SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Distribution of Rheb and tuberin. *A* - *C*. Neurons were stained at stage 2 (1 d.i.v.) and stage 3 (3 d.i.v.) with an anti-MAP2 (A-C; red) or the Tau-1 (C; blue) antibody and an anti-Rheb (A; green), anti-tuberin (B, C; green), or anti-phospho-tuberin antibody (p-tuberin; phosphorylated at T1462) (B, C; green). Rheb and p-tuberin were present in all growth cones of stage 2 neurons and became enriched in the axon of stage 3 neurons (asterisks). Total tuberin was detected throughout the neuron at both stages. The scale bar is 20 μm.

Fig. S2. Rheb and mTOR are required for neuronal polarity. *A.* HEK 293T cells were transfected with vectors for EGFP, HA-Rheb, and pSuper or vectors for shRNAs directed against Rheb (pSb357, pSb102). Lysates were analyzed by Western blot with antibodies specific for HA, EGFP (transfection control), and β-tubulin (loading control). Expression of pSb102 but not pSuper or pSb357 resulted in a significant knock-down of Rheb. *B.* Neurons were transfected on 0 d.i.v. with vectors for EGFP and pSuper or pSb102, and stained on 3 d.i.v. with the Tau-1 (blue) and anti-Rheb (red) antibodies. Expression of pSb102 strongly reduced Rheb in the EGFP-positive cell (arrow). *C.* 293T cells were transfected with vectors for EGFP, mouse GFP-Rheb, rat HA-Rheb, and pSuper or a vector for an shRNAs directed against rat Rheb (pSb102). Lysates were analyzed by Western blot with antibodies specific for Rheb and EGFP (transfection control). Expression of pSb102 resulted in a significant knock-down of rat Rheb while mouse Rheb was resistant to suppression by pSb102. *D* & *E*. Neurons were transfected on 0 d.i.v. with a vector for EGFP (green), the shRNA directed against Rheb (Rheb RNAi), and mouse GFP-Rheb as indicated. Neurons were stained on 3 d.i.v. with the Tau-1 (blue) and an anti-Rheb antibody (red). The percentage of neurons positive for Rheb staining is shown (I) (means ± s.e.m; * $P<0.001$ compared to control; n= 60-100 neurons from 3 experiments). The scale bar is 20 μm. *F* - *I.* Neurons were transfected on 0 d.i.v. with a vector for EGFP (green), the shRNA directed against Rheb (Rheb RNAi), and mouse GFP-Rheb as indicated. Neurons were stained on 3 d.i.v. with the Tau-1 (blue) and an anti-MAP2 antibody (red). The number of axons per cell (G), the percentage of neurons without an axon (0), with a single axon (1), and with multiple axons (>1) (H), and the length of axons are shown (I) (means ± s.e.m; * $P<0.001$ compared to controll; n= 180-250 neurons from 3 experiments). The scale bar is 20 μm. *J.* Solvent (DMSO) or 200 nM rapamycin (Rpmc) were added to neurons 3-5 hours after plating. Neurons were stained on 5 d.i.v. with the Tau-1 (axonal marker) and anti-MAP2 antibodies (minor neurites). The percentage of polarized neurons is shown (means ± s.e.m; n= 150-250 neurons per condition from 3 experiments; * $P<0.001$ compared to control (DMSO)).

Fig. S3. Distribution of 4E-BP1 phosphorylated at S65 in hippocampal neurons. *A.* Hippocampal neurons express both 4E-BP1 and 4E-BP2. Hippocampal neurons were lysed on 5 d.i.v. in lysis buffer (50 mM Tris pH 7, 150 mM, 1 mM DTT, 1.5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, 1% Triton X-100, 1x proteinase inhibitor cocktail, 50 mM NaF), proteins immunoprecipitated overnight with anti-4E-BP1 or anti-4E-BP2 antibodies as indicated, and analyzed by Western blot using antibodies specific for 4E-BP1 phosphorylated at Ser65 (p-4E-BP1), 4E-BP1, or 4E-BP2. Anti-p-4E-BP1 specifically detected phosphorylated 4E-BP1 but showed no

signal in anti-4E-BP2 immunoprecipitates. *B - H*. Early stage 2 (B; 24 hours in vitro), late stage 2 (C, D; 36 hours in vitro), and stage 3 neurons (C, D; 72 hours in vitro) were stained with phalloidin-rhodamine (B; red) or an anti-MAP2 antibody (C, D; red), the Tau-1 antibody (C, D; blue), and antibodies specific for 4E-BP1 (C, D; green) or 4E-BP1 phosphorylated at Ser65 (B-D; p-4E-BP1; green). Growth cones positive for p-4E-BP1 are marked by arrows. In morphologically unpolarized late stage 2 neurons, p-4E-BP1 is present in the cell body and enriched in a single neurite (arrow). In stage 3 neurons, p-4E-BP1 is restricted to the axon (arrow). *E*. Quantification of relative p-4E-BP1 and 4E-BP1 fluorescence intensity in the distal 5 μm of axons and minor neurites at stage 3 (means ± s.e.m; n=10 axons and 20 minor neurites per measurement, * P<0.001 compared to control). The fluorescence intensity of the distal part of axons and minor neurites was measured at 2 pixel intervals, the intensity of adjacent cell-free areas (background fluorescence) subtracted, and the values averaged. The fluorescence intensity at the tips of minor neurites as normalized to a value of 1. *F*. The percentage of neurons is shown where p-4E-BP1 was not enriched in any neurite (white bars), was enriched in one (black bars), or more than one of the neurites (grey bars) at early stage 2 (24 hours in vitro), late stage 2 (36 hours), or stage 3 (48 hours). At early stage 2 (24 hours), p-4E-BP1 was distributed uniformly in 51 ± 2% (n=348) of the neurons and enriched in the growth cone of a single neurite in 37 ± 2% of the cells. At late stage 2 (36 hours), p-4E-BP1 became enriched in the growth cone of a single neurite in 49 ± 2% (n=514) of the neurons and 72 ± 2% (n=260) of the polarized neurons showed an enrichment of p-4E-BP1 in the axonal growth cone at stage 3 (48 hours). *G & H*. Intensity profiles of p4E-BP1 or 4E-BP1 fluorescence in arbitrary units in stage 3 neurons. Fluorescence intensity was determined at intervals of 2 pixels along the axon and minor neurites. Background fluorescence was determined as the average fluorescence intensity of cell-free areas adjacent to the analyzed neuron. The scale bar is 20 μm.

