SUPPLEMENTAL DATA

SUPPLEMENTAL EXPERIMENTAL PRECEDURES

Constructs- pUAST-GFP-Ada-FL, *pUAST-GFP-Ada-ΔEar*, *pUAST-GFP-Ada-Trunk*, *pUAST-GFP-Ada-N2*, and *pUAST-GFP-Ada-ΔNΔEar* were generated by fusing EGFP in frame at the 5' terminus of the *α-ada* coding sequence (Ada-FL [aa 1-940]; Ada-ΔEar [aa 1-713]; Ada-Trunk [aa 1-621], Ada-N2 [aa 1-400], Ada-ΔNΔEar [aa 81-713], respectively), followed by subcloning of the resulting fusion constructs into the *pUAST* vector. *α-ada* coding sequences were obtained by PCR. Primer sequences were available upon request. Transgenic flies for these *GFP-α-Ada* fusion constructs were generated in our lab or by BestGene Inc. USA. *pUAST-Numb-PTB-GFP-α-Ada* or *pUAST-Numb-PTB-GFP* was generated by inserting Nb-PTB sequence (aa 1-203) at the 5' terminus of GFP-Ada fusion or EGFP, respectively, followed by subcloning into the *pUAST* vector and microinjection, as described above.

ΔNm-Spdo was generated by replacing the YTNPAF motif [6-11aa] in the N-terminus of Spdo with TG (creating an AgeI restriction site). *pUAST-Spdo-GFP* and *pUAST-ΔNm-Spdo-GFP* were generated by inserting EGFP coding sequence in frame at the 3' terminus of Spdo and ΔNm-Spdo, respectively, followed by subcloning of the resulting fusion constructs into the *pUAST* vector.

To generate constructs for co-immunoprecipitation experiments, cDNA fragments encoding Ada-FL (aa 1-940), Ada-ΔEar5 (aa 1-850), Ada-ΔEar (aa 1-713) or Ada-Trunk (aa 1-621) were cloned in-frame into a *pcDNA3* expression vector harboring a Flag tag at the N-terminus. Similarly, Nb-FL (aa 1-557), Nb-N (aa 1-267), Nb-C (aa 268-557), Nb-ΔPTB (Δ79-203), Nb-ΔCT (aa 1-426), Nb-PTB (aa 1-203) or Nb-M (aa 204-364) tagged with a Flag epitope at the N-terminus were cloned into *pcDNA3* vector. *pcDNA3-Myc-Ada-FL* contains a N-terminal Myc tag in frame with the coding region of Ada-FL, while *pcDNA3-Numb-Myc* contains a C-terminal Myc tag in frame with the coding region of full-length Numb (1).

Cell culture and co-immunoprecipitation - HEK293T cells were maintained in DMEM medium (GIBCO) supplemented with 10% newborn calf serum (Lonza). For co-immunoprecipitation experiments, HEK293T cells were transfected with Fugene 6 transfection reagent (Roche) following the manufacturer's protocol. 48 hours after transfection, cells were harvested, washed with ice-cold PBS and incubated for 20 minutes with 450 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 1 (Sigma). The cell lysate was centrifuged for 5 minutes at 13,000 rpm and the supernatant was collected. The lysate was incubated with mouse anti-FLAG M2 antibody coupled to agarose beads (Sigma), with gentle mixing at 4^{o}C for 3-4 hours. Beads were washed with lysis buffer three times for 5 min each. Proteins were eluted from agarose beads by the addition of sample buffer (Bio-Rad), boiled for 5 min, and analyzed by Western blotting with the indicated antibodies.

Antibodies and Immunohistochemistry - For larval brain immunostaining, the primary antibodies used were: chicken anti-GFP (1:2000, Abcam), mouse anti-Elav (1:200, Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Pros (1:200, DSHB), mouse anti-N^{ECD} C458.2H (1:200, DSHB), mouse anti-Dl^{ECD} (1:200, DSHB), rabbit anti-Miranda (1:1000, F. Matsuzaki), mouse anti-Miranda (1:10, F. Matsuzaki), guinea pig anti-Dpn (1:1000, J. Skeath), guinea pig anti-

Sanpodo (1:1000, J. Skeath), guinea pig anti-Numb (1:1000, J. Skeath), rabbit anti-PKCζ C-20 (1:500, Santa Cruz Biotechnology, Inc.). Images were obtained on a Zeiss LSM510 confocal microscope.

Antibodies used for Western blotting were: rabbit anti-Ada (1:2000, NJ. Gay), rabbit anti-GFP (1:2000, Abcam), mouse anti-Flag M2 (1:1000, Sigma), rabbit anti-Flag (1:4000, Sigma), mouse anti-Myc (1:1000, Millipore), rabbit anti-Myc (1:1000, Cell Signaling Technology), guinea pig anti-Numb (1:20,000, J. Skeath).

