Supporting Information

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SI Materials and Methods

Antibodies and Chemicals. Primary antibodies were monogenic audiogenic seizure susceptible 1 [MASS1; Santa Cruz Biotechnology and from Uwe Wolfrum, Johannes Gutenberg University of Mainz, Germany (1)], myelin-associated glycoprotein (MAG; Zymed), NeuN/GFAP/myelin basic protein (MBP) (Chemicon), proteolipid protein (PLP)/2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase) (Abcam), PKC/p-CREB-S133/CREB (CREB, cAMP responsive element binding protein) (Cell Signaling), HA/M2-Flag (Sigma), Fluoro-myelin (Molecular Probes) (2), and myelin oligodendrocyte glycoprotein/GST (Santa Cruz Biotechnology). All chemicals were purchased from Sigma unless otherwise stated. *Mass1*-specific siRNA duplex and control siRNAs were obtained from Invitrogen.

Mice. Experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of University of California, San Francisco (UCSF). *Frings* mice were from an in-house colony at UCSF. SWR/Bm mice were purchased from the Jackson Laboratory. Because the *Frings* mice have been maintained as an inbred line for more than 60 y, SWR/Bm mice were used as controls because they are also derived from the Swiss family and are the most closely related known line to *Frings* (3).

Immunohistochemistry. Cryosections were prepared from 4% paraformaldehyde-perfused control (SWR/Bm) and *Frings* mice brains, permeabilized with Triton X-100, and blocked and incubated overnight with primary antibodies. After washing with PBS, sections were incubated with secondary Cy2- or Cy3-labeled anti-mouse or -rabbit IgGs (Amersham). Samples were washed with PBS (5 min each three times), and coverslips were mounted with Vectashield (Vector Laboratories) mounting medium and DAPI.

Transmission EM. EM for the analysis of myelin structure was carried out as described previously (4). Briefly, mice were first anesthetized with 2.5% avertin and then perfused with 4% paraformaldehyde and 2.5% glutaldehyde in phosphate buffer and incubated in fixative for 1 wk. After being postfixed, spinal cords were dissected and embedded in Epon. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate and then collected on grids. The pictures were taken with the Tecnai electron microscope at the San Francisco VA Medical Center EM Lab.

In Vitro Binding and Ubiquitylation Assay. For in vitro assays to identify G_α protein(s) binding to MASS1, HEK293T cells were transfected with each FLAG-tagged G_α and GST-tagged MASS1 C terminus (amino acid residues 6148–6298) and the third cytoplasmic loop (amino acid residues 6074–6097) or GST alone (Figs. S2*B* and 4*A*). Twenty-four hours after transfection, lysates were incubated for 1 h at room temperature with Glutathione Sepharose 4B coated beads (Amersham) in lysis buffer [50 mM Tris·Cl (pH 7.4), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 1% Triton X-100, phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors], washed three times with wash buffer (lysis buffer containing 0.1% Triton X-100), and subjected to Western blot analyses against FLAG and GST. To assess ubiquitylation, HEK293T cells were transfected with HA-tagged ubiquitin, MAG, and MASS1 (Fig. 3*D*). After transfection, cells were serum-starved in DMEM devoid of calcium chloride (Invitrogen) for 24 h and incubated for 4 h with 10 μM MG132. Cell lysates were immunoprecipitated with anti-MAG antibody, washed three times with the above wash buffer, and subjected to Western blot analysis against HA. The band density of each lane was evaluated using TINA software (Fuji).

