Supporting Information

Hurtado de Mendoza et al. 10.1073/pnas.1114226108

SI Materials and Methods

Generation of the HeLa LFG Cell Line and Fas Apoptosis Assays. The HeLa-LFG cell line was generated by transducing HeLa cells with a lentivirus expressing a Flag tagged LFG cDNA. Four days posttransduction the Fas agonistic antibody CH11 was added to the culture media to select for resistant cells. After two weeks of selection cells were tested for LFG expression by WB with the FlagM2 antibody (Sigma Aldrich, St Louis, MO). To determine whether LFG was the only factor conferring resistance to Fas-mediated apoptosis in this system HeLa-LFG cells were transduced by Lenti-shLFG or by a control shRNA against beta-secretase 1 (BACE1). Knockdown of LFG was confirmed by WB with Flag antibody.

For the apoptosis assays cells were plated into 6 well plates and when 50% confluency was reached they were challenged with the mouse anti-human Fas mAb CH11 at 0.5 μg/mL (Upstate Biotech, Billerica, MA). After 24 h, cell death was measured by FACS analysis of Annexin V staining (BD Biosciences, San Jose CA). Assays for each cell line and condition were performed in triplicate.

Cloning and Validation of the shRNAs by WB. The shRNA constructs were generated using a 94-mer oligonucleotide that contains a 22-nucleotide sense and a 22-nucleotide antisense strand separated by a 9-nucleotide loop. At the 5′ end an XbaI site followed by a stretch of five adenines that serve as template for the Pol III promoter termination signal, and 25 nucleotides complementary to the 3′ end of the mouse U6 pol III promoter. A PCR using this 94 mer and a SP6 primer amplified a fragment containing the whole mouse U6 promoter and the shRNA sequences. XbaI and SpeI digestion allows ligation of the shRNA cassette into the NheI site located at the U3 region of a lentiviral vector containing an eGFP cassette driven by the mouse PGK promoter, as a transduction marker. PCR conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 40 seconds and 72 °C for 50 seconds. Primer sequences (sense and antisense sequence in bold, loop underlined): shmLFG1, 5′-GCT GTC TAG ACA AAA **AGC CGT GTT CTT TGC AAC TTA CCT** CTC TTG AA**G GTA AGT TGC AAA GAA CAC GGC** AAA CAA GGC TTT TCT CCA AGG GAT A-3′; shmLFG2- 5′-GCT GTC TAG ACA AAA **AGC CCT CAA CAT CTA CTT AGA CAT** CTC TTG AA**T GTC TAA GTA GAT GTT GAG GGC** AAA CAA GGC TTT TCT CCA AGG GAT A-3′; shLFG, 5′-GCT GTC TAG ACA AAA **AGC TGT GTT CTT TGC AAC CTA CCT** CTC TTG AA**G GTA GGT TGC AAA GAA CAC AGC** AAA CAA GGC TTT TCT CCA AGG GAT A-3′. Primers for shBACE and shGFP were the same as described (1, 2). Validation of the shRNA constructs was performed by calcium phosphate transfection of HEK293T cells together with a LFG cDNA that contains a C' V5 tag in a 5:1 ratio followed by protein extraction and WB using V5 antibody (Invitrogen; Carlsbad, CA) and p65 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) as a loading control.

Lentivirus Production. Recombinant lentiviruses were produced by calcium phosphate transfection of HEK293T cells as described (3, 4). After 48 and 72 h infectious lentiviruses were harvested, filtered through a 0.22 μm pore cellulose acetate filter and concentrated by ultracentrifugation (2h at 50,000 × *g*) followed by purification on a 20% sucrose gradient (2h at 46,000g) as described (3, 4). The viral titer was assessed using an immunocapture p24-gag ELISA (Alliance; DuPont-NEN) and by quantification of GFP positive cells after transduction of HEK293T cells by flow cytometry as described (5).