Fig. S4. The role of 4E-BP1. *A & B*. p-4E-BP1 is reduced in growth cones after inhibition of PI3K and mTOR. 100 μM LY294002 (LY) or 200 nM rapamycin (Rpmc) were added 45 minutes before the neurons were fixed and stained on 3 d.i.v. with the Tau-1 antibody (blue) and antibodies specific for MAP2 (red) and p-4E-BP1 (green). *B*. The percentage of neurons with p-4E-BP1-positive growth cones after incubation with PI3K or mTOR was determined (means ± s.e.m; n=100-150, 3 experiments; * P<0.001 compared to solvent). *C*. Neurons were transfected with vectors for HA-4E-BP1 or HA-4E-BP1-AA on 0 d.i.v., fixed on 3 d.i.v., and stained with antibodies specific for the HA tag (green), MAP2 (red), or the Tau-1 antibody (blue) to analyze the effect of expressing mTOR-insensitve 4E-BP1 mutants. *D - F*. Neurons were transfected with vectors for the indicated proteins on 0 d.i.v., fixed on 3 d.i.v. and stained with antibodies specific for the myc or HA-tag, MAP2, or the Tau-1 antibody. The number of minor neurites is shown. The scale bar is 20 μm.

Fig. S5. 4E-BP1, Rap1B, and translational regulation. *A*. 4E-BP1 phosphorylation is induced in supernumerary axons after treatment with insulin. 200 nM insulin was added 3-5 hours after plating, neurons were fixed on 3 d.i.v., and stained with anti-MAP2 (red), the Tau-1 (blue), and anti-p-4E-BP1 (green) antibodies. P-4E-BP1 is enriched in the growth cones of the three Tau-1 positive axons (1-3). A higher magnification of the growth cones is shown. *B*. Regulation of Rap1B expression by insulin. The indicated concentrations (nM) of insulin (Ins), rapamycin (Rpmc), and LY294002 (LY) were added to neurons shortly after plating, the cells

lysed on 2.5 d.i.v., and the expression of Rap1B analyzed by Western blot. The effect of 500 nM insulin was blocked by 200 nM Rpmc or 100 μM LY294002. The loading of comparable amounts of protein was confirmed by staining with an anti-tubulin antibody (3 experiments). *C.* Incubation of polarized neurons with Rpmc results in the loss of Rap1B from axons. DMSO or 200nM Rpmc was added on 2 d.i.v., neurons fixed on 3 d.i.v. and stained with the anti-MAP2 (red), Tau-1 (blue) and anti-Rap1 antibodies (green). Rap1B is restricted to the axonal growth cone in control neuron (DMSO), while it was lost from the growth cone after treatment with Rpmc. Higher magnifications of growth cones (1 and 2) are shown. The scale bar is 20 μm.

Fig. S6. Rap1B acts downstream of mTOR and Rheb. *A* - *D*. Neurons were transfected with expression vectors for EGFP (green) or myc-tagged Rap1BV12 and solvent (DMSO) or 200 nM Rpmc was added shortly after the transfection. Neurons were fixed on 3 d.i.v., and stained with an anti-MAP2 (red), the Tau-1 (blue), and an anti-myc antibody (green). The length of axons (B; total: average length of axons, single: length of axons from neurons that formed a single axon (DMSO/GFP: 87 ± 5%, Rpmc/GFP: 29 ± 3%, DMSO/Rap1BV12: 56 ± 5%, Rpmc/Rap1BV12: 62 ± 7%), multiple: length of axons from neurons that formed multiple axons (DMSO/GFP: 9 ± 3%, Rpmc/GFP: 1 ± 1%, DMSO/Rap1BV12: 40 ± 3%, Rpmc/Rap1BV12: 32 ± 5%)), the number of minor neurites per cell (C), and the length of minor neurites are shown (D) (n = 30-57 neurons). *E* - *G*. Neurons were transfected on 0 d.i.v. with a vector for EGFP (control) or myc-tagged Rap1BV12 and pSHAG (control) or an shRNA vector directed against Rheb (Rheb RNAi) as indicated (see Fig. 7), fixed on 3 d.i.v., and stained with the Tau-1 and an anti-MAP2 antibody. The length of axons (E; total: average length of axons, single: length of axons from neurons that formed a single axon (control: 87 ± 4%, GFP/Rheb RNAi: 35 ± 5%, Rap1BV12/Rheb RNAi: 57 ± 7%), multiple: length of axons from neurons that formed multiple axons (control: 9 ± 4%, GFP/Rheb RNAi: 3 ± 2%, Rap1BV12/Rheb RNAi: 48 ± 8%)), the number of minor neurites per cell (F), and the length of minor neurites are shown (G) (means ± s.e.m; * $P<0.001$ compared to control or between the values indicated by brackets). The scale bar is 20 μm.

