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# Supplementary Information for

## CAMSAP3 maintains neuronal polarity through regulation of microtubule stability

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**This PDF file includes:**

Supplementary text
Figs. S1 to S5
References for SI reference citations



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**Supplementary Information Text**

## Materials and Methods

### Knockout Mice.

A target vector to knockout *Camsap2* (Accession No. CDB1062K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) was designed to insert a loxP flanked neo selection cassette into the 5$^{th}$ exon (Fig. S2A). Genotyping was performed by PCR using the following primers to detect wild-type (428F/1033R) or *Camsap2* knockout (428F/734R) mice: 428F, 5'-TTGAAAAGGGCAGCATGGAG -3'; 1033R, 5'-GTGGCGTATGAGGAGGTCTTTC-3'; 734R, 5'-TGATCGGAATTCGATAGCGG-3'. *Camsap3* knockout mice were described previously (1). *Camsap3*$^{tm1a(EUCOMM)Wtsi}$ mice were obtained from the Wellcome Trust Sanger Institute (2-4). CAMSAP protein expression in these knockout mice was examined using rabbit polyclonal antibodies raised against the a.a. 551–904 region for CAMSAP2 (Proteintech, 17880-1-AP), and the a.a. 596–1076 region for CAMSAP3 (5). The experiments using mice were performed in accordance with protocol(s) approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch.

### Culture of Neurons and Cell Lines.

Embryonic hippocampi were dissected from E16-E17 mice, unless otherwise specified. Tissues were washed twice with a Hanks' balanced salt solution (HBSS), supplemented with 1 mM sodium pyruvate (Invitrogen), and incubated twice with 16U papain (Nacalai) at 37°C for 10 min. These tissues were placed in DMEM containing 1 mM DNase I (Sigma-Aldrich) at room temperature for 1 min, then washed twice with DMEM prior to suspension in Neurobasal Medium (Thermo Fisher Scientific) containing 5% fetal calf serum (Gibco), 5% B-27 (Invitrogen), 1% L-glutamine and 1% penicillin/streptomycin. We cultured 5 x $10^4$ hippocampal neurons on a 12-mm coverslip coated with 0.5 mg/ml poly-D-lysine (Sigma-Aldrich), and incubated for various periods.

Neuro-2a (N2a) and Caco2 cells were purchased from JCRB Cell Bank and the American Type Culture Collection, respectively. Cells were maintained in DMEM/F12 media (Wako) containing 10% fetal bovine serum (Wako), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen). To treat cells with nocodazole, its optimal concentration required for depolymerizing microtubules was preliminarily determined by observing α-tubulin networks in cells to be used. 2 and 10 μM nocodazole were chosen for treating N2a and hippocampal neurons, respectively. As a control, 0.1 % DMSO (the solvent for nocodazole) was added to cultures.

**RNA Interference and Plasmid Transfections.**

Hippocampal neurons or N2a cells were transfected with stealth siRNAs specific for the target proteins or control siRNAs, using Lipofectamine™ RNAiMAX Transfection Reagent according to the manufacturer's instructions (Invitrogen). Stealth siRNAs for CAMSAPs, αTAT1 and control were purchased from Invitrogen, which have the following sequences: siCAMSAP3 (MSS229833), 5'-ACAGUGGCAGCAGUUCUCCUGUCUU-3'; siCAMSAP2 (MSS228747), 5'-CCCUUCAGAAGAGACUUUAAAUGAA-3'; and siαTAT1 (MSS292331), 5'-GAGCCAUUAUUGGUUUCCUCAAAGU-3'. Lipofectamine™ RNAiMAX and 60 nM siRNA were diluted separately in Opti-MEM medium (Invitrogen), and the siRNA solutions were incubated with Lipofectamine™ RNAiMAX at room temperature for 15 min. The mixture was then dropped to the cell cultures containing antibiotic-free medium, and incubated for 6 hrs for N2a cells or 2 hrs for neurons at 37 °C. After 72 hrs, cells were subjected to further experiments.

The CAMSAP2 and CAMSAP3 plasmids were constructed as previously described (Tanaka et al., 2012). To construct the αTAT1 plasmid, αTAT1-wt and αTAT1-D157N cDNA were amplified from pEF5B-FRT-GFP-αTAT1 (Addgene, 27099) and pEF5B-FRT-GFP-αTAT1-D157N (Addgene, 27100), respectively. The Not1 site and kozak sequences were added to the 5' terminus, and the Sal1 site at the 3' end of *αTAT1-wt* and *αTAT1-D157N* gene. To obtain pCA-αTAT1-wt-GFP and pCA-αTAT1-D157N

plasmids, DNA oligo was integrated into Not1 and Sal1 sites of pCA-sal-GFP vector. For plasmid transfection, N2a cells and hippocampus neurons were transfected with Amaxa Nucleofector (Invitrogen), according to the manufacturer's instructions.