Transduction of NIE 115 Mouse Neuroblastoma Cells and Q-PCR for Knockdown Validation. NIE115 cells were transduced with lentiviruses expressing two different shRNAs specific for LFG and an shRNA that targets BACE1 using an MOI of 50. Total RNA was extracted using TRIzol reagent (Invitrogen) and followed by reverse transcription using SuperScript III First-strand Synthesis System (Invitrogen). Quantitative PCR was performed using SYBR green in an ABI PRISM 7700 (Applied Biosystems; Foster City, CA) with the following primers: LFG1 5′-TGC CCT TCC AAT ACG TCC C-3′; GAA CAC ACC CGC TCC CAG T; LFG2 5′-GGC TAA CCC CGG CTG GTA-3′; GCA AGC CAG AGT CAG GTA AGT TG; Cyclophyllin 5′-GGC CGA TGA CGA GCC C-3′; TGT CTT TGG AAC TTT GTC TGC AAA T. These primer sets were also used for validation of LFG knockdown and KO in cerebellar cDNA.

Lentiviral Transgenesis. The basic methodology has been described by our laboratory (1).

Briefly, 6-wk-old B6D2 F1 females were superovulated by injecting 5 units of pregnant mare serum gonadotropin (Sigma), followed by injection of 5 units of human CG (Sigma) 48 h later. At this time B6D2 F1 females were mated to B6D2 F1 males and the following day female oviducts were flushed with FHM medium (Specialty Media, Lavellette, NJ) to isolate fertilized eggs at the two-cell stage. Removal of the zona pellucida was performed by acidic tyrode treatment (Sigma) followed by transduction with 2,500 ng of p24 per ml in 5 μL of KSOM medium (Specialty Media) covered with light mineral oil. Forty-eight hours after transduction, blastocysts were transplanted into the uterus of ICR pseudopregnant females.

Tissue Preparation. All procedures were approved by the Institutional Animal Care Committee of The Salk Institute for Biological Studies. Mice were euthanized by Nembutal overdose, and their brains were dissected. Brains were either fixed with Bouin and Paraffin embedded or fixed with 4% PFA followed by 30% sucrose and frozen in OCT. Sagittal or coronal sections were cut using a microtome at 10μm for the Paraffin blocks or with a cryostat at 20 μm for the frozen samples.

DISC Immunoprecipitation and Immunoblots. HeLa and HeLa LFG cells were either untreated or incubated for 15 min with 2 μg/mL of anti-Fas antibody APO1-3 (Alexis Biochemicals, Farmingdale, NY). Then, cells were lysed in 30 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and complete protease inhibitors (Roche, Palo Alto, CA). Protein-A Sepharose beads (Amersham Pharmacia, Piscataway, NJ) were precoated with rabbit-anti mouse antibody and were then added to the samples and incubated for 3 h rotating at 4 °C. Fas antibody was added to the control lysates at this step. Samples were washed three times with lysis buffer, and then the beads were resuspended in standard WB sample buffer. After boiling, the immunoprecipitates, together with the whole cell extracts, were resolved by SDS/PAGE and subjected to immunoblot analysis with FADD (BD Transduction Laboratories, Lexington, KY), caspase 8 (Cell Signaling, Boston, MA), and Flag M2 (Sigma) antibodies. The intensity of the procaspase 8 and cleaved caspase 8 bands was calculated using Image J, and the results were expressed as the percentage of cleaved caspase 8.

ISH, Nissl Staining, TUNEL Assay, and Immunohistochemistry. The full-length LFG cDNA was cloned into pGEMTeasy and linearized by digestion with SpeI. The linear template was gel-purified and used

to generate a riboprobe by in vitro transcription in the presence of [35S]-UTP. Radioactive ISH was performed as described (6). Nissl staining was performed by Cresyl Violet staining.

TUNEL immunoassays were performed using the cell death detection kit (Roche). Immunohistochemistry was performed as described (7). The following antibodies were used: mouse anti-parvalbumin (Chemicon; Billerica, MA), rabbit and mouse anti-calbindin (Swant; Bellinzona, Switzerland), anti-active caspase 3 (Cell Signaling), anti-active caspase 8 (Novus Biologicals, Littleton, CO).