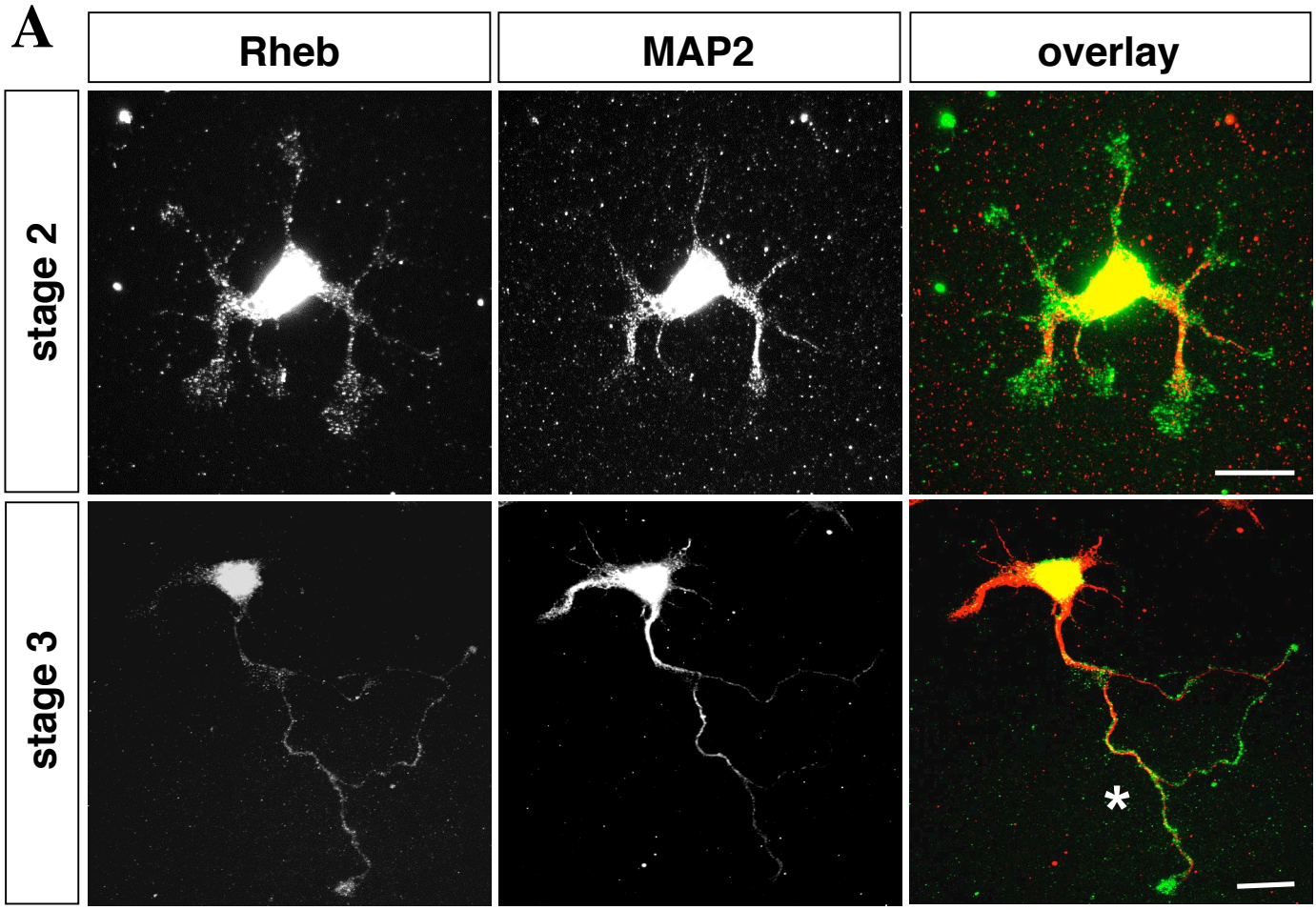