Animal Cell Culture. HEK293T, COS-7, and S16 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS. Primary oligodendrocyte progenitor cell (OPC) cultures were performed according to the immunopanning method (4, 5). Briefly, brains were obtained from postnatal day 7 mice, and the cerebellum and midbrain/hindbrain structures were removed by crude dissection, digested in papain, sequentially immunopanned on Thy1.2 (Serotech), anti-galactocerebroside (GalC; Chemicon), and then O4 antibody (Chemicon)-coated plates to select $GalC^{-}O4^{+}$ OPCs. Purified OPCs were cultured on poly-D-lysine–coated tissue culture dishes or chamber slides at 37 °C, 10% CO_2 in DMEM containing human transferrin, BSA, putrescine, progesterone, sodium selenite, *N*-acetyl-L-cysteine, D-biotin, forskolin, bovine insulin (all from Sigma), glutamine, sodium pyruvate, penicillin–streptomycin, B-27 supplement (all from Invitrogen), Trace Elements B (Mediatech), and ciliary neurotrophic factor (10 ng/mL; PeproTech). Proliferation medium also contained OPC mitogens such as platelet-derived growth factor AA and neurotrophin 3 (both from PeproTech), whereas differentiation medium included triiodothyronine (T3; Sigma) without OPC mitogens. Oli-neu cells were maintained in SATO medium (DMEM containing N1, biotin, insulin, L-glutamine, and horse serum). For differentiation to mature OLs, 1 mM dbcAMP (Calbiochem, catalog no. 28745) was added to the SATO medium for 5 d.

Plasmids. The cDNAs encoding MASS1, MAG, $G_{\alpha z}$, $G_{\alpha 13}$, $G_{\alpha olf}$, $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, $G_{\alpha t2}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15}$, $G_{\alpha s}$, $G_{\alpha q}$, and $G_{\alpha 12}$ were amplified from mouse brain total RNAs by RT-PCR, and ligated into pCMVTag4A (Stratagene) or pEBG (mammalian GST fusion vector) (6) (Fig. 5*A* and Fig. S2). We also used four previously reported G_α plasmids ($G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha i2}$, and $G_{\alpha 12}$), which were obtained from American Type Culture Collection and Henry Bourne (UCSF) (7, 8).

Quantification of cAMP. Intracellular cAMP was quantified using a cAMP Assay kits (R&D Systems and Thermo Scientific) according to the manufacturer's protocols.

1. Maerker T, et al. (2008) A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. *Hum Mol Genet* 17(1):71–86.
2. Shin D, Shin J-Y, McManus MT, Ptácek LJ, Fu YH (2009) Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. *Ann Neurol* 66(6):843–857.
3. Skradski SL, et al. (2001) A novel gene causing a mendelian audiogenic mouse epilepsy. *Neuron* 31(4):537–544.
4. Shin D, Howng SYB, Ptáček LJ, Fu Y-H (2012) miR-32 and its target SLC45A3 regulate the lipid metabolism of oligodendrocytes and myelin. *Neuroscience* 213:29–37.
5. Dugas JC, Tai YC, Speed TP, Ngai J, Barres BA (2006) Functional genomic analysis of oligodendrocyte differentiation. *J Neurosci* 26(43):10967–10983.
6. Chen P, Hutter D, Liu P, Liu Y (2002) A mammalian expression system for rapid production and purification of active MAP kinase phosphatases. *Protein Expr Purif* 24(3):481–488.
7. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR (1993) Substitution of three amino acids switches receptor specificity of Gq α to that of Gi α. *Nature* 363(6426):274–276.
8. Wong YH, et al. (1991) Mutant alpha subunits of G_{i2} inhibit cyclic AMP accumulation. *Nature* 351(6321):63–65.

