Close-up images of control and *zld* IR type II NB lineages (induced with *wor-Gal4, ase-Gal80*) marked by membrane-bound GFP and stained for Dpn (red), Ase (blue) and Mira (grey). (**d, e, f**) Close-up images of type II NB lineages marked with nuclear RFP (red) of *mCherry* (control) and *zld* shmiR (induced with wor-Gal4, ase-Gal80) stained for Dichaete (**d**, green), Grainy head (Grh) (**e**, green) and Eyeless (Eye) (**f**, green). Note that NB lineages of *zld* shmiR contain less Dichaete, Grainy head and Eyeless-positive INPs but their sequential location (highlighted with white arrows) is not affected. White asterisks and arrowheads designate type II NB.

Pictures and plots are representative of three independent experiments. Error bars represent standard deviation. Scale bars, 10µm.

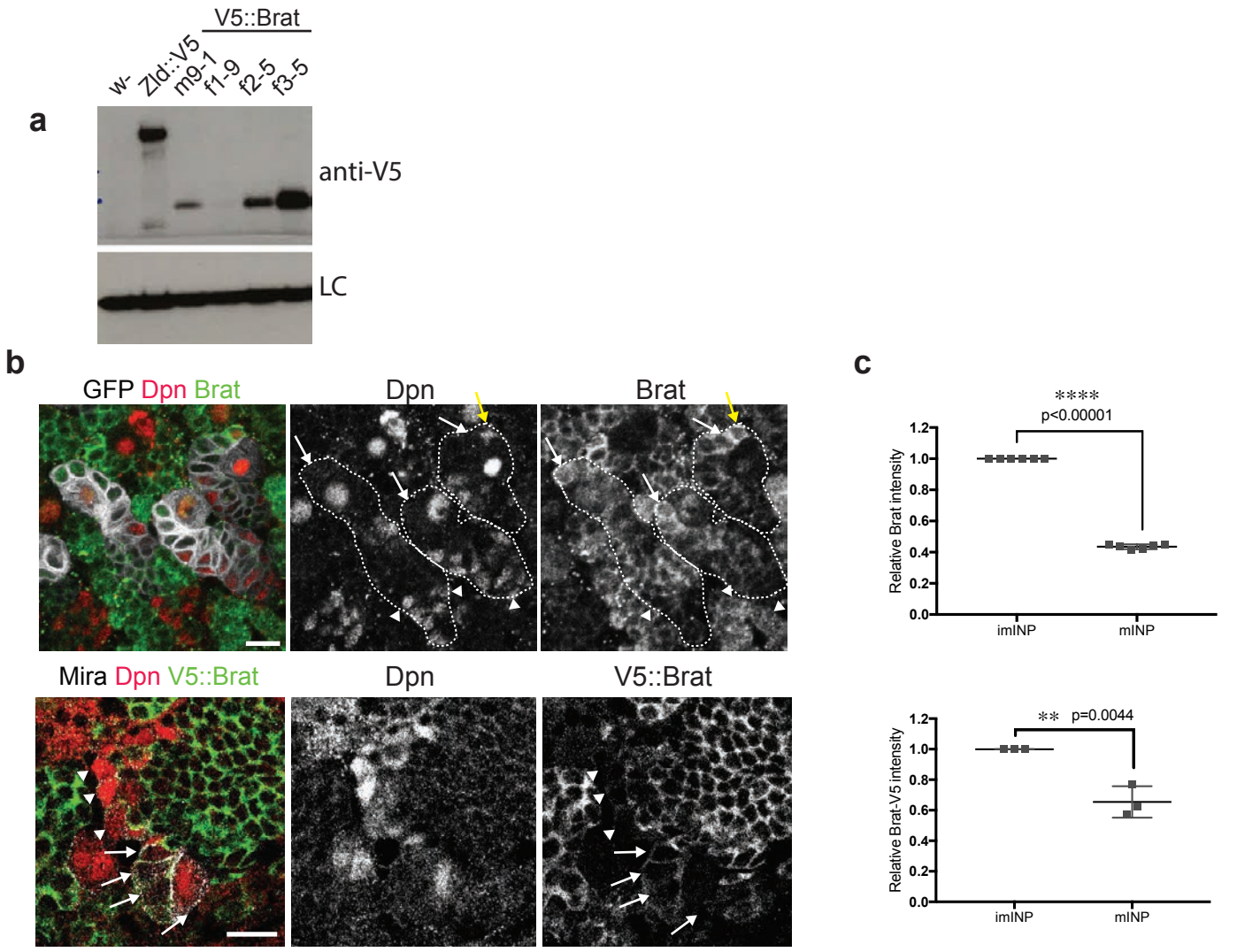



**Appendix Figure S7 (related to Figure 3).**
(**a**) Snapshot of RNA-seq tracks for NBs (upper track) and neurons (lower track) of the *Zld* locus. Note that *zld-RB* is specifically expressed in NBs, whereas *zld-RD* is expressed in neurons. (**b**) Schematic representation of *Zld* isoforms and FISH probes used to detect *zld-RB* and *-RD*. The oligo used for CRISPR/Cas9 targeted V5-tag insertion into *Zld-PB* locus is indicated. (**c**) Left panel shows brain lobe double-stained with FISH probes against *Zld-RB* (red) and *Zld-RD* (green). Remaining panels exhibit close-up images of type II NB lineages expressing membrane-bound GFP (green) and stained with either *Zld-RB* or *Zld-RD* FISH probe (red). *type I NB, **type II NB. (**d**) Western Blot showing Zld-PB::V5 expression. LC, loading control anti-Lamin. (**e**) Larval brain lobe expressing endogenous Zld::V5 and *zld* IR expressed in NB lineages by *insc-Gal4* marked by membrane-bound GFP and stained for Dpn and V5. Note that Zld::V5 is absent from NBs. Scale bars, 20µm. All pictures and blots are representative of three independent experiments.