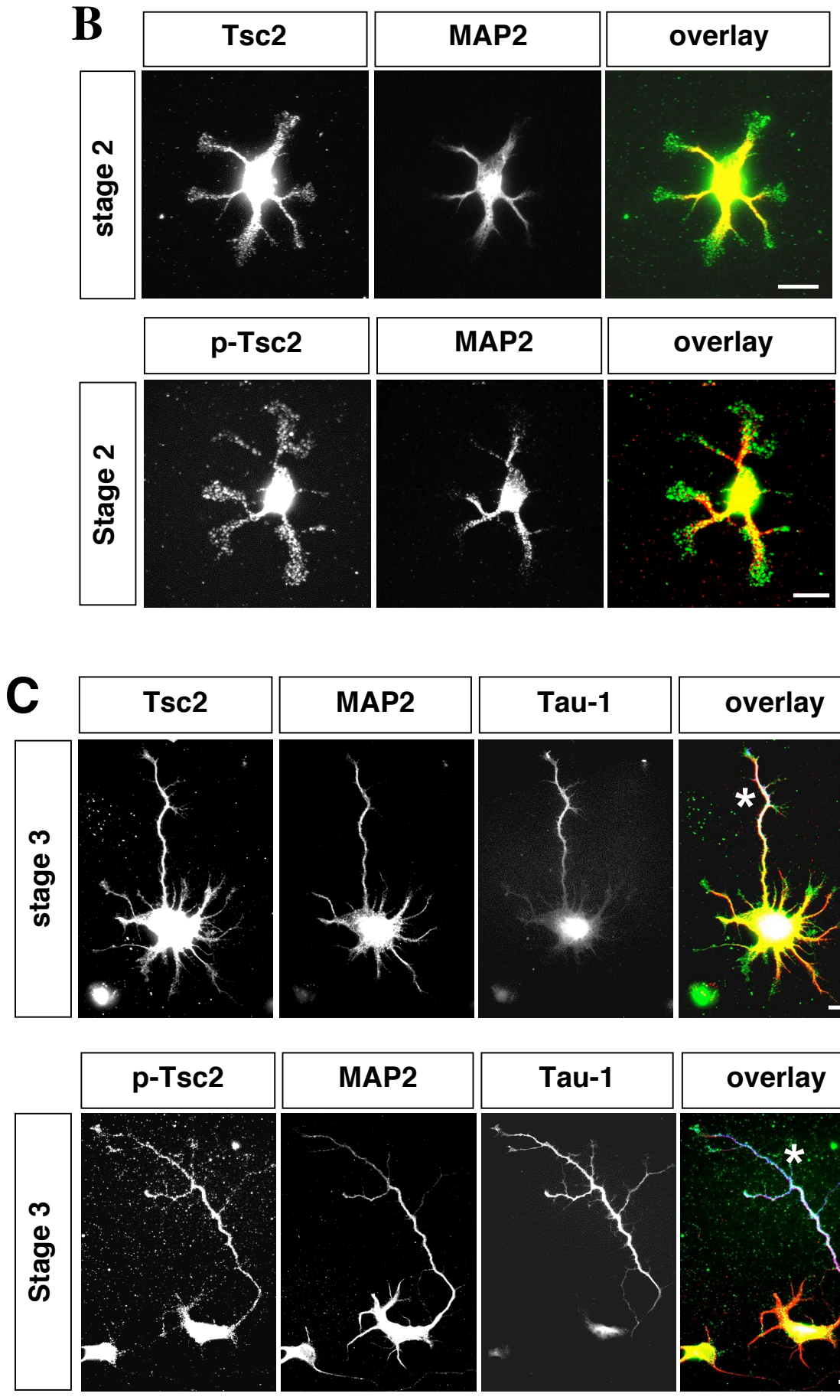