**Reagents and Antibodies.**

Nocodazole (M1404) and DAPI (D9542) were purchased from Sigma-Aldrich. Antibodies specific for CAMSAP3 were previously described (5). Other primary antibodies were purchased as follows: rabbit antibodies for CAMSAP2 (1:1000 dilution for immunoblotting; 1:500 dilution for immunostaining, Proteintech, 17880-1-AP), MAP2 (1:1000 dilution for immunostaining, Millipore, MAB3418), acetylated α-tubulin (1:1000 dilution for immunoblotting, Cell Signaling, 5335), GFP (1:500 or 1000 for immunostaining and immunoblotting, MBL, 598), Synapsin (1:500 dilution for immunostaining, Chemicon, AB1543), and His (1:400 for immunostaining, Santa Cruz, sc-804); mouse antibodies for Ankyrin-G (1:50 dilution for immunostaining, Invitrogen, 33-8800), GAPDH (1:1000 dilution for immunoblotting, Santa Cruz, 32233), Tau (1:1000 for immunostaining, Millipore, MAB3420), α-tubulin (1:1000 or 5000 for immunostaining and immunoblotting, Sigma-Aldrich, clone DM1A, T6199 and T9026), SMI312 (1:1000 dilution for immunostaining, Covance, SMI-312R), PSD95 (1:250 dilution for immunostaining, Thermo Fisher Scientific, MA1045), tyrosinated tubulin (1:500 for immunostaing, Sigma-Aldrich, T9028), acetylated tubulin (1:5000 dilution for immunoblotting, 1:500 or 1000 dilution for immunostaining, Sigma-Aldrich, clone 6-11B-1, T7451 and T6793), and βIII-tubulin (1:1000 for immunoblotting, Covance, MMS-435P) ; and rat antibodies for α-tubulin (1:1000 dilution for immunostaining, Merk Millipore, MAB1864). The secondary antibodies used were goat Alexa Fluor 488-, 568-, 555- and 647-conjugated anti-mouse, rabbit or rat IgG (1:1000 dilution for immunostaining, Invitrogen), sheep HRP-conjugated anti-mouse or anti-rabbit IgG (1:2500 dilution for immunoblotting, GE Healthcare).

**Immunoblotting.**

Hippocampal tissues or N2a cells were lysed in a lysis buffer containing 1% Nonidet 40, 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl, a protease inhibitor cocktail

(Roche) and 10% glycerol for 30 min on ice. Lysates were then centrifuged at 20,000*g* at 4°C for 15 min. Protein content was measured by Pierce BCA assay kit (Pierce). We dissolved 80 μg protein in a 6X SDS buffer, separated by SDS-PAGE, and transferred to PVDF membranes. Blots were blocked using 5% non-fat milk at room temperature for 30 min, and incubated with specific primary antibody at 4°C overnight, followed by incubation with a HRP-tagged secondary antibody at room temperature for 2 hrs. Proteins were detected using Western lightning plus ECL (PerkinElmer), Chemi-Lumi One Super (Nacalai), or Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen).

**Immunofluorescence Staining.**

For immunostaining for CAMSAPs, cells were fixed with 1 or 2% PFA and 4% sucrose in HBSS at room temperature for 20 min, and washed three times with 0.1% Tritox-100. 2% PFA fixation was also used for double-immunostaining for α-tubulin and acetylated tubulin. For double-immunostaining for CAMSAPs and α-tubulin or acetylated tubulins, cells were fixed in MeOH at -20 °C for 5 to 10 min. Cells were blocked with 3% BSA at room temperature for 30 min, incubated with specific antibodies at room temperature for 2 hrs, and subsequently with proper secondary antibodies. Coverslips were then mounted with FluorSave reagent (Millipore).

**Golgi Staining.**

Postnatal brains were subjected to Golgi staining using FD Rapid GolgiStain$^{TM}$ Kit (FD NeuroTechnologies), according to the manufacturer's protocol. Brains were sectioned into 200-μm slices by a cryostat. Images were acquired by Axioplan2 equipped with a cooled CCD camera (ORCA-R2, HAMAMATSU) through a Fluar 10x or Plan-Apochromat 20x or objective lens.