Sequence analysis: The protein sequence of α-Ada Trunk domain (A) or Numb-N (B) were aligned using Multalin (2).

SUPPLEMENTARY REFERENCES

1. Ouyang, Y., Petritsch, C., Wen, H., Jan, L., Jan, Y. N., and Lu, B. (2011) *Development* **138**(11), 2185-2196
2. Corpet, F. (1988) *Nucleic Acids Res* **16**(22), 10881-10890

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. **Quantification of the relative distribution of Spdo immunofluorescence at the cell surface or cytoplasm of *α-ada*^{1} vs. control NBs or IPs.**

FIGURE S2. **AP2σ regulates Spdo endocytosis in NB lineages.** In an *AP-2σ*^{KG02457} mutant type II NB clone (encircled by dashed line), Spdo (red) localized predominantly to the plasma membrane of Mira^{+} primary NB (bracket), ectopic NBs or IP cells (yellow arrowheads), whereas a control NB outside of the clone (white open arrowheads) or its differentiating daughter cells (marked by dotted line) showed cytoplasmic Spdo localization. Scale bar: 10 μm.

FIGURE S3. **Supporting evidence for a functional significance of the novel interaction between α-Ada and Numb in regulating NB homeostasis.** *A*, schematic representations of Numb domain structures and various Numb deletion constructs. *B*, interaction between α-Ada and fragments of Numb. Both Nb-N and Nb-ΔCT interacted with Ada-FL, but with reduced affinity compared to Nb-FL. *C*, NBs in single larval brain lobes of various genotypes are marked with Mira. Quantification of data from (*C*) is shown in (*D*). Scale bars: 100 μm (*C*).

FIGURE S4. **Ada-Trunk is the minimal functional subunit in mediating NB homeostasis.** N-terminal or C-terminal deletions in Ada-Trunk (*A*) abolished the ability of Ada-Trunk to rescue the NB overproliferation (*C-E*) or Spdo cortical localization (*C'-E'*) phenotypes of *α-ada*^{1}mutants. Quantification of central brain NB number is shown in (*B*). Scale bars: 100 μm (*C-E*); 5 μm (*C'-E'*).

FIGURE S5. **A mutant form of Nb, Nb-TS4D, showed unusually tight interaction with Ada.** *A*, NB-specific overexpression of Nb-TS4D transgene resulted in strong NB (marked with Mira)

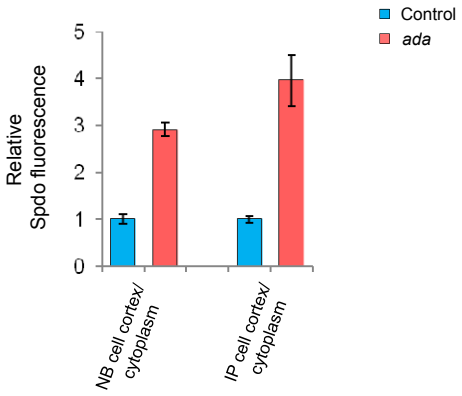
overproliferation phenotype. *B*, interaction between α-Ada and WT, TS4D or TS4A forms of Numb proteins. Nb-TS4D interacted with Ada-FL with extraordinarily stronger affinity, compared to Nb-WT or Nb-TS4A. Green arrowheads indicate the Numb protein bands. Scale bar: 100 μm.

FIGURE S6. **NB overproliferation phenotypes in *numb* mutant clones were completely suppressed by Numb-PTB-Ada.** Single type II lineage NB clones of $numb^{15}$ (*A*, *A'*), $numb^{15}$; *Elav*>Ada-FL (*B*, *B'*) or $numb^{15}$; *Elav*>Nb-PTB-Ada (*C*, *C'*) genotypes were marked with CD8-GFP (encircled by dashed line). Scale bars: 40 μm.

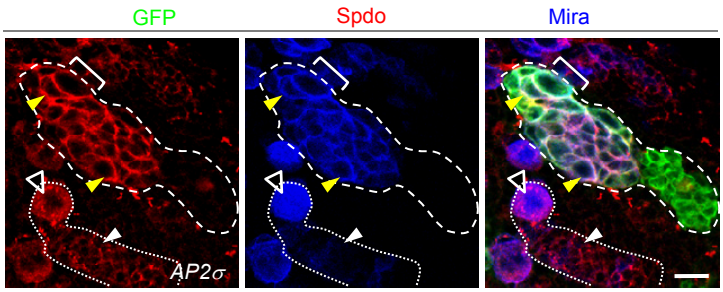
FIGURE S7. **Dl protein trafficking is not affected by Ada inactivation.** WT (*A-A''*) or α-ada^{1} (*B-B''*) mutant NBs at metaphase stage were triple labeled with Dl^{ECD}, Mira, and DNA. Delta showed cytoplasmic and vesicular distribution in both WT and *ada* mutant NBs. Scale bar: 10 μm.