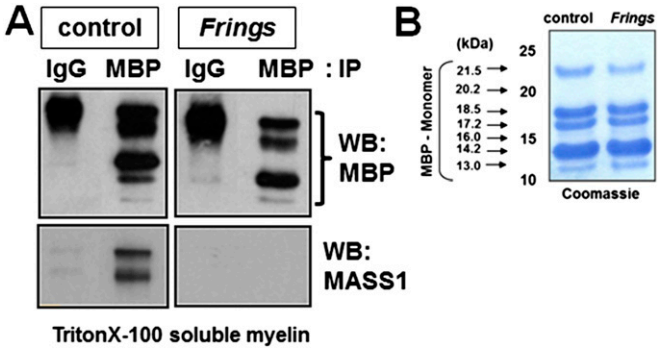

Fig. S1. (*A*) The myelin fractions were purified from control and *Frings* mice and then the myelin protein complex was immunoprecipitated by using anti-MBP. The precipitates were analyzed by Western blot against MBP and MASS1. It showed that MASS1 is present in the myelin fraction associated with MBP. The myelin from the *Frings* mice did not show any MASS1 signals. (*B*) The purified myelin fractions were stained with Coomassie blue for the normalization of myelin.

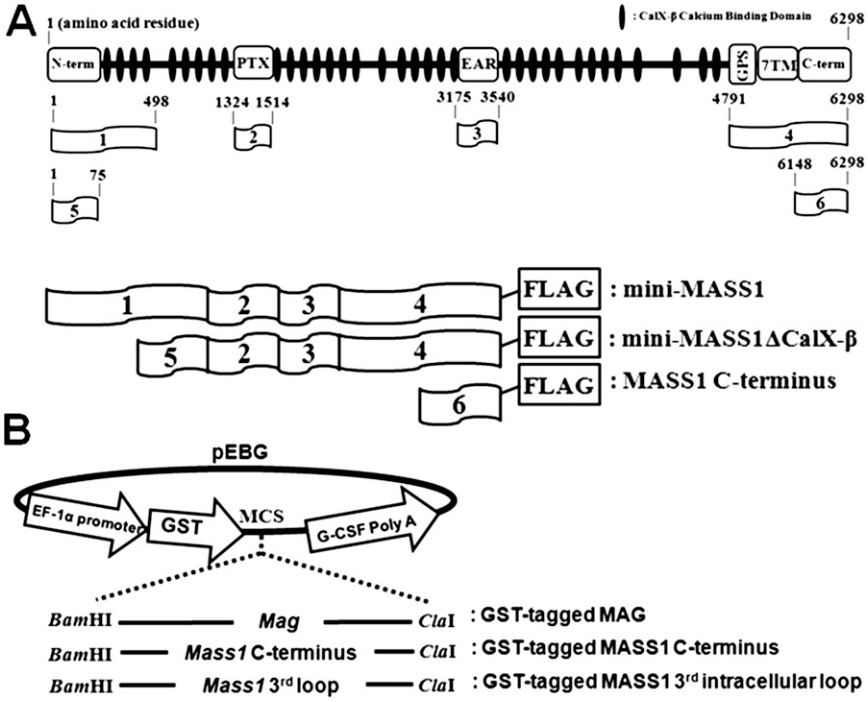

Fig. S2. Exogenous MASS1 and MAG constructs. (*A*) MASS1 has a large ectodomain with 35 CalX-β repeats, 7 EAR repeats forming a putative β-propeller folding structure, a pentraxin domain, a G protein coupled receptor (GPCR)-proteolytic site (GPS), and B-family 7 TM domains. Mini-MASS1 contains the N terminus, four copies of CalX-β, one Pentraxin, seven EAR repeats, a GPS, a B-family 7 TM domain, and whole C terminus of endogenous MASS1. We have also created constructs lacking four copies of the CalX-β domain (mini-MASS1ΔCalX-β) or having only the C terminus (MASS1 C terminus). (*B*) Full coding sequences of MAG, MASS1 C terminus, and third cytoplasmic loop regions were subcloned into pEBG plasmids for the GST-tagged protein expressions in mammalian cells.