**Image Analysis.**

The images were obtained by an epi-fluorescence microscope (Axioplan2 or AxioImagerZ2, Carl Zeiss, Inc.) equipped with a cooled CCD camera (ORCA-R2, HAMAMATSU) or a LSM880 Airyscan confocal microscope (LSM880; Carl Zeiss, Inc.) through a Plan Apochromat 63x /1.40 NA oil emersion objective. Images were captured

by Axioplan2 or Zen2 for LSM880, and processed by Fiji software. Live imaging was performed by a laser confocal microscope IX71 (Olympus) with spinning-disc unite CSU-X1 (Yokogawa). Composite images were shown to cover an entire neuron, when necessary.

**Statistical Analysis**

The data were obtained from at least four independent experiments with indicated sample numbers, unless specified. Statistical analysis was performed using Student's t-tests (two-tailed) for Figs. 3A, 4B, 5C, and S3E, and Mann-Whitney test with GraphPad Prism 4 software for Figs. 2A, 2C, 3E, 5D, S3A, and S3C.

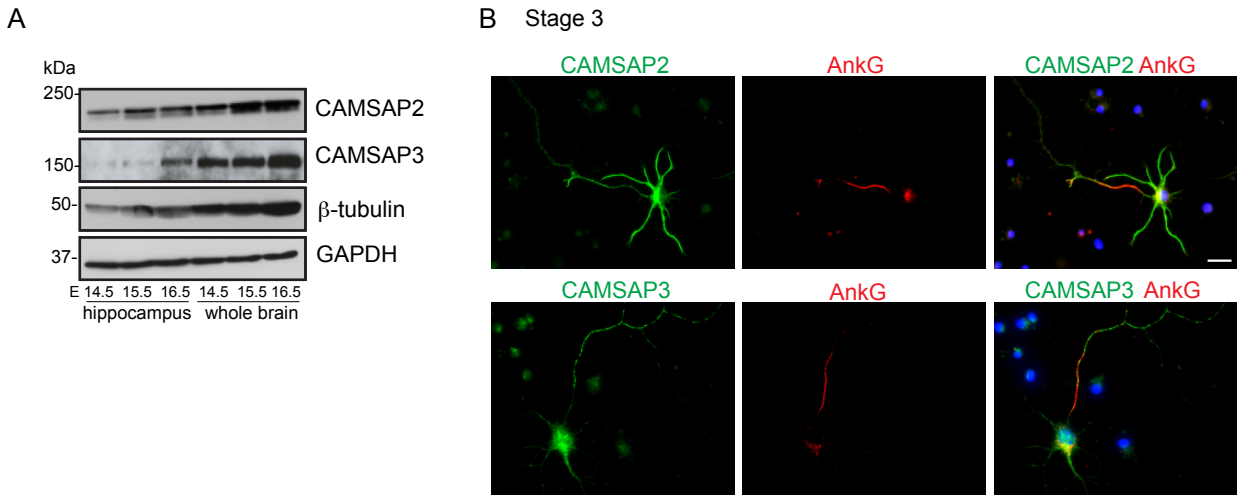
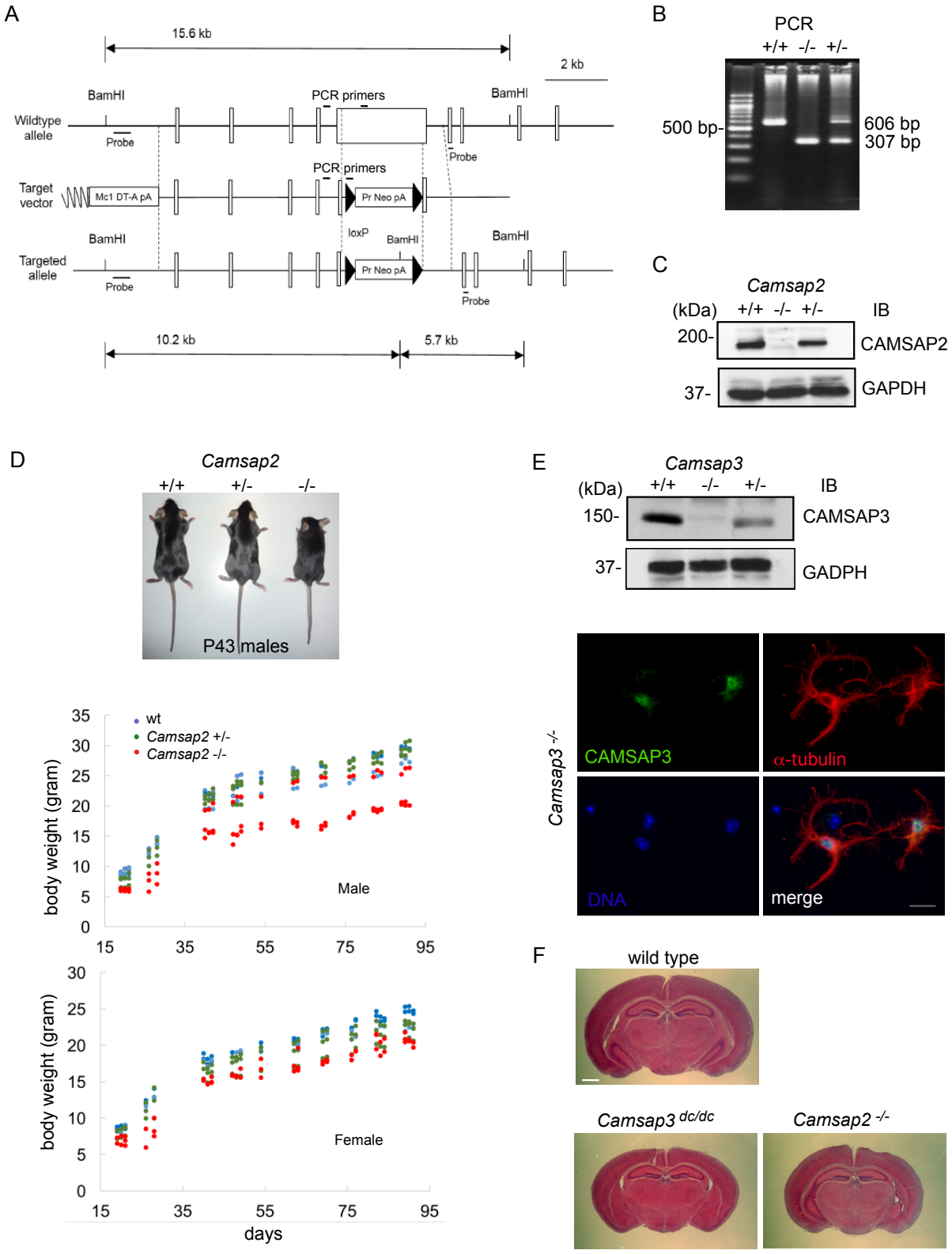