FIGURE S8. **Sequence alignments of the newly identified interacting domains in α-Ada and Numb.** The protein sequence of α-Ada Trunk domain (A) or Numb-N (B) were aligned using Multalin. The alignments include orthologs from Drosophila (*Drosophila melanogaster*), mouse (*Mus musculus*) and human (*Homo sapiens*). Violet or green letters indicate residues with high (90%) or low (50%) consensus levels, respectively. Grey letters denote neural residues.

Supplemental Figure S1



Supplemental Figure S2



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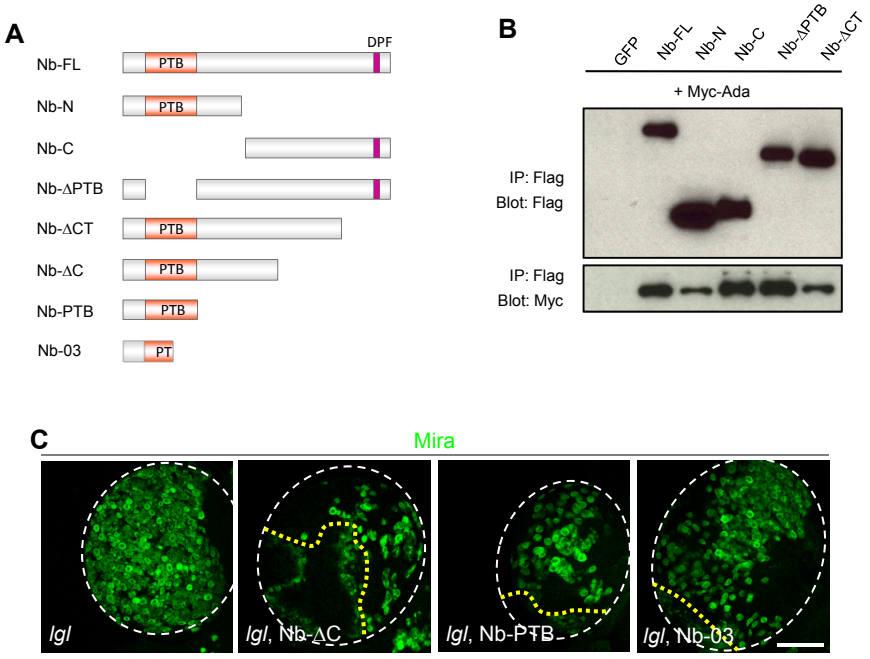

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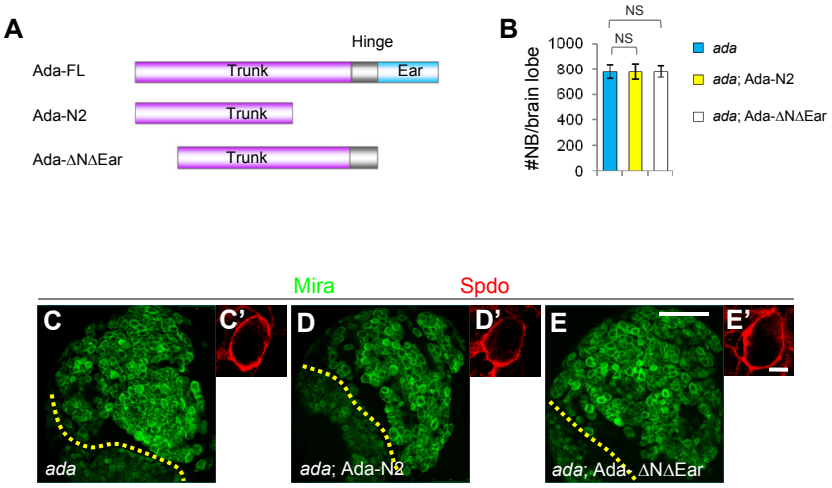

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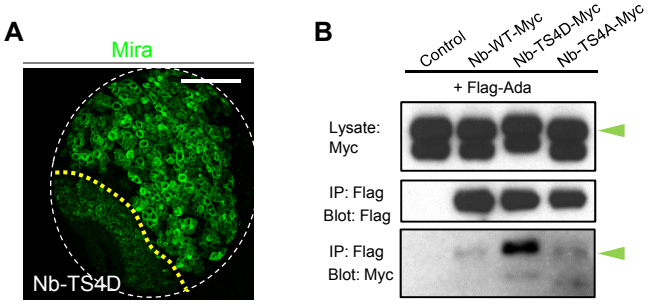