Slice Imaging and Statistical Analysis. The slices were imaged with a Leica TCS SP2 confocal microscope using the 488 and 568 excitation settings for calbindin and TUNEL, respectively, with a 20× objective. For image quantification, *z* stacks of four images over 15 μm were taken. We analyzed eight representative images per animal, four untreated and four Jo2 treated. Within each image, we defined two types of 3×3 fields: (*i*) the PC field, based on the CB positive signal, and (*ii*) the IGL field, adjacent to the PC field with no CB-positive cells. Three different fields of each type were analyzed in each image to determine the number of TUNEL-positive cells, for a total of 12 PC fields and 12 IGL fields per mouse and per treatment. Statistical analysis was performed with SigmaStat software and analyzed by two-way ANOVA followed by Tukey's post hoc test for multiple comparisons. ANOVA results were considered significant if $P < 0.05$.

Morphometric Analysis. Morphometric analysis was performed in sagittal sections with matching levels along the medial-lateral axis by focusing in olfactory bulb, septum, hippocampus, and colliculus as neuroanatomical matching points. We took high-magnification pictures and delineated the cerebellum using a morphometric program (AxioVision) that gave us values in μm^2. For the IGL, we used the same program to draw lines that covered the thickness of the IGL at three different points and made an average to get the value per lobule. Thickness was measured in μm. The results were expressed in percent reduction compared with WT.

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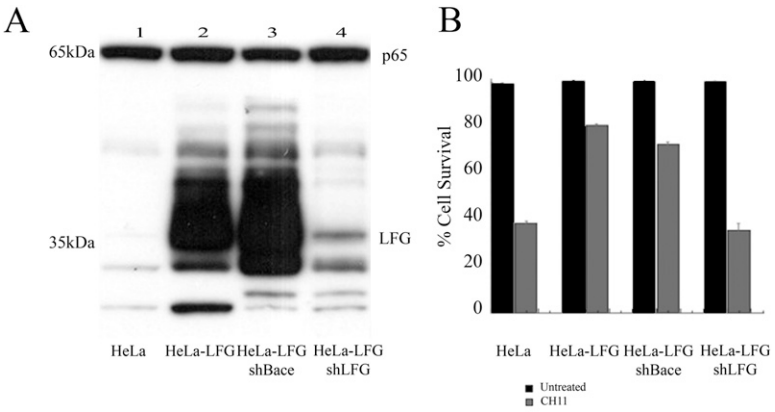

Fig. S1. LFG protects HeLa cells from Fas-mediated apoptosis. (*A*) SDS/PAGE/immunobloting of protein lysates from WT or LFG expressing HeLa cells alone or transduced with lentiviruses expressing shRNAs against LFG or BACE1 analyzed by using the Flag M2 antibody specific for Flag-tagged LFG and a p65 antibody as a loading control. Lane 4 shows effective down-regulation with the shRNA specific for LFG. (*B*) Histograms representing the percentage of live cells after treatment with 0.5 μg/mL of the CH11 Fas agonistic antibody for 24 h, showing how HeLa cells expressing LFG are protected from Fas-mediated cell death and how this protection is abolished by using a LFG specific shRNA. Cell death was measured by Annexin V staining.