**Fig. S1. Expression and localization of CAMSAP2 and 3.** (A) Western blot analysis of CAMSAP 2 and 3 expressions in mouse embryonic brains. Lysates of whole brain or hippocampus were collected at the indicated embryonic days. (B) Hippocampal neurons (stage 3) were triple-stained for CAMSAP2 or 3 (green), Ankyrin G (AnkG, red) and DNA (blue) at DIV4. Scale bar, 20 μm.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2 kb
BamHI
PCR primers
BamHI
Wildtype allele
Probe
Probe
PCR primers
Target vector
Mc1 DT-A pA
Pr Neo pA
loxP
BamHI
BamHI
BamHI
Targeted allele
Probe
Pr Neo pA
Probe
10.2 kb
5.7 kb
B
PCR
+/+ -/- +/-
500 bp-
606 bp
307 bp
C
Camsap2
(kDa) +/+ -/- +/- IB
200-
CAMSAP2
37-
GAPDH
D
Camsap2
+/+ +/- -/-
P43 males
wt
Camsap2 +/-
Camsap2 -/-
body weight (gram)
Male
Female
days
E
Camsap3
(kDa) +/+ -/- +/- IB
150-
CAMSAP3
37-
GADPH
Camsap3 -/-
CAMSAP3
α-tubulin
DNA
merge
F
wild type
Camsap3 dc/dc
Camsap2 -/-

**Fig. S2. Genetic design and characterization of *Camsap* knockout mice.** (A) A map of homologous recombination for targeting the *Camsap2* gene. A LacZ-neo selection cassette was inserted into the 5$^{th}$ exon. (B) Genotyping of mice by PCR. (C) Western blot analysis of CAMSAP2 protein expression in wild-type (+/+), and *Camsap2* homozygous (-/-) and heterozygous (+/-) mice at P2. (D) Growth of wild-type, *Camsap2* heterozygous, and homozygous mice. (E) CAMSAP3 protein expression in *Camsap3*$^{tm1a(EUCOMM)Wtsi}$ mice. *Upper*, Western blot analysis of brain lysates obtained from P2 mice. *Lower*, Immunostaining for CAMSAP3 (green), α-tubulin (red) and DNA (blue) in hippocampal neurons derived from *Camsap3* homozygous (-/-) mice. Nuclear staining is likely due to non-specific reaction of the anti-CAMSAP3 antibodies. (F) Hematoxylin-eosin staining of paraffin sections of P25 brains. Scale bars, 20 μm in E, and 1 mm in F.