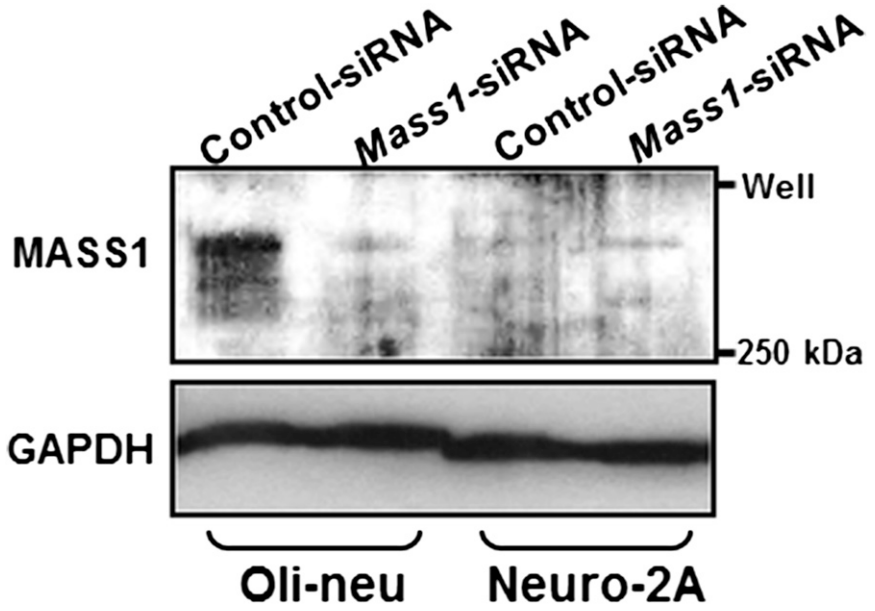

Fig. S3. *Mass1* siRNAs dramatically suppressed the expression of MASS1 in oli-neu (MASS1 present) cells. Neuro-2A neuronal cells (MASS1 absent) were used as a control to show the protein band is specific to MASS1.

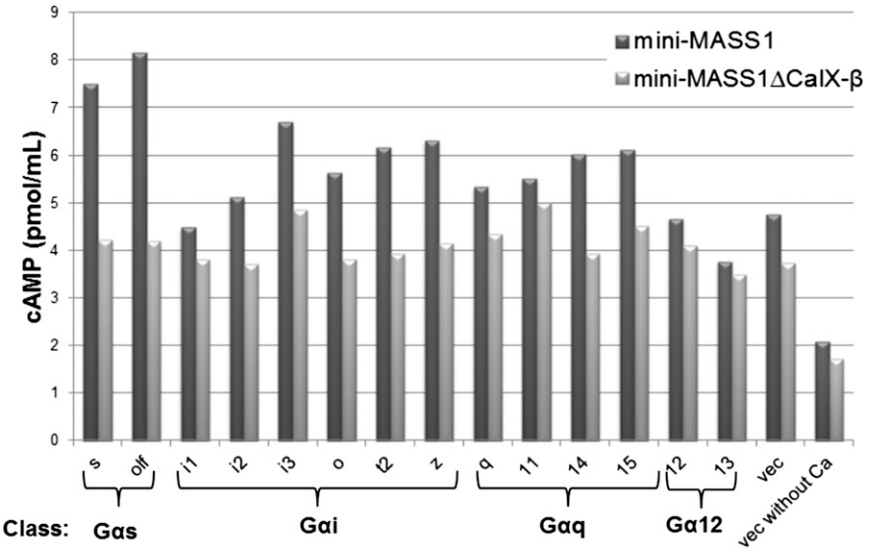

Fig. S4. The evaluation of immunoenzymatic colorimetric cAMP detection. Fourteen G_{α} proteins (classified by $G_{\alpha s}$, $G\alpha_{i2}$, $G_{\alpha q}$, and $G_{\alpha 12}$ classes) and empty control vector (with or without calcium) were cotransfected into COS-7 with mini*Mass1* or mini*Mass1*ΔCalX-β. Colorimetric enzymatic immunoassay against cAMP revealed that calcium-activated mini-MASS1 increases intracellular cAMP primarily via $G_{\alpha s}$ class significantly more than controls (empty vector and mini*Mass1*ΔCalX-β).