## Appendix Figure S8 (related to Figure 5).

(**a**) Control and brat mutant MARCM clones marked by membrane-bound GFP (green) stained for Dpn (red) and Ase (blue) 48 h after clone induction. Arrows depict imINPs in control and *brat*$^{G774D}$ clones. White asterisks designate type II NB. (**b, c**) Quantification analysis of cell composition of control and *brat* mutant clones (five or more independent experiments). Error bars represent standard deviation. (**d**) Close-up images of type II NB lineages expressing *pum* RNAi or shmiR and *pum*$^{ET1}$ MARCM type II NB clone stained for Dpn (red) and Zld::V5 (blue). Dpn and Zld display a normal expression pattern. (**e**) Close-up images of control and *brat*$^{G774D}$ type II NB 48h clones marked with membrane-bound GFP (green) and stained for Dpn (red), Ase (blue) and Mira (grey). Note that *brat*$^{G774D}$ NB give rise to Ase-negative Dpn-positive cells (yellow arrows). Arrow-head: Ase-negative Dpn-negative imINP; White arrows: Ase-positive Dpn-negative imINP. **(f)** Close-up images of control and *brat*$^{G774D}$ type II NB 72h clones marked with membrane-bound GFP (green) and stained for Dpn (grey), Prospero(red) and PH3(blue). Blue arrows: PH3-positive Dpn-positive INP. **(g)** Quantifications of WT and *brat*$^{G774D}$ MARCM clones (n=4): Total cells, Dpn+ progeny cells and PH3+ INP cells (Dpn+ progenies) counts. Scale bars, 10um. Error bars represent standard deviation. T-test. *p<0.05. All pictures are representative of three independent experiments.