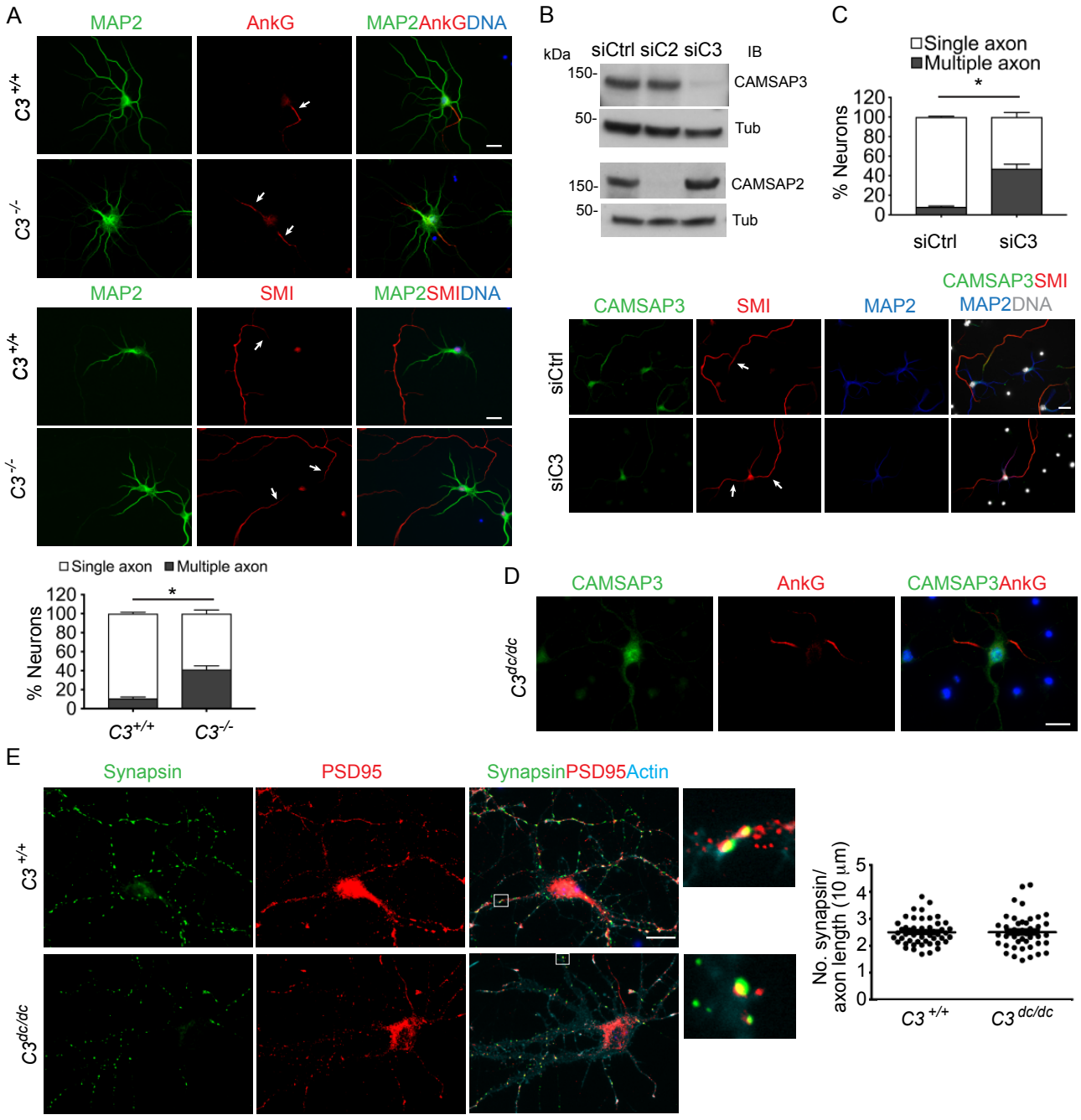
A
MAP2
AnkG
MAP2AnkGDNA
C3+/+
C3-/-
MAP2
SMI
MAP2SMIDNA
C3+/+
C3-/-
Single axon
Multiple axon
% Neurons
120
100
80
60
40
20
0
*
C3+/+
C3-/-
B
kDa
siCtrl siC2 siC3
IB
150-
CAMSAP3
50-
Tub
150-
CAMSAP2
50-
Tub
C
Single axon
Multiple axon
% Neurons
120
100
80
60
40
20
0
*
siCtrl
siC3
CAMSAP3
SMI
MAP2
CAMSAP3SMI
MAP2DNA
siCtrl
siC3
D
CAMSAP3
AnkG
CAMSAP3AnkG
C3dc/dc
E
Synapsin
PSD95
SynapsinPSD95Actin
C3+/+
C3dc/dc
No. synapsin/
axon length (10 µm)
5
4
3
2
1
0
C3+/+
C3dc/dc