Figure S1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MAP2
overlay
stage 2
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MAP2
overlay
Stage 2
C
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stage 3
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overlay
Stage 3

Figure S1

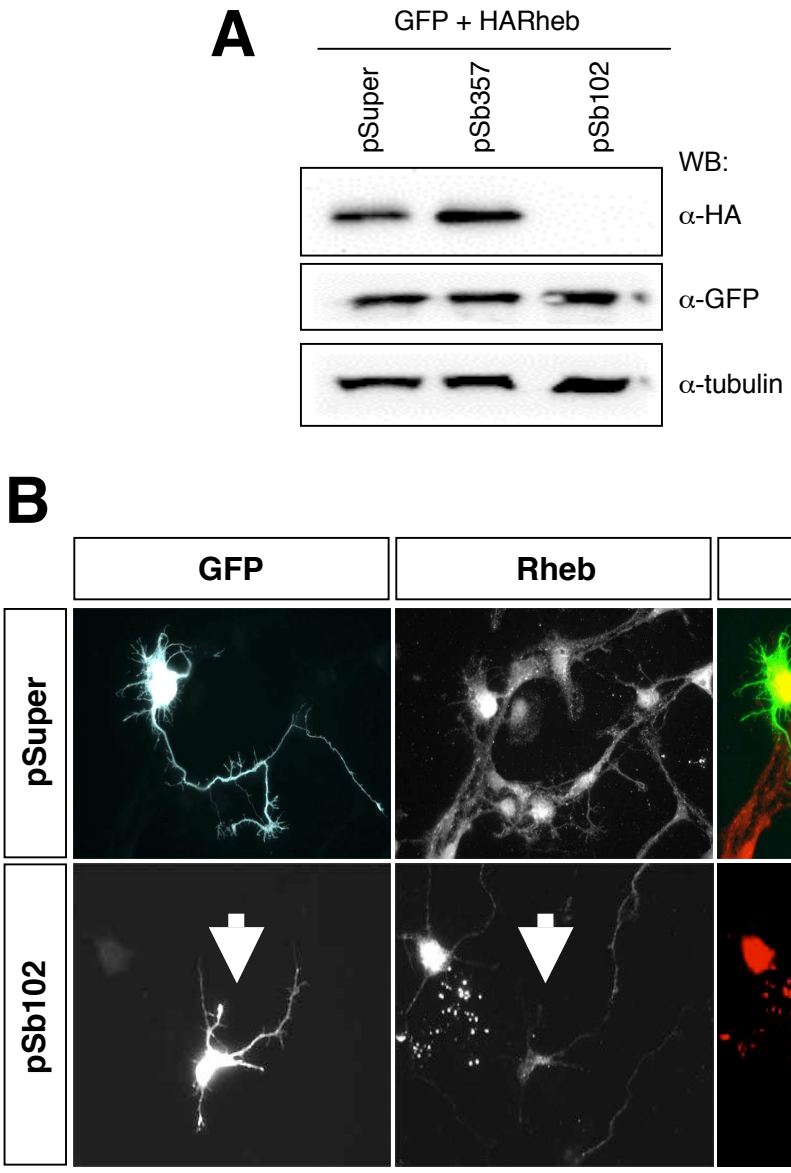