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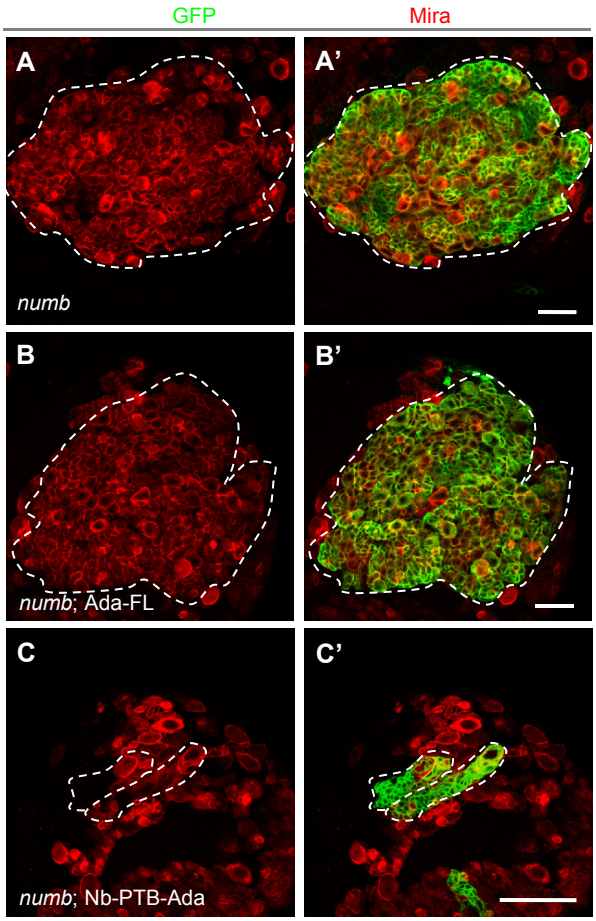
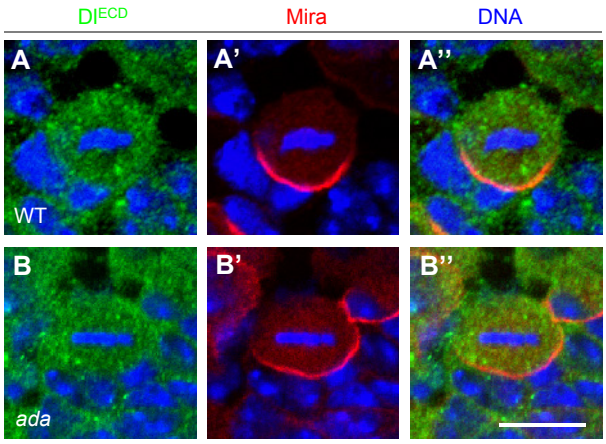


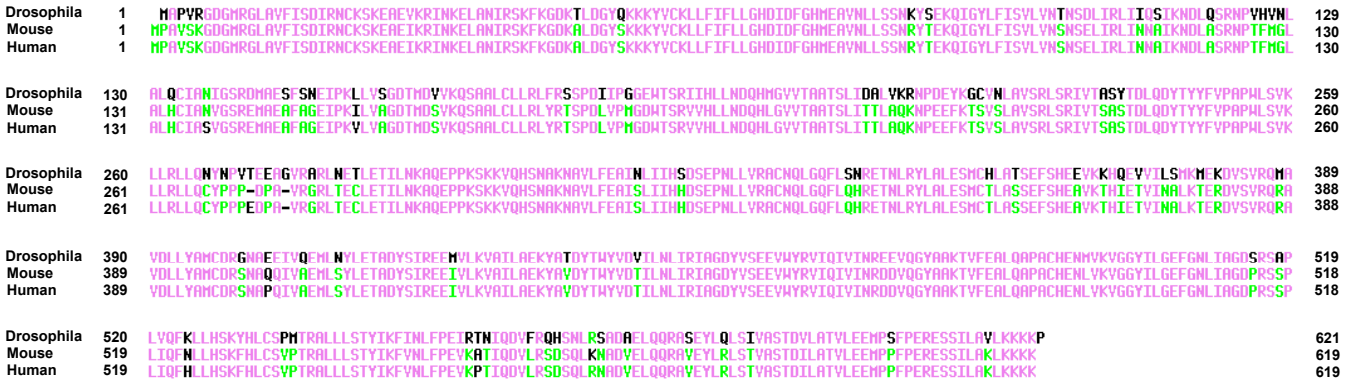
Supplemental Figure S7



Supplemental Figure S8

A

The Trunk domain of α-Adaptin



B

Numb-N