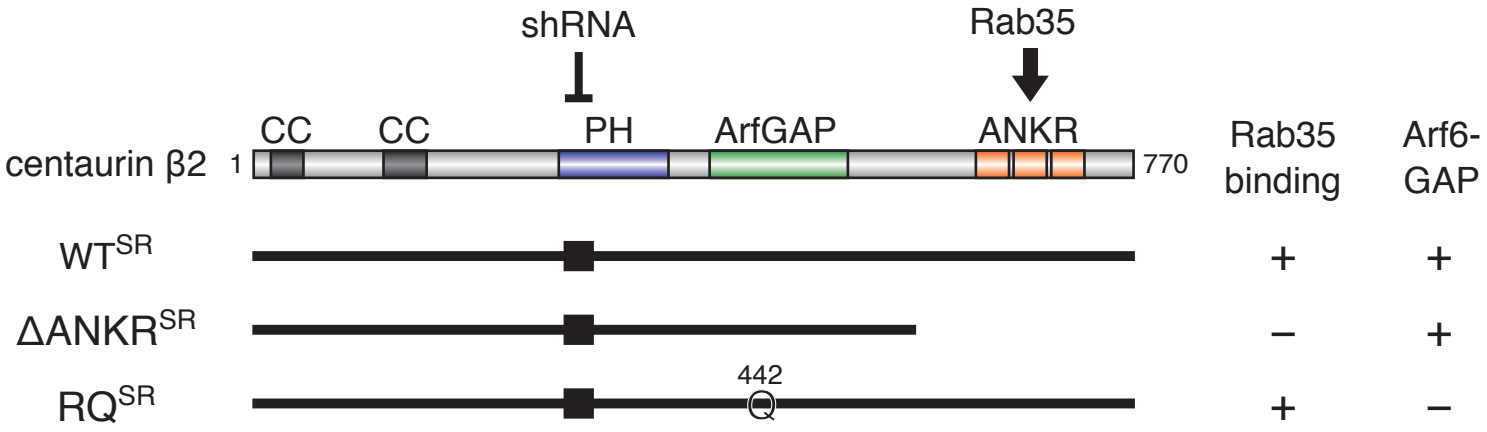

Fig. S1. Schematic representation of centaurin β2 and the centaurin β2 mutants used in this study. Centaurin β2 contains two coiled-coil (CC) domains in the N-terminal region (black boxes), a PH domain and an ArfGAP domain in the middle region (blue box and green box, respectively), and an ankyrin repeat domain (ANK), which specifically binds GTP-Rab35 (Kanno et al., 2010), in the C-terminal region (orange boxes). The closed boxes represent resistance to *centaurin β 2*-shRNA (#1) (SR) without an amino-acid substitution. The Q in the ArfGAP domain represents R442Q substitution, which causes loss of Arf6-GAP activity, and the ΔANKR represents deletion of the ANKR domain, which causes loss of Rab35-binding activity. The effects of each mutant on Rab35-binding ability and Arf6-GAP activity (−, +) are indicated on the right.