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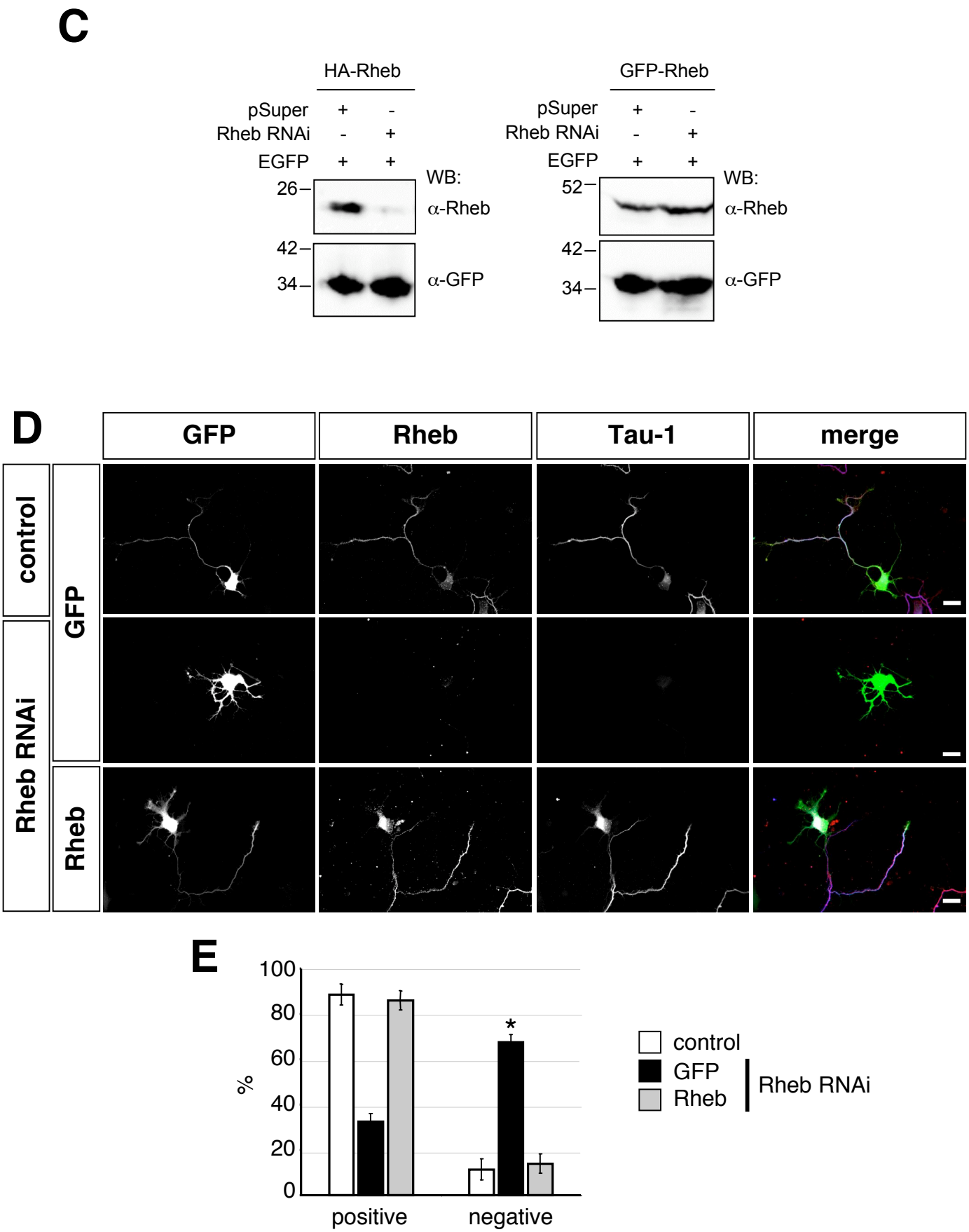
Figure S2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Rheb
Tau-1
merge
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positive
negative
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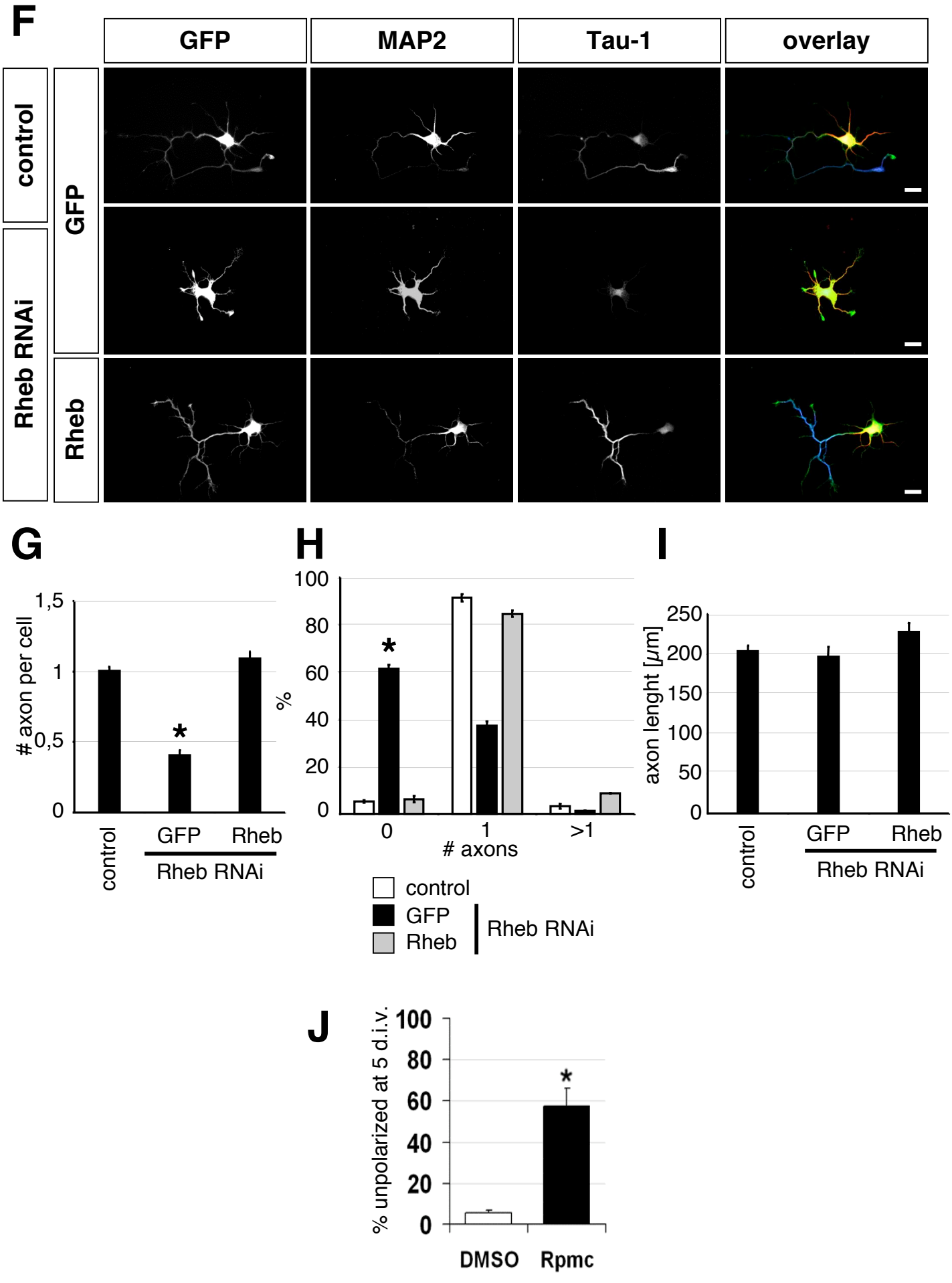
Rheb RNAi