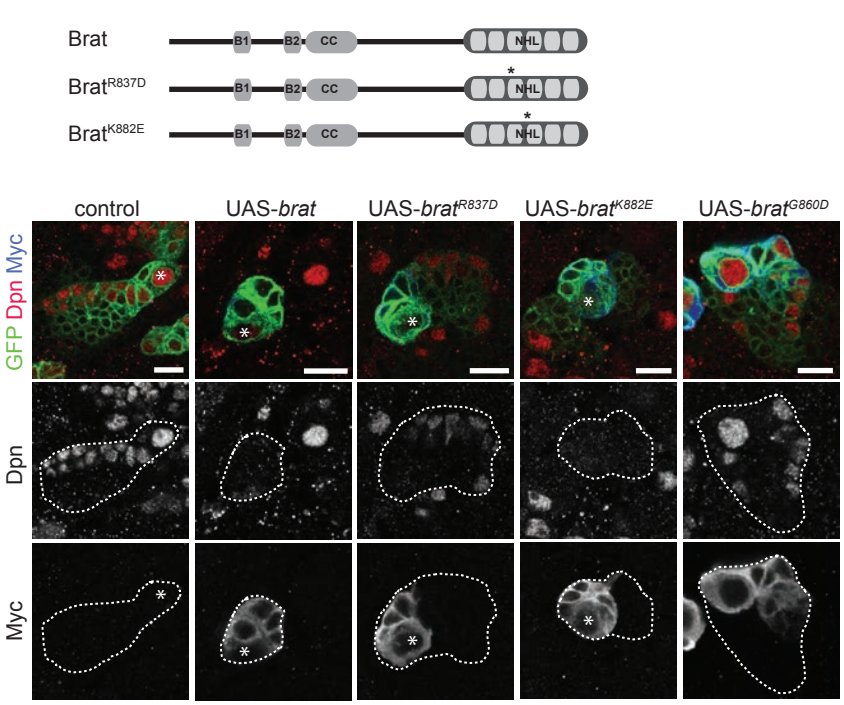

**Appendix Figure S9 (related to Figure 5).**

(**a**) Western Blot showing four V5::Brat transformants expression, in addition to Zld::V5 as positive control. f3-5 transformant was selected and further labeled as V5::Brat. LC, loading control anti-Lamin. (**b**) Close-up images of larval brain NB lineages marked by membrane-bound GFP (white, upper pannels) or endogenously V5-tagged Brat (lower pannels) and stained for Mira (white, lower pannels), Dpn (red) and Brat (green, upper pannels) or V5 (green, lower pannels). Brat accumulates in new born imINPs when Dpn disappears (white arrows) but is low in mINPs when Dpn reappears (arrow-heads). The yellow arrow indicates a very young new-born imINP in which half of the mother NB Dpn signal was inherited. (**c**) Quantification analysis of Brat fluorescence intensity measurement from larval brain sections (three or more independent experiments). Error bars represent standard deviation. All pictures and blots are representative of three independent experiments. Statistical analysis was done using T-test. **p<0.01, ****p<0.0001. Scale bars, 10 μm.