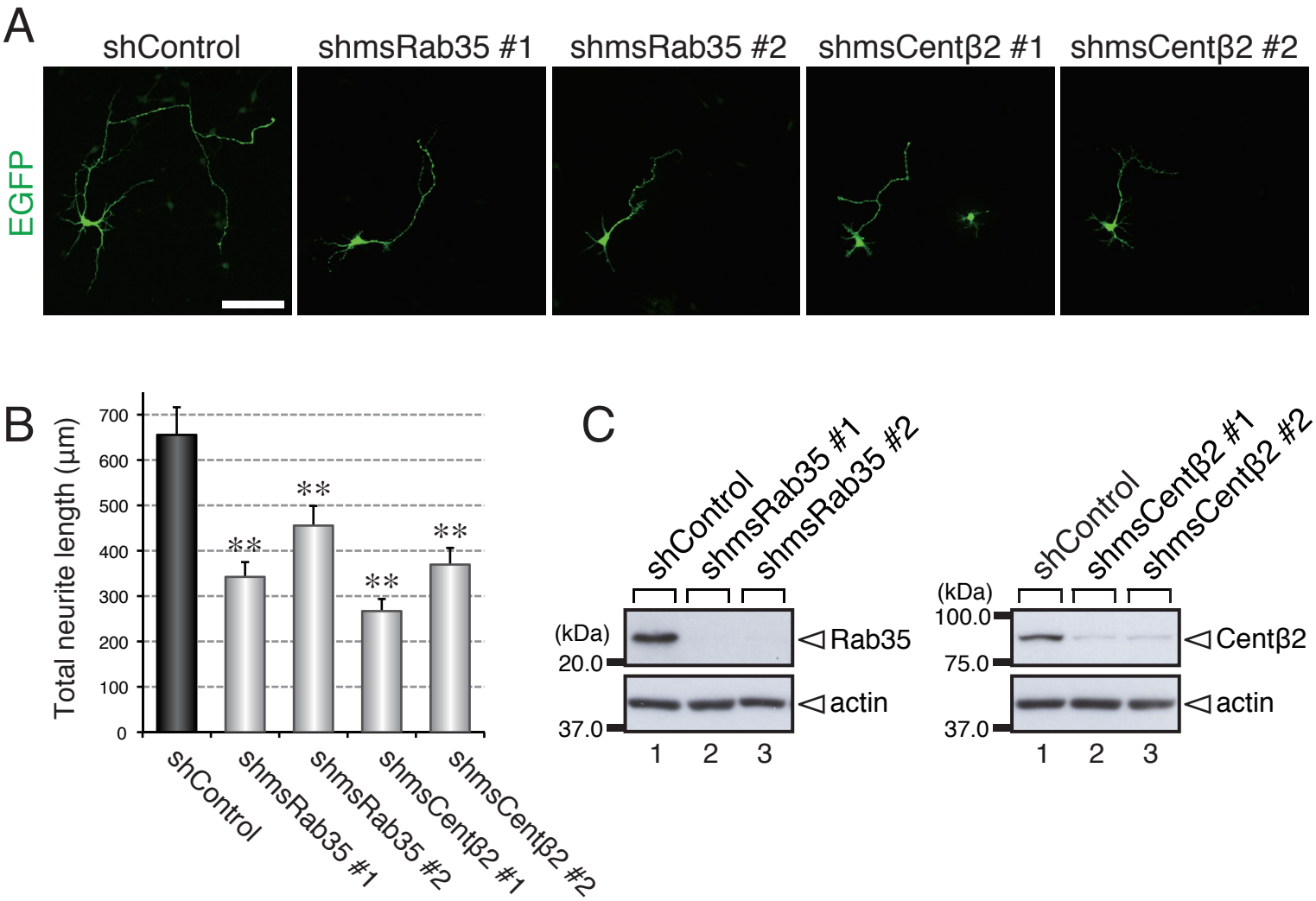

Fig. S2. Rab35 and centaurin β2 are required for neurite outgrowth of primary hippocampal neurons. **(A)** Typical images of control shRNA (shControl)-treated, mouse *Rab35* shRNA (shmsRab35)-treated, and mouse *centaurin β2* shRNA (shmsCentβ2)-treated primary hippocampal neurons at 4 DIV. shRNA-treated cells were identified by the green fluorescence of EGFP. Scale bar, 100 μm. **(B)** Effect of *Rab35*-knockdown or *centaurin β2*-knockdown on neurite outgrowth of primary hippocampal neurons. Bars represent the total neurite length values (mean and SE) of shControl-treated (control; black bar), shmsRab35#1-treated (1st white bar), shmsRab35#2-treated (2nd white bar), shmsCentβ2#1-treated (3rd white bar), and shmsCentβ2#2-treated (4th white bar) cells (n = 60). $^{**}p < 0.01$, in comparison with the control cells (Student's unpaired *t*-test). Note that shRNA-mediated knockdown of *Rab35* or *centaurin β2* in primary hippocampal neurons dramatically inhibited neurite outgrowth, the same as in PC12 cells (Figs. 1, 2). **(C)** Reduced expression of endogenous Rab35 or centaurin β2 in shmsRab35-treated or shmsCentβ2-treated mouse neuroblastoma, Neuro2a cells. Cell lysates of Neuro2a cells treated with either shControl, shmsRab35#1, shmsRab35#2, shmsCentβ2#1, or shmsCentβ2#2 were subjected to 10% SDS-PAGE followed by immunoblotting with anti-Rab35 antibody (upper panel on the left; 1/500 dilution) or anti-centaurin β2 antibody (upper panel on the right; 1/200 dilution), and anti-actin antibody (lower panels; 1/200 dilution). The positions of the molecular mass markers (in kDa) are shown on the left.