Figure S2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control
GFP
Rheb
Rheb RNAi
H
%
100
80
60
40
20
0
*
0
1
>1
axons
control
GFP
Rheb
Rheb RNAi
I
axon lenght [μm]
250
200
150
100
50
0
control
GFP
Rheb
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60
40
20
0
*
DMSO
Rpmc

Figure S2

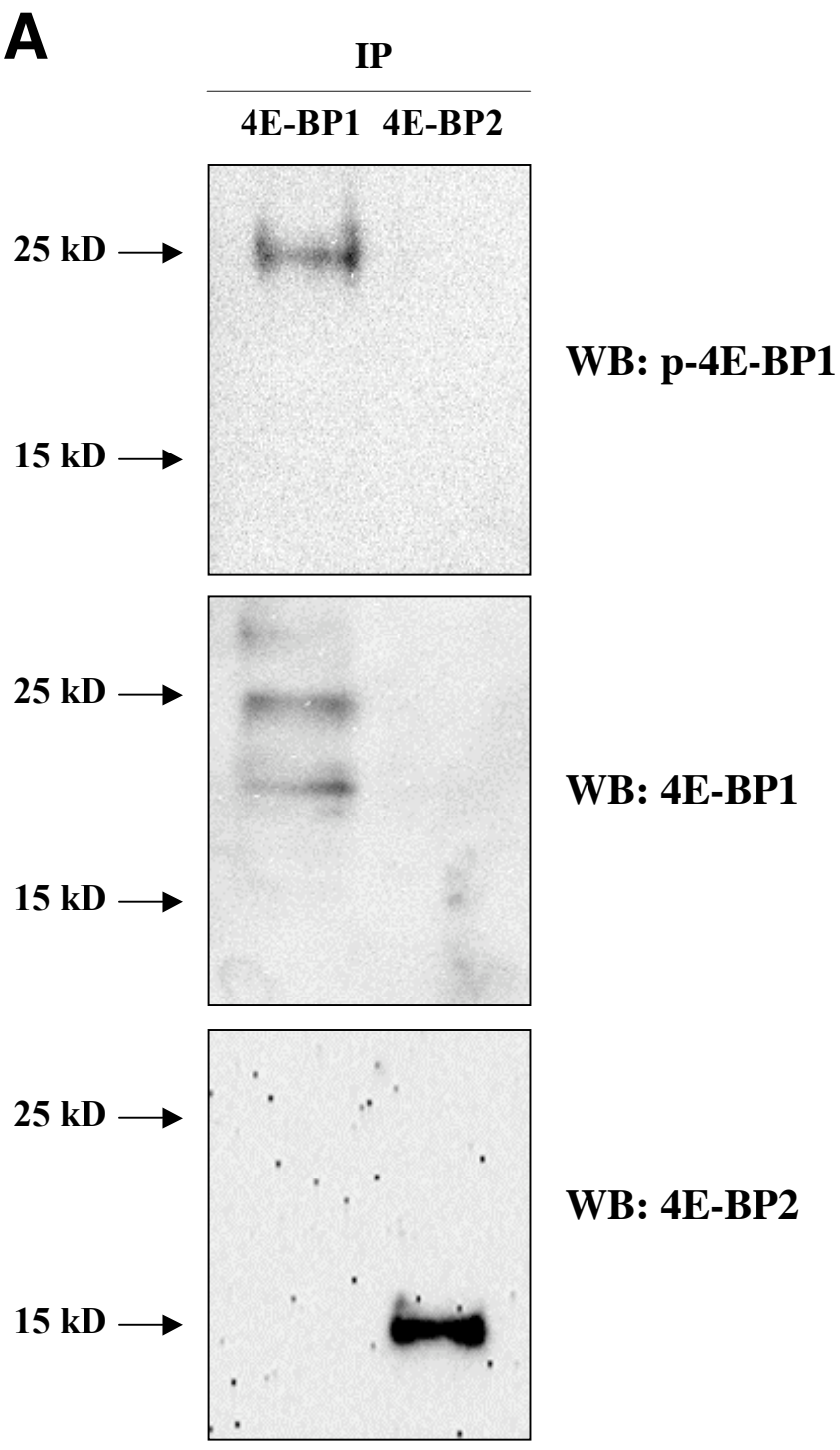
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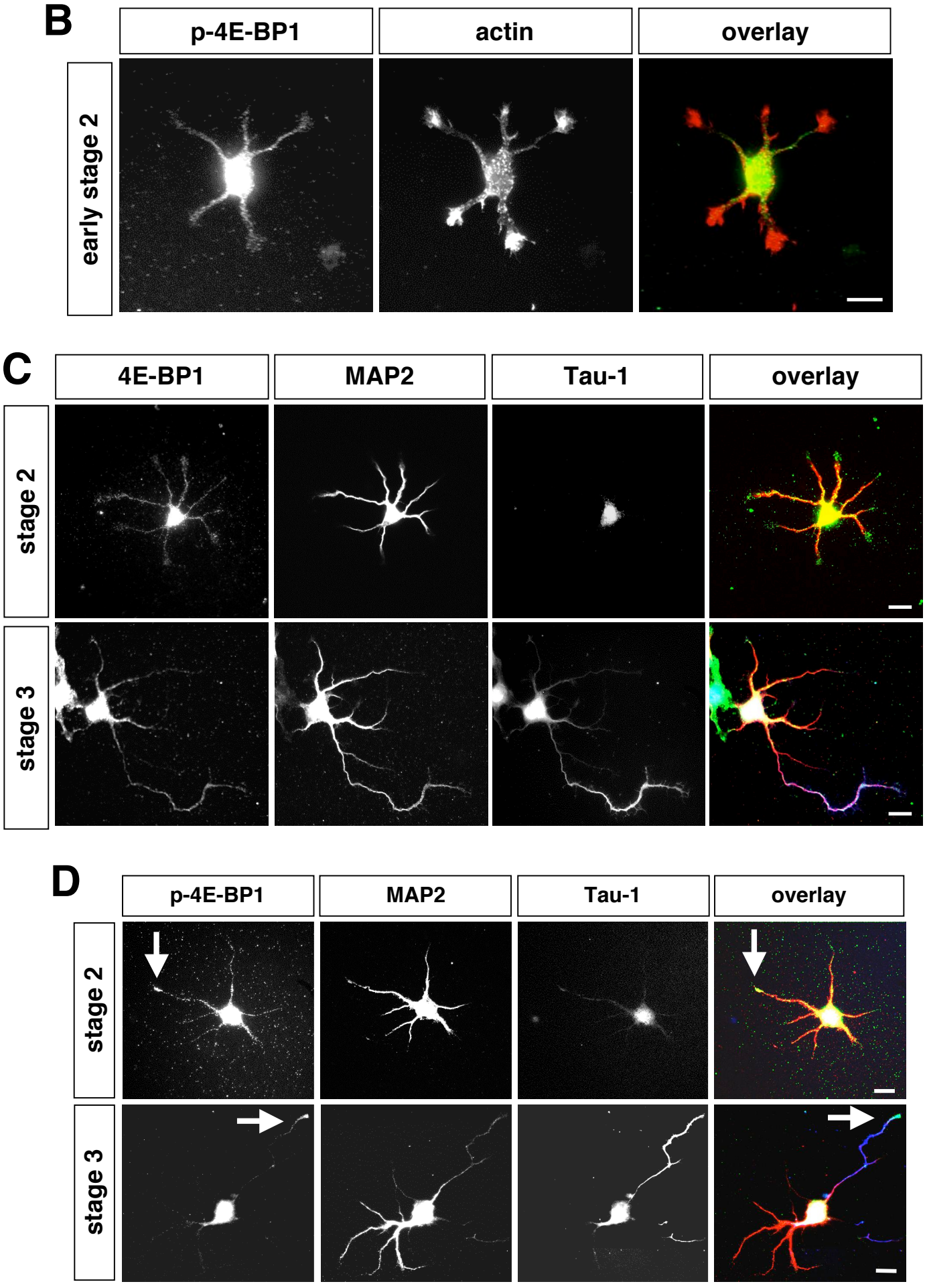
Figure S3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MAP2