**Fig. S3. Neurite patterning and synapse formation after CAMSAP3 removal.** (A) Axon and dendrite patterns in *Camsap3*$^{tm1a(EUCOMM)Wts}$ neurons. Neurons derived from wild-type and homozygous *Camsap3*$^{tm1a(EUCOMM)Wts}$ (-/-) neurons were stained for MAP2 (green), DNA (blue), and AnkyrinG or SMI312 (SMI) (red) at DIV8. The ratio of neurons with single to those with multiple axons is also shown below. More than 300 neurons were analyzed. * $p < 0.005$ versus the wild-type cells. (B) Knockdown efficiency of control (siCtrl), CAMSAP3-specific (siC3) or CAMSAP2-specific (siC2) siRNA in N2a cells, assessed by Western blotting. Tub, α-tubulin. (C) Axon and dendrite patterns in neurons treated with siCtrl or siC3 siRNA. Wild-type neurons were transfected with siCtrl or siC3 at DIV1, and then triple-stained for CAMSAP3 (green), MAP2 (blue) and SMI312 (red) at DIV6. The ratio of neurons with single to those with multiple axons is shown at the upper right. At least 300 neurons were analyzed. * $p < 0.005$ versus the siCtrl group. (D) Immunostaining for CAMSAP3 (green) and AnkyrinG (red) in *C3*$^{dc/dc}$ neurons at DIV6. (E) Distribution of synapsin (green), PSD95 (red), and actin (blue) in wild-type and *C3*$^{dc/dc}$ neurons at DIV14. Boxed areas are enlarged at the right. Number of synapsin-positive puncta was counted along axons that were identified by SMI immunostaining, and their density in axons was calculated. Approximately 50 neurons were analyzed. Scale bars, 20 μm.