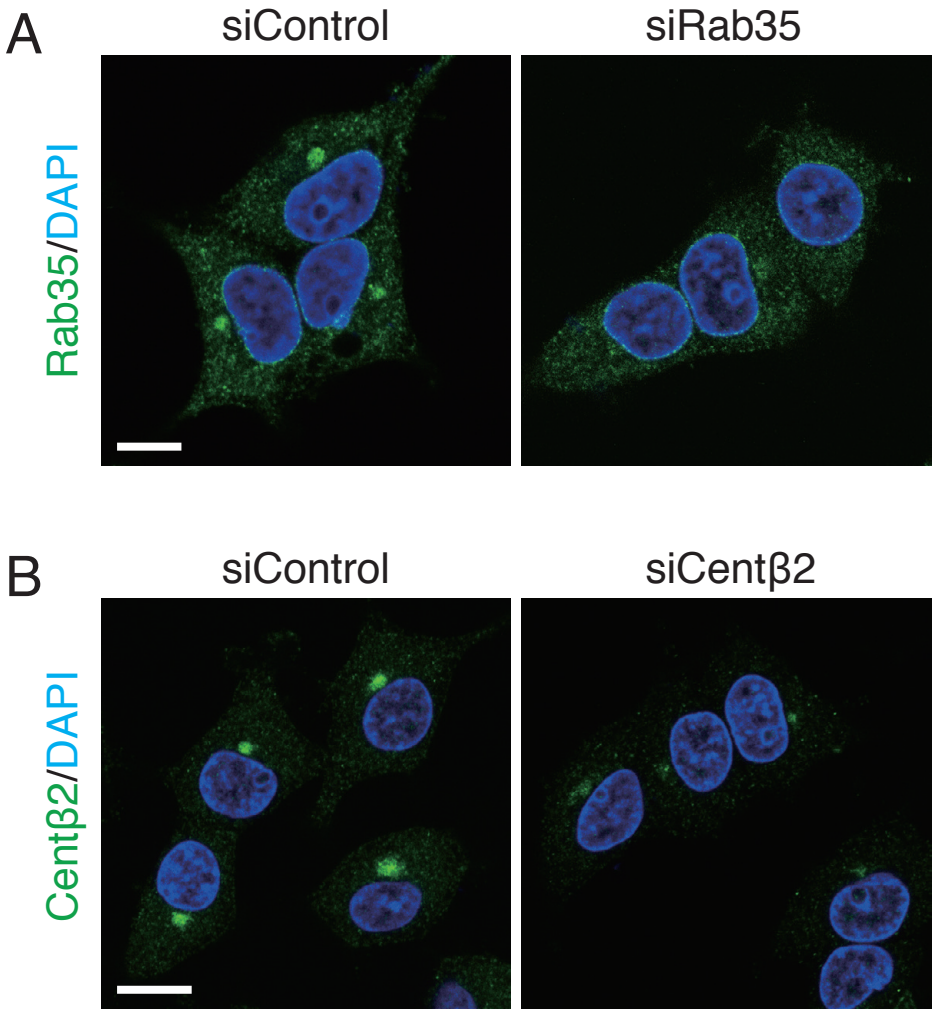

Fig. S3. Anti-Rab35 antibody and anti-centaurin β2 antibody specifically recognize Rab35 and centaurin β2, respectively. **(A)** Immunofluorescence signals of endogenous Rab35 in *Rab35* siRNA-treated PC12 cells after NGF stimulation for 6 hr. PC12 cells transfected with control siRNA (siControl) or *Rab35* siRNA (siRab35) were fixed and stained with anti-Rab35 antibody (green; 1/100 dilution) and DAPI (nuclei; blue). Note that the peri-nuclear signals of Rab35 disappeared in the siRab35-treated cells. Scale bar, 10 μm. **(B)** Immunofluorescence signals of endogenous centaurin β2 in *centaurin β2* siRNA-treated PC12 cells after NGF stimulation for 6 hr. PC12 cells transfected with control siRNA (siControl) or *centaurin β2* siRNA (siCentβ2) were fixed and stained with anti-centaurin β2 antibody (green; 1/100 dilution) and DAPI (nuclei; blue). Note that the peri-nuclear signal of centaurin β2 almost disappeared in the siCentβ2-treated cells. Scale bar, 10 μm.