**a**

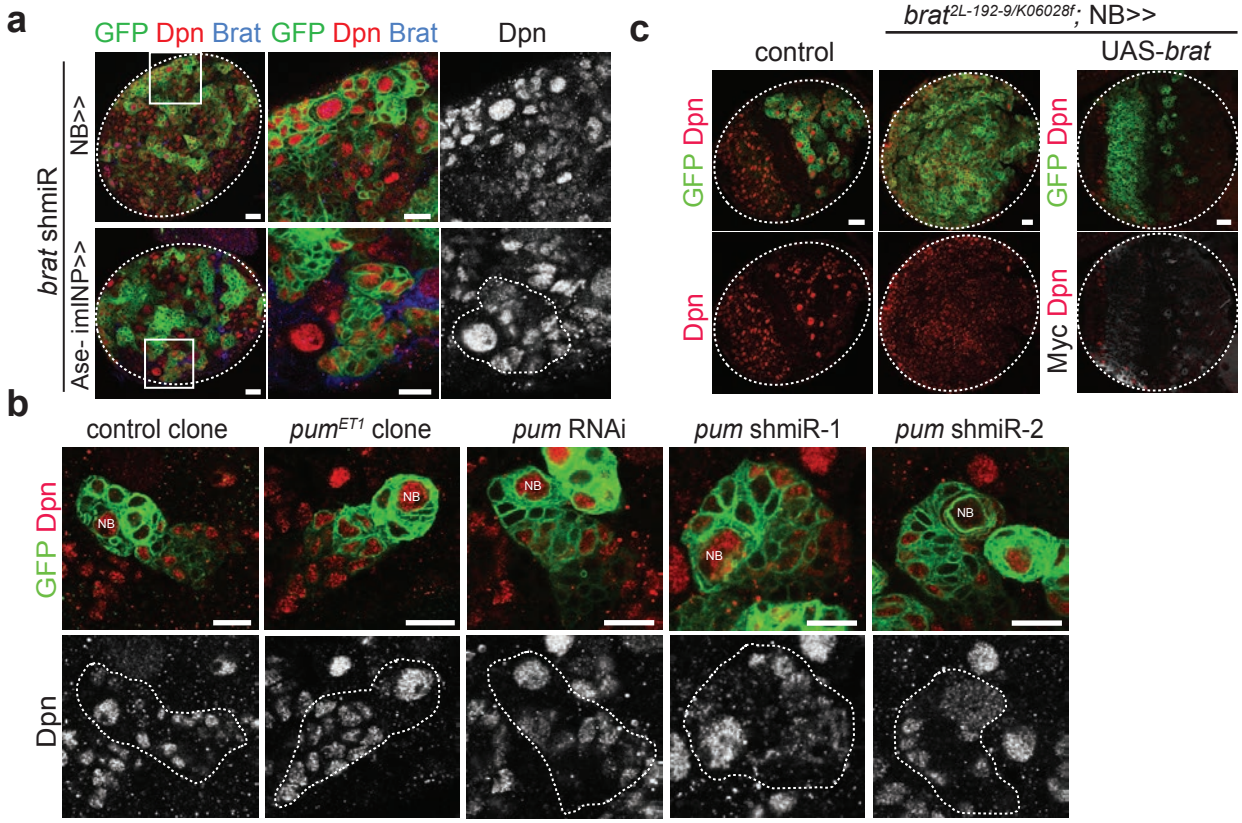
| [AU]UGUU[AGU] | padj | log2FoldChange | fpkm | hpergeomtric.p.val | drawn.expressed.m | n.expressed.m | n.expressed.noM | drawn.expressed |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.1 | 1 | 1 | 0.000149237 | 30 | 2883 | 1749 | 34 |
| 2 | 1.1 | 1 | 1 | 4.90E-05 | 23 | 1684 | 2948 | 34 |
| 3 | 1.1 | 1 | 1 | 3.96E-06 | 19 | 1029 | 3603 | 34 |
| 4 | 1.1 | 1 | 1 | 2.48E-06 | 15 | 633 | 3999 | 34 |
| 6 | 1.1 | 1 | 1 | 0.0005491 | 7 | 268 | 4364 | 34 |
| 8 | 1.1 | 1 | 1 | 0.000226828 | 5 | 123 | 4509 | 34 |
| 10 | 1.1 | 1 | 1 | 0.001414215 | 3 | 68 | 4564 | 34 |

**b**




**Appendix Figure S10 (related to Figure 5).**

(**a**) Summary of Fisher's exact test indicating significant enrichment of genes bearing one or more Brat binding motifs to be upregulated upon *brat* RNAi using the data from the transcriptome analysis. (**b**) Schematic representation of *brat* constructs, which have been used for overexpression in type II NB lineages. Close-up images of type II NB lineages marked with membrane-bound GFP expressing Myc-tagged wild-type *brat*, *brat*$^{R837D}$, *brat*$^{K882E}$ or *brat*$^{G860D}$ with *wor-Gal4, ase-Gal80* driver and stained for Dpn (red) and Myc (blue). White asterisks designate type II NB. Scale bars, 10 µm. All pictures are representative of three independent experiments.