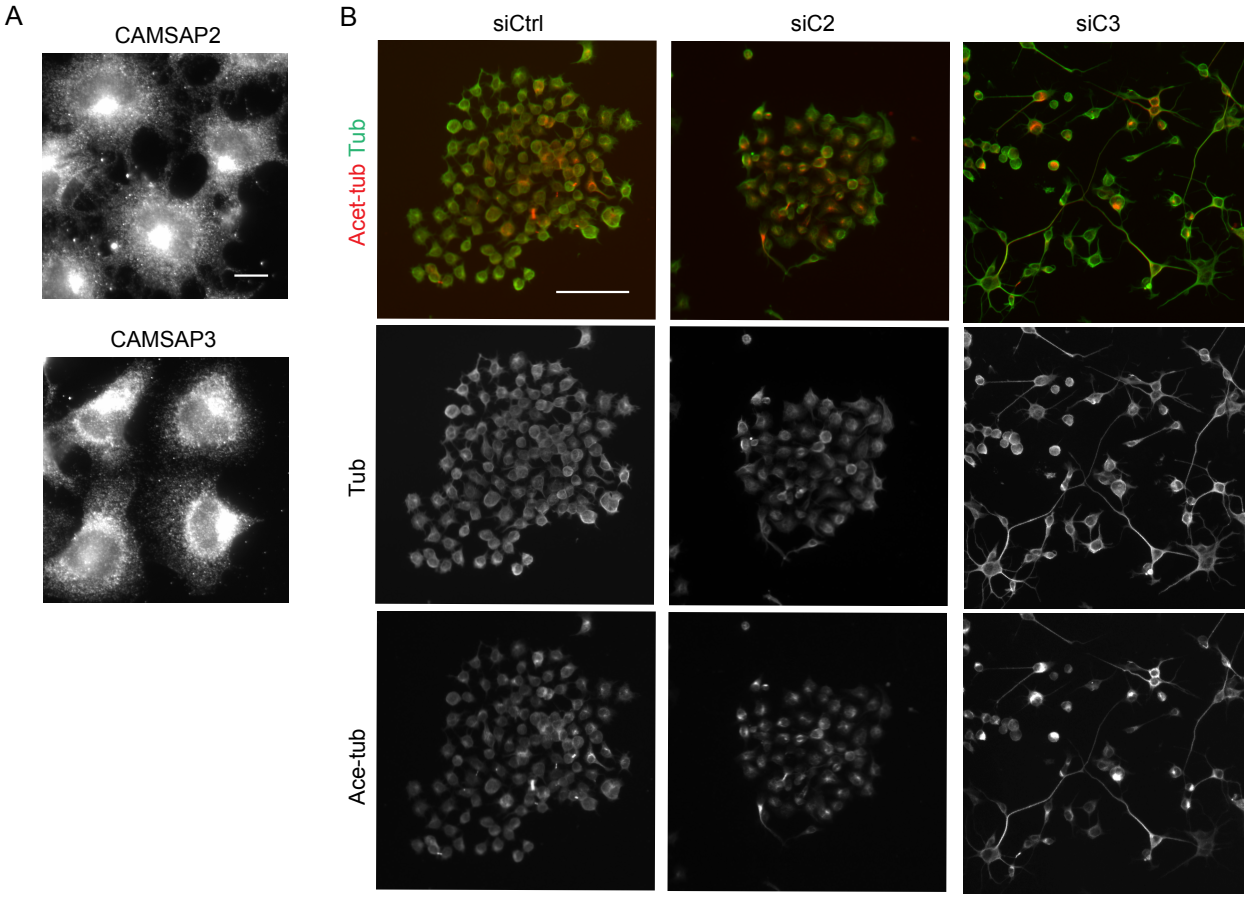
A
CAMSAP2
CAMSAP3
B
siCtrl
siC2
siC3
Acet-tub Tub
Tub
Ace-tub
C
DMSO
nocodazole
siCtrl
siC3
Acet-tub Tub
Ace-tub
D
C3 Ace-tub
C3
Ace-tub

**Fig. S4. CAMSAPs and tubulin acetylation in cell lines.** (A) Immunostaining for CAMSAP2 and CAMSAP3 in N2a cells. (B) N2a cells were treated with control siRNA (siCtrl), CAMSAP2 siRNA (siC2), or CAMSAP3 siRNA (siC3) for 3 days, and then co-immunostained for α-tubulin (Tub, green) and acetylated tubulin (Ace-tub, red). Images were collected from three independent experiments, and a representative set of images are shown. (C) N2a cells were treated with siCtrl or siC3 as in B, and then incubated with 2 μM nocodazole or 0.1% DMSO for 60 min. Representative cells are shown. (D) CAMSAP3 (red) and acetylated tubulin (green) are co-immunostained in Caco2 cells. Arrowheads point to examples of CAMSAP3 puncta attaching to an end of acetylated microtubules. Arrows indicate examples of acetylated microtubules that are not anchored to CAMSAP3. Boxed region is enlarged at the right. Scale bars, 10 μm in A, C and D; 100 μm in B.