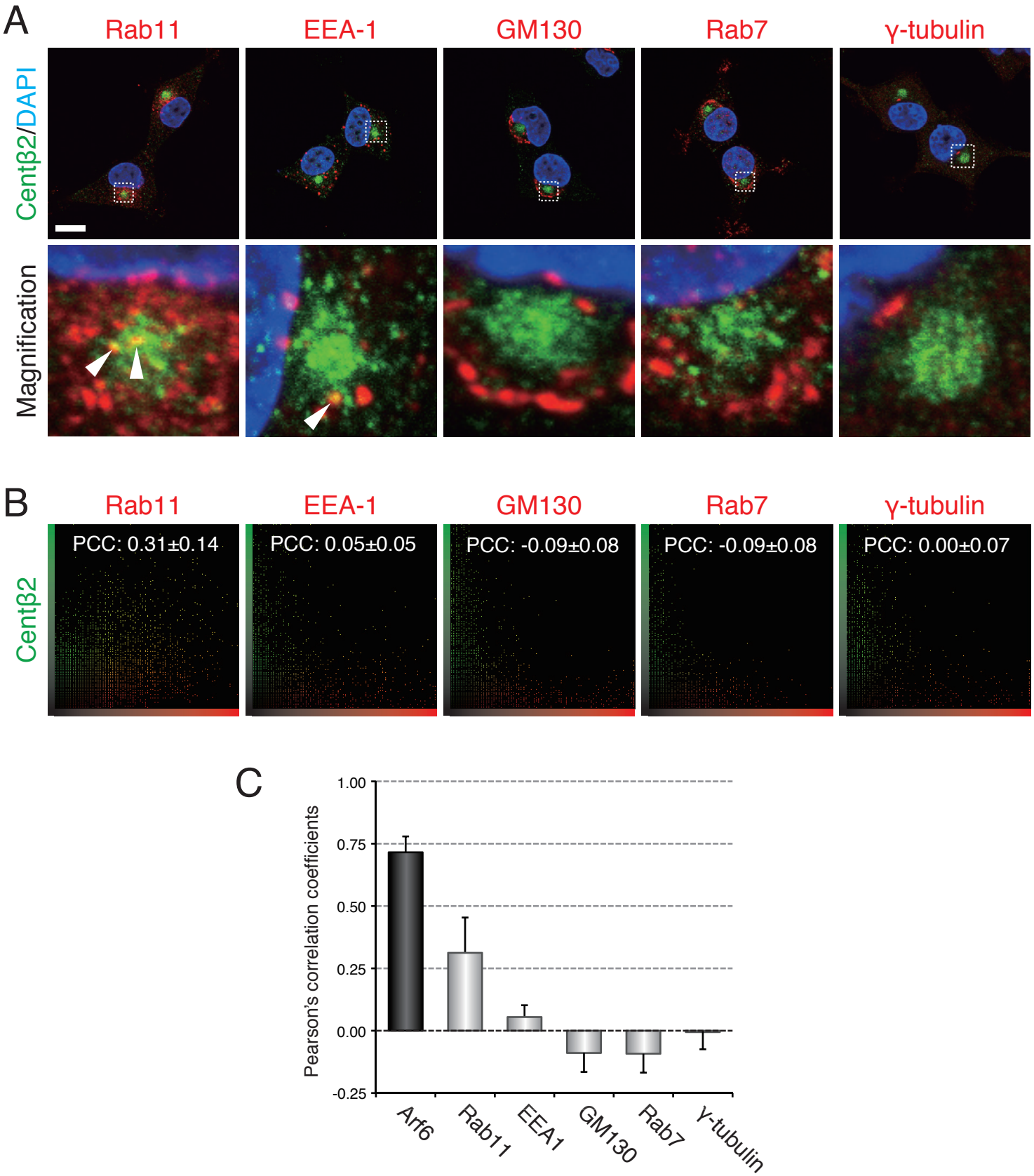

Fig. S4. Co-localization analyses of centaurin β2 with organelle markers (Rab11, EEA-1, GM130, Rab7, and γ-tubulin) in NGF-stimulated PC12 cells. **(A)** After NGF stimulation for 6 hr, PC12 cells were fixed, and then co-stained with anti-centaurin β2 antibody (green; 1/100 dilution) and antibodies against the organelle markers indicated (red; 1/100 dilution). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. **(B)** Intensity scatterplots of centaurin β2 signals and the organelle marker signals indicated. **(C)** Pearson's correlation coefficients (PCCs) for the relations between the centaurin β2 