**Appendix Figure S11 (related to Figure 6).**

(**a**) Overview and close-up images of larval brains expressing *brat* shmiR and membrane-bound GFP from type II NB driver (*wor-Gal4, ase-Gal80*) or Ase-negative imINP driver (*erm-Gal4* at 2nd chromosome) and stained for Dpn (red) and Brat (blue). Ectopic Dpn-positive type II NB-like cells are formed when *brat* shmiR is expressed in NBs and Ase-negative imINPs, whereas *brat* shmiR has no effect when expressed in Ase-positive imINPs. (**b**) Close-up images of type II NB lineages marked with membrane-bound GFP (green) and stained for Dpn (red). INPs of control, $pum^{ET1}$ mutant and *pum* RNAi lineages exhibit normal Dpn expression pattern. (**c**) Overview images of control and $brat^{2L\text{-}192\text{-}9/K06028}$ trans-heterozygous brain lobes expressing membrane-bound GFP (green) and stained for Dpn (red). Overexpression of Myc-tagged brat in NB lineages with *inscuteable-gal4* driver strongly diminished the formation of ectopic NB-like cells. Scalebars, 10 µm (a close-ups, b) and 20 µm (a overviews, c). All pictures and blots are representative of three independent experiments.

**Appendix Material and Methods**

*RNA Immunoprecipitation*

- dissect 100 control and 50 *brat*$^{K06028}$ mutant 3$^{rd}$ instar larval brains
- wash brains 2x in PBS
- fix brains in 0.5 % Formaldeyde/ PBS 20' rocking at RT
- quench fixed brains or cells in 0.125 M (final) Glycin, 0.01 % Triton X-100/ PBS 5' rocking RT
- wash brains 2x in PBS
- homogenize brains in 0.5 ml in **RIP lysis buffer**, incubate 10' on ice
- briefly sonicate brains with a microtip sonicator (Omni-Ruptor 250, Omni International; microtip, power output: 20) for 2 cycles, 20 s on, 60 s off on ice-water bath
- perform DNA digest: 25 mM $MgCl_2$
  5 mM $CaCl_2$
  6 µl DNase I
  3 µl RNase Inhibitor
  incubate 15' at 37°C
- centrifugate sample 20' at 16.000 g 4°C
- transfer supernatant into fresh tube
- freeze down 1/ 10 Input samples of lysate at -20°C
- incubate samples with 7.5 µl rabbit ant-Brat antibody overnight at 4°C
- wash 100 µl Protein-G-dynabeads (Life Technologies) 3x in RIP lysis buffer
- incubate antibody-protein complexes with 50 µl Protein-G-dynabeads 1 h at 4°C
- washings: 2x 10' with RIP lysis buffer
  1x 10' with **LiCl buffer**
  1x 10' with TE + 100 mM NaCl
- completely remove wash buffer and add 150 µl of **RIP elution buffer**
- incubate 15' at 65°C
- transfer supernatant to fresh tube
- thaw INPUT samples and add 100 µl of RIP elution buffer
- add 200 mM (final) NaCl and 20 µg Proteinase K to all samples and incubate 1 h at 42°C and 1 h at 65°C
- add 100 µl $H_2O$/ DEPC and 250 µl Phenol:Chloroform, invert and centrifuge 10' at 13000 rpm at 4°C
- transfer aqueous phase into new tube

- add 3 M NaAc, 1 µl GlycoBlue (Life Technologies, Ambion), 2.5 Vol 100 % EtOH and incubate 2 h at -20°C
- centrifuge 10' at 13000 rpm at 4°C
- wash pellet with 80 % EtOH
- air-dry pellet
- resuspend pellet in 20 µl nuclease-free $H_2O$
- perform First-strand synthesis (Superscript II) and qRT-PCR

**RIP lysis buffer**

50 mM Hepes pH 7.5
140 mM NaCl
1 mM EDTA
1 % Triton X-100
0.1 % Sodium-deoxycholate
fresh: Protease Inhibitor, 40 U/ ml RNasin

**LiCl buffer**

10 mM Tris pH 8
250 mM LiCl
1 mM EDTA
0.5 % NP-40
0.1 % Sodium-Deoxycholate
fresh: 40 U/ ml RNasin

**RIP elution buffer**

100 mM Tris pH 8
10 mM EDTA
1 % SDS
fresh: 40 U/ ml RNasin