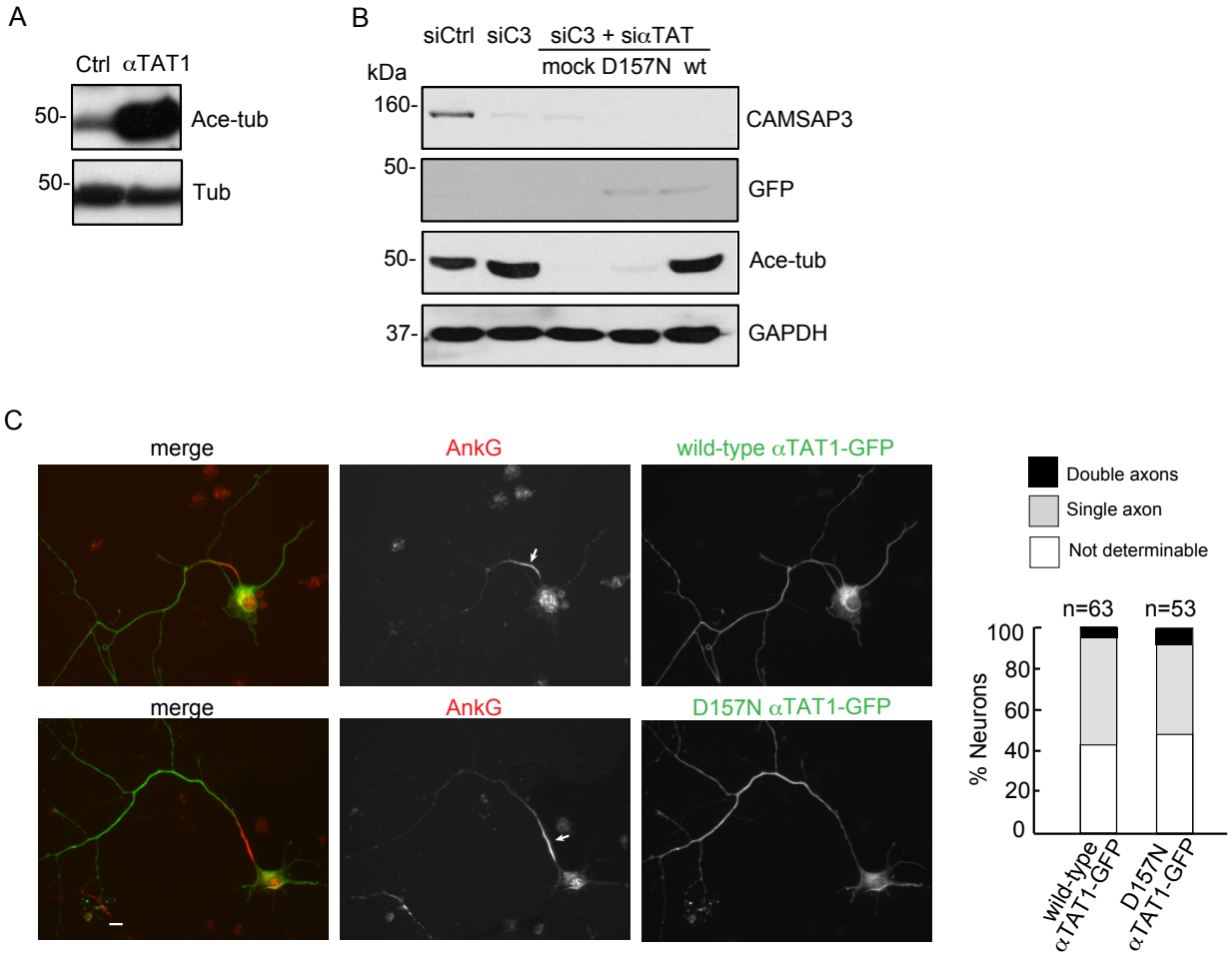
A
Ctrl αTAT1
50-
Ace-tub
50-
Tub
B
siCtrl siC3 siC3 + siαTAT
mock D157N wt
kDa
160-
CAMSAP3
50-
GFP
50-
Ace-tub
37-
GAPDH
C
merge
AnkG
wild-type αTAT1-GFP
merge
AnkG
D157N αTAT1-GFP
Double axons
Single axon
Not determinable
n=63
n=53
% Neurons
100
80
60
40
20
0
wild-type αTAT1-GFP
D157N αTAT1-GFP
D
Tub
wild-type αTAT1-GFP
Tub
D157N αTAT1-GFP
Ace-tub
wild-type αTAT1-GFP
Ace-tub
D157N αTAT1-GFP
merge
GFP
Tub
merge
GFP
Ace-tub

**Fig. S5. Effects of αTAT1 or its mutant expression on tubulin acetylation and neuronal polarity.** (A) N2a cells were transfected with an αTAT1 plasmid, and cultured for 72 hrs before Western blot analysis. Ctrl, untransfected control. (B) N2a cells were transfected with indicated siRNAs. For cDNA transfection, cells were initially incubated with siRNAs for 48 hrs, and then transfected with a wild-type (wt) αTAT1, mutant (D157N) αTAT1, or mock plasmid. After another 24-hr incubation, protein levels were analyzed by immunoblotting. (C) Neurons were transfected with αTAT1-GFP or D157N αTAT1-GFP at DIV1, and fixed at DIV6, followed by co-immunostaining for GFP and Ankyrin G (AnkG). A representative image is shown for each experiment. The graph shows the ratio of neurons with single to double axons. Neurons whose axons could not be identified due to diffusion of AnkG immunostaining signals are categorized as 'not determinable'. Samples were collected from two independent experiments. (D) N2a cells were transfected with αTAT1-GFP or D157N αTAT1-GFP, and cultured for 3 days, followed by immunostaining for α-tubulin (Tub) and acetylated tubulin (Ace-tub). Scale bar, 10 μm.

**References**


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