signals and the organelle marker signals. Bars represent the PCC values (mean and SD, n = 30 from 3 independent experiments).

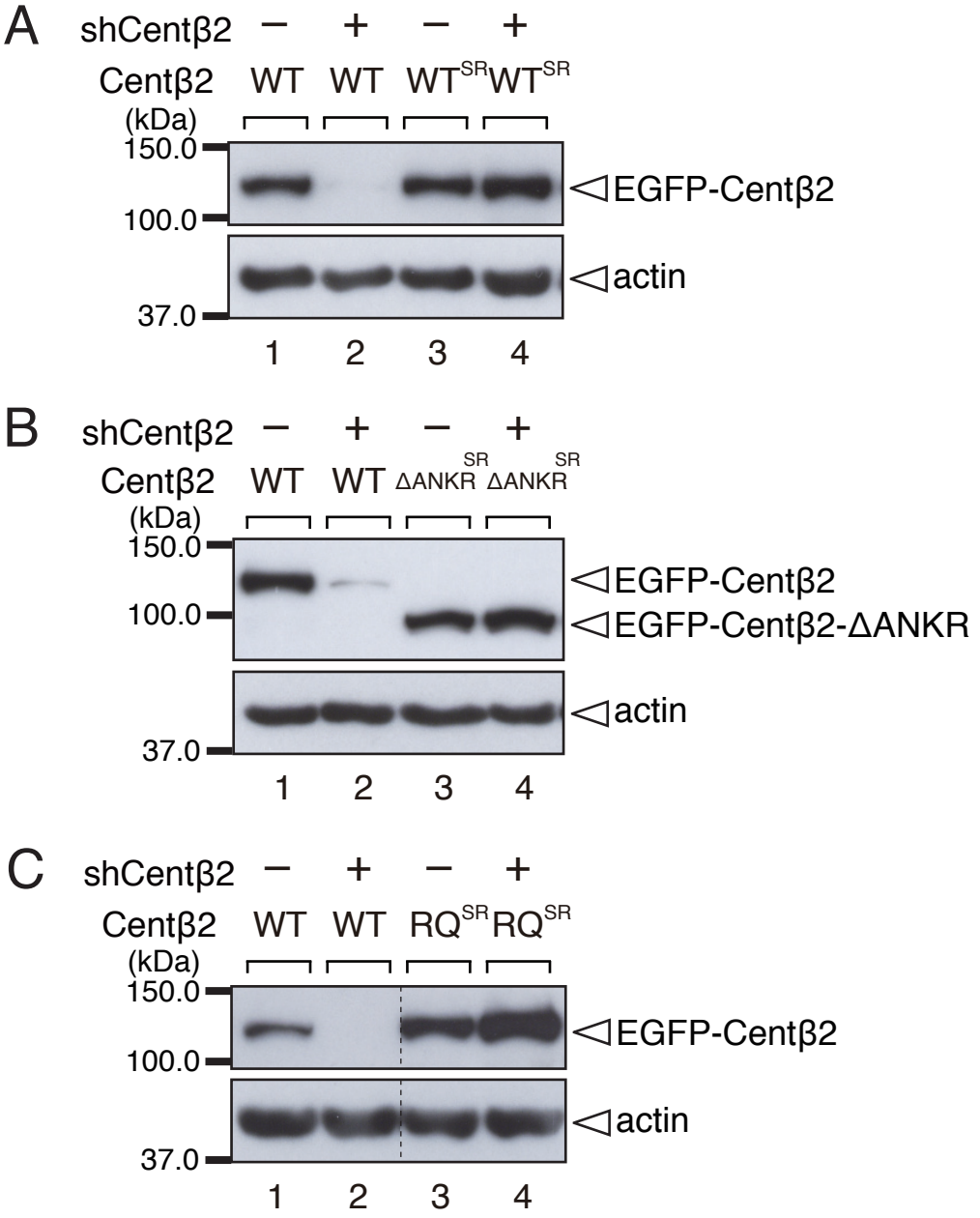

Fig. S5. shRNA resistance of centaurin β2^{SR} mutants. (A) An shRNA-resistant (SR) mutant of wild-type centaurin β2 (Centβ2-WT). Cell lysates of PC12 cells expressing *centaurin β2* shRNA (shCentβ2) and shRNA-resistant EGFP-Centβ2-WT^{SR} were subjected to 10% SDS-PAGE followed by immunoblotting with anti-GFP antibody (upper panel; 1/500 dilution) and anti-actin antibody (lower panel; 1/200 dilution). **(B)** An shRNA-resistant mutant of Centβ2-ΔANKR. Cell lysates of PC12 cells expressing shCentβ2 and EGFP-Centβ2-ΔANKR^{SR} were subjected to 10% SDS-PAGE followed by immunoblotting with anti-GFP antibody (upper panel; 1/500 dilution) and anti-actin antibody (lower panel; 1/200 dilution). The positions of the molecular mass markers (in kDa) are shown on the left. **(C)** An shRNA-resistant mutant of Centβ2-RQ. Cell lysates of PC12 cells expressing shCentβ2 and EGFP-Centβ2-RQ^{SR} were subjected to 10% SDS-PAGE followed by immunoblotting with anti-GFP antibody (upper panel; 1/500 dilution) and anti-actin antibody (lower panel; 1/200 dilution). The positions of the molecular mass markers (in kDa) are shown on the left.