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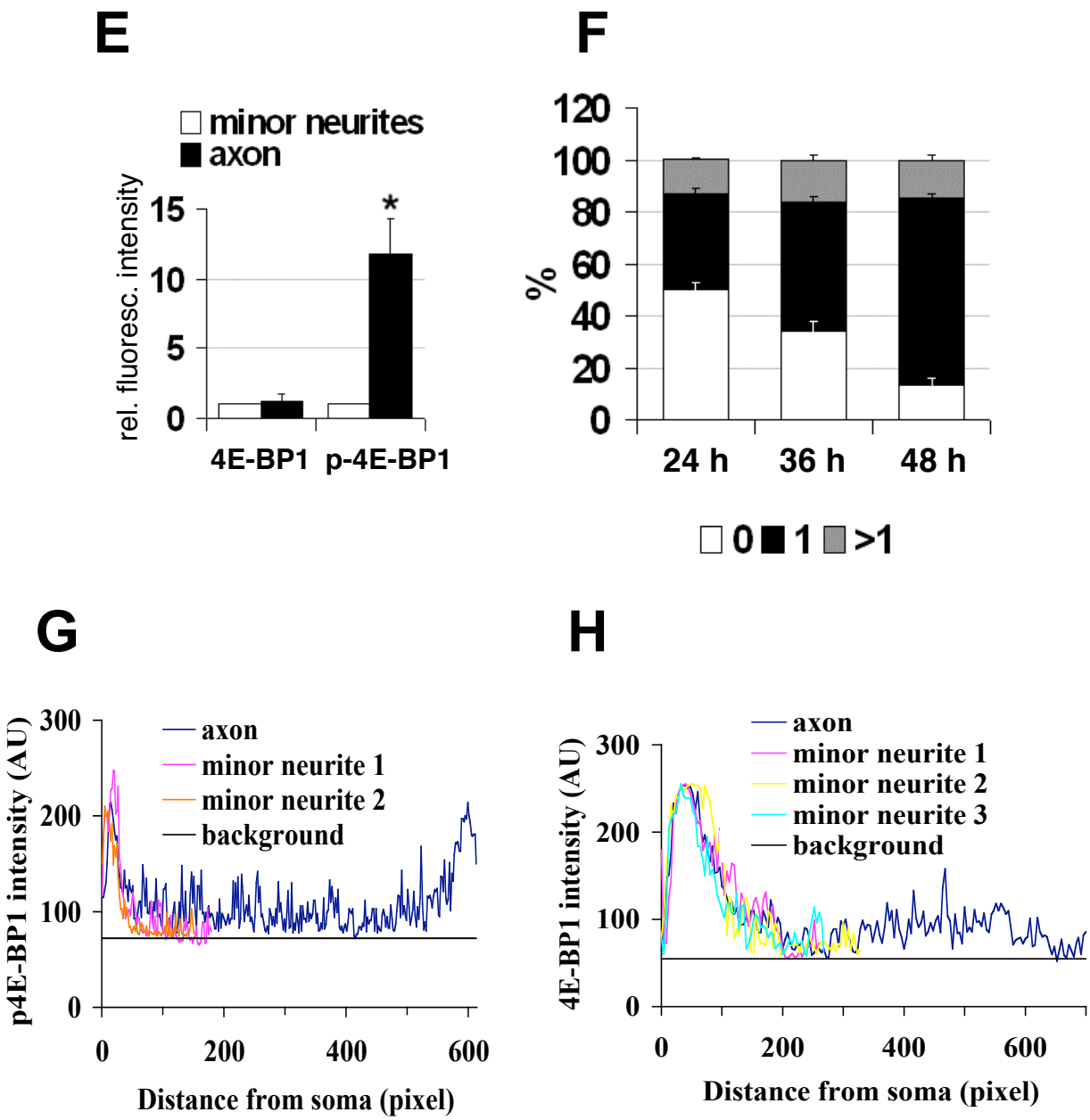
overlay
stage 2
stage 3

Figure S3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60
40
20
0
%
24 h
36 h
48 h
0
1
>1
G
p4E-BP1 intensity (AU)
300
200
100
0
axon
minor neurite 1
minor neurite 2
background
0
200
400
600
Distance from soma (pixel)
H
4E-BP1 intensity (AU)
300
200
100
0
axon
minor neurite 1
minor neurite 2
minor neurite 3
background
0
200
400
600
Distance from soma (pixel)

Figure S3