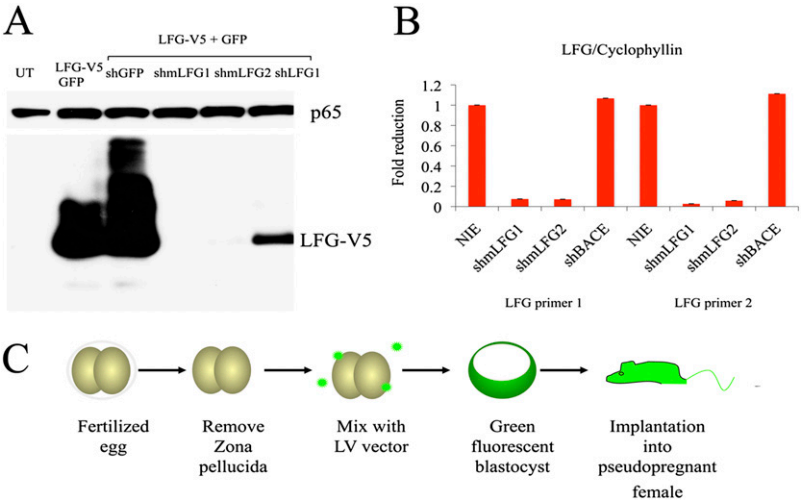

Fig. S2. Validation of the knockdown construct. (*A*) WB of protein extracts from 293T cells untransfected (UT) or transfected with LFG-V5 and GFP alone or in combination with shRNAs against BACE1 or LFG. The upper panel is probed with p65 antibody as a loading control and the lower panel with V5 antibody for detection of LFG-V5 showing the down-regulation of LFG expression. (*B*) Histograms showing LFG mRNA levels in NIE-115 cells. mRNA levels were determined by real time PCR in uninfected cells (NIE) and in cells transduced with lentiviruses containing shRNAs against LFG (90–95% knockdown) and BACE 1 as indicated. Two different primer sets were used for LFG detection; LFG levels were normalized to cyclophyllin. (*C*) Schematic representation of the lentiviral transgenesis technique.

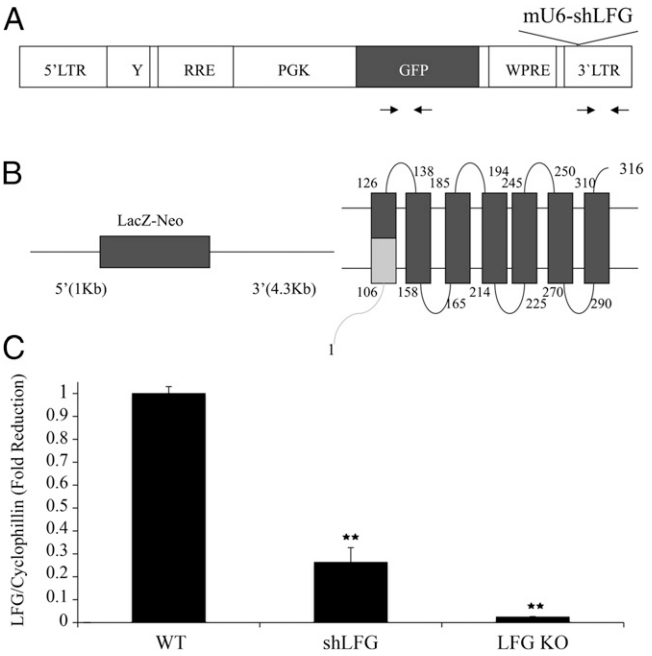

Fig. S3. LFG knockdown and KO mice. (*A*) Schematic representation of the lentiviral construct used to generate the shLFG mice. LTR, long terminal repeat; **Ψ**, packaging sequence; RRE, rev response element; PGK, phosphoglycerate kinase (promoter); GFP, green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; mU6, mouse U6 (promoter). Arrows indicate the position of genotyping primers. (*B*) Schematic representation of the LFG KO targeting vector containing the LacZ-Neomicin cassette and a topology map of LFG showing where the insertion takes place and the portion of LFG that forms part of the fusion protein with LacZ. Numbers represent amino acid position. (*C*) Histograms representing the QPCR performed on cerebellar cDNA from WT, shLFG and KO mice. LFG was normalized against cyclophyllin. $*P \leq 0.05$, $**P \leq 0.01$, ± SEM, $n = 3$ mice per group.

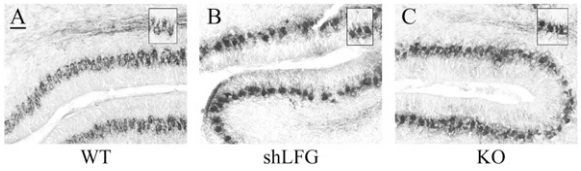

Fig. S4. Calbindin expression confirms the presence of shLFG and KO PCs. CB immunostaining of cerebellar sagittal sections of WT (*A*), shLFG (*B*), and KO (*C*) mice at P5 are shown. The upper left corner of each panel contains high-magnification insets of the PCs. (Scale bars: 50 μm.)