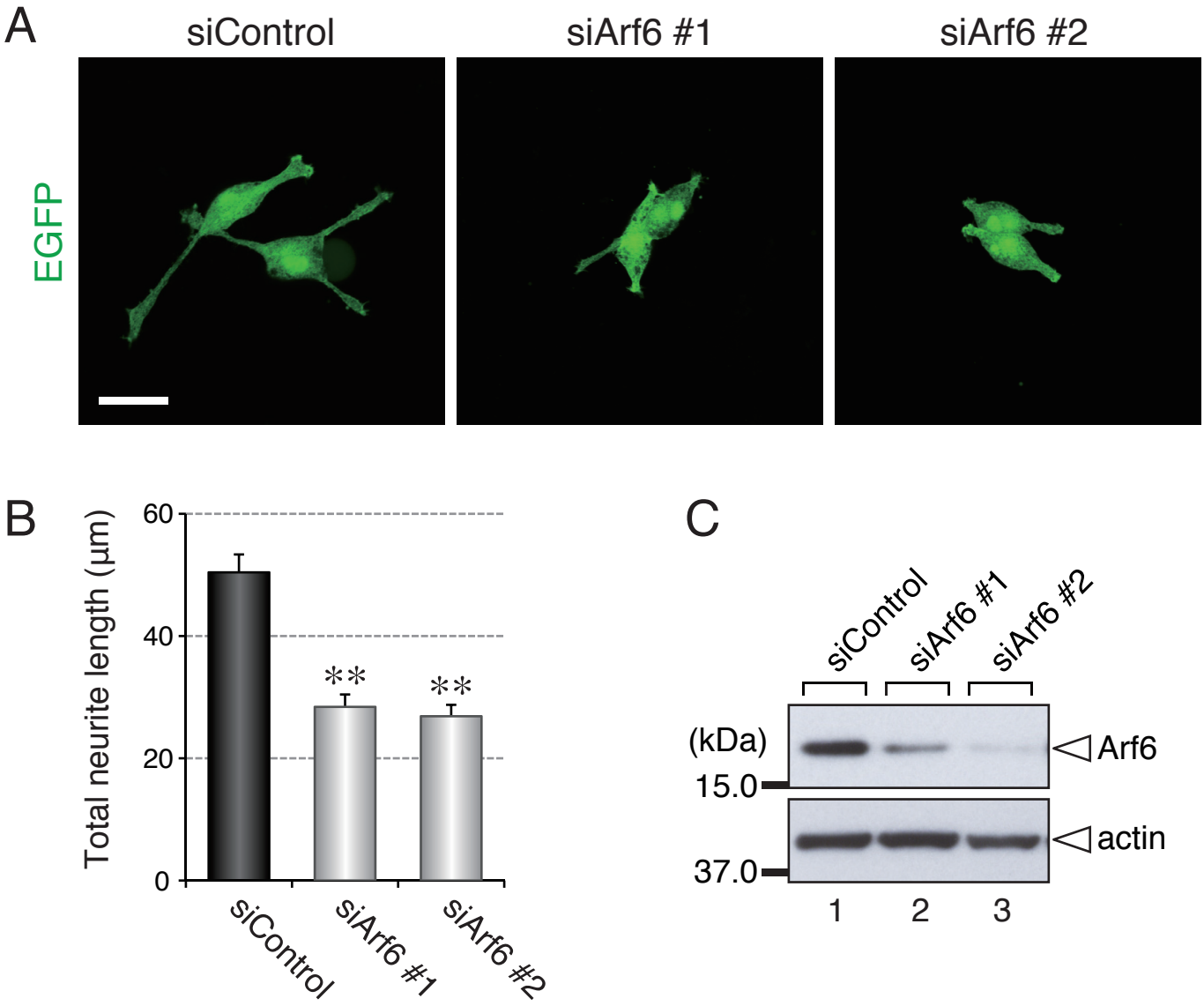

Fig. S6. Arf6 is required for NGF-induced neurite outgrowth of PC12 cells. (A) Typical images of control siRNA (siControl)-treated and *Arf6* siRNA (siArf6)-treated PC12 cells after NGF stimulation for 36 hr. siRNA-treated cells were identified by the green fluorescence of EGFP. Scale bar, 30 μm. **(B)** Effect of *Arf6*-knockdown on neurite outgrowth of PC12 cells. Bars represent the total neurite length values (mean and SE) of siControl-treated (control; black bar), siArf6#1-treated (left shaded bar), and siArf6#2-treated (right shaded bar) cells (n >100). $^{**}p$ <0.01, in comparison with the control cells (Student's unpaired *t*-test). Note that siRNA-mediated knockdown of *Arf6* dramatically inhibited neurite outgrowth of PC12 cells, the same as knockdown of *Rab35* did (Fig. 1A,B). **(C)** Reduced expression of endogenous Arf6 in siArf6-treated PC12 cells. Cell lysates of PC12 cells treated with either siControl, siArf6#1, or siArf6#2 were subjected to 12.5% SDS-PAGE followed by immunoblotting with anti-Arf6 antibody (upper panel; 1/200 dilution) and anti-actin antibody (lower panel; 1/200 dilution).