## SUPPLEMENTAL FIGURES

**Figure S1, related to Figure 1. AnkG is not required for axon specification.**

(A-C) Rat cortical neurons electroporated at E16 using ankG-shRNA immunostained at P4 (A) or P28 (B, C) using antibodies against ankG (red) and GFP. Spine-like protrusions can be seen along axons at P28 (B, C, arrowheads). Scale bars: A, B, 10 µm; C, 5 µm.

**Figure S2, related to Figure 2. Axonal enrichment of MAP1a and NF-M require ankG.**

(A, B) MAP1a is enriched in axons at 7 DIV, but is lost after silencing of ankG expression by ankG-shRNA. Scale bar, 20 µm.

(C, D) NF-H is enriched in axons at 7 DIV, but is lost after silencing of ankG expression by ankG-shRNA. Scale bar, 20 µm.

(E, F) Acetylated tubulin is found in axons at 7 DIV, and is not affected by silencing of ankG by ankG-shRNA. Scale bar, 20 µm.

(G) Immunostaining of stage 5 cultured hippocampal neuron using antibodies against αII spectrin, ankG, and MAP2. Scale bar, 10 µm.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

*shRNA adenovirus*

The shRNA adenoviral constructs were made as previously described (Hedstrom et al., 2008). The sense sequences of the shRNA used here are: for ankB 5'-GACAAGCAGAAGTTGTCAA-3'; for αII-specrtrin 5'-AGCATGATGTTCAAACACT-3'; for βII-spectrin silencing we used two shRNAs (β2r2) 5'-GCATGTCACGATGTTACAA-3' and (β2r5) 5'-GGATGAAATGAAGGTGCTA-3'; the NF-shRNA and ankG-shRNA have been previously described (Hedstrom et al., 2007).

*Hippocampal culture and Transfection*

Primary hippocampal neurons were prepared from E18 rat embryos as previously described (Ogawa et al., 2006). Briefly, hippocampi were dissected and collected in HBSS without calcium or magnesium. The collected tissue was incubated with 0.25% Trypsin in HBSS for 15 min after which it was mechanically dissociated and the suspension was centrifuged for 5 min at 1000 rpm. The pelleted cells were briefly washed and resuspended in growth medium (Neurobasal medium, 2% B27 and 1% Glutamax). All media reagents were purchased from Invitrogen. Neurons were plated at low density for immunocytochemistry (200 cells/$mm^2$) and high density for Western blot (400 cells/$mm^2$). The stage analysis of hippocampal neurons followed established criteria (Dotti et al., 1988).

Transfection of hippocampal neurons was performed by electroporation before plating using the Neon transfection system according to the manufacturer's instructions (Invitrogen). 1 mg/ml of each cDNA was used for the electroporation of 200cells/$mm^2$. Cell cultures were maintained in 5% $CO_2$ and 37$^oC$ for the indicated times. Three hours after plating, fresh growth media was added to the cells and subsequently half of the media was replaced every 4 days.

For transduction of neurons by adenovirus, the adenovirus ($10^5$-$10^6$ pfu) was added to cultures 1 hr after plating. After 2 hr of incubation with the adenovirus, the media was replaced with fresh growth media. After 7 days, cells were fixed and immunostained to evaluate the knockdown effects.

*Antibodies*

Mouse monoclonal pan-Nav and pan-Neurofascin antibodies have been previously described (Hedstrom et al., 2008; Schafer et al., 2004). Mouse monoclonal antibodies against ankB (N105/17) and ankG (N106/36, N106/20) were purchased from the UC Davis/NIH NeuroMab mouse monoclonal antibody resource. Other mouse monoclonal antibodies included: αII spectrin (Millipore), βII spectrin and KIF3A (BD Biosciences), KAP3 (SantaCruz Biotechnology), Neurofilament H (Covance). The rabbit polyclonal βIV spectrin antibody was previously described (Ogawa et al., 2006). The affinity-purified rabbit polyclonal anti-ankB and anti-ankG were made against the same immunogen used for the mouse monoclonal antibodies against ankB and ankG. Specificity for these

antibodies was tested against knockout tissue (ankB) and against neurons with ankG silenced by shRNA (data not shown). Rabbit anti-GFP and Rat anti-GFP were purchased from Invitrogen and Nacalai Tesque Inc., respectively. The chicken polyclonal MAP2 antibody was purchased from EnCor Biotechnology. The rabbit anti-neurofilament M (NFM) antibodies were purchased from Millipore. All fluorescent secondary antibodies were purchased from Invitrogen except for AMCA-conjugated anti-chicken antibody (Accurate Chemical).

*Immunofluorescence and imaging*

Cultured neurons were fixed in 2% PFA, pH 6.0 (for αII spectrin immunostaining) or 4% PFA, pH 7.2 (for all other antibody combinations) and immunolabeled as previously described (Hedstrom et al., 2007). For immunostaining of nervous system tissues, brains were dissected at the indicated times, fixed in 4% paraformaldehyde for 1 hr (embryos) to 1 hr 30 mins (postnatal times) on ice followed by immersion in 20% sucrose overnight at 4$^{o}$C. After this, tissues were sectioned using a freezing stage Microtome (Microm KS 34, Thermo Scientific), collected and suspended in 0.1M PB and finally spread out on glass coverslips for immunostaining.

Immunofluorescence labeling was visualized and images were collected on an Axio-imager Z1 microscope (Carl Zeiss MicroImaging) fitted with an AxioCam digital camera (Carl Zeiss MicroImaging). Images were taken using 20X (0.8 NA), 40X (1.0 NA), 40X (0.75 NA), or 63X (1.4 NA) objectives. AxioVision (Carl Zeiss MicroImaging) acquisition software was used for collection

of images. Measurement of fluorescence intensity was performed using ImageJ (NIH) and AxioVision analysis software. In some images, linear contrast and brightness adjustments were performed using Adobe Photoshop (Adobe). In some instances of *in vivo* immunostaining, Z-stacks and 3-D reconstruction was performed using AxioVision. No other processing of the images was performed. All figures were assembled using Adobe Illustrator.

*Generation of βII spectrin conditional knockout mice.*

The SPNB2 gene has multiple alternatively spliced mRNAs. The conditional knockout strategy was based on the major transcript (ID: ENSMUST00000006629) and a targeting construct was generated in which loxP sites were designed to flank exon 3, resulting in disruption of the reading frame upon Cre mediated excision. A genomic bacterial artificial chromosome clone (bMQ-431M24) containing the SPNB2 locus was purchased from Geneservice Ltd. Cambridge Park, UK. Genomic DNA fragments harboring exon 3 of SPNB2 were subcloned in pBluescript (Stratagene). The targeting vector comprised a 6.5 kb long arm and a 1.7 kb short arm, with a PGK–neomycin resistance ($Neo^r$) selection cassette flanked by two FRT sites and the herpes simplex thymidine kinase gene. The targeting vector was linearized and electroporated into mouse embryonic stem (ES) cells (129SV/EV). Positive clones were identified by PCR and confirmed by Southern blot analysis using external and internal probes. Three correctly targeted ES cell clones were injected into C57BL/6J blastocysts to produce germline transmitting chimeric mice, which then were mated with C57BL/6J mice for generation of $SPNB2^{fl/+}$ animals. These mice were then bred

to mice bearing a flp-recombinase transgene (ROSA-Flp) to remove the neomycin selection marker. The resulting SPNB2$^{fl/+}$ offspring were crossed with Nestin-Cre mice to excise the floxed exon, resulting in βII spectrin-deficient mice.

**REFERENCES**

Dotti, C. G., Sullivan, C. A., and Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. J Neurosci 8, 1454-1468.

Hedstrom, K. L., Ogawa, Y., and Rasband, M. N. (2008). AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. J Cell Biol 183, 635-640.

Hedstrom, K. L., Xu, X., Ogawa, Y., Frischknecht, R., Seidenbecher, C. I., Shrager, P., and Rasband, M. N. (2007). Neurofascin assembles a specialized extracellular matrix at the axon initial segment. J Cell Biol 178, 875-886.

Ogawa, Y., Schafer, D. P., Horresh, I., Bar, V., Hales, K., Yang, Y., Susuki, K., Peles, E., Stankewich, M. C., and Rasband, M. N. (2006). Spectrins and ankyrinB constitute a specialized paranodal cytoskeleton. J Neurosci 26, 5230-5239.

Schafer, D. P., Bansal, R., Hedstrom, K. L., Pfeiffer, S. E., and Rasband, M. N. (2004). Does paranode formation and maintenance require partitioning of neurofascin 155 into lipid rafts? J Neurosci 24, 3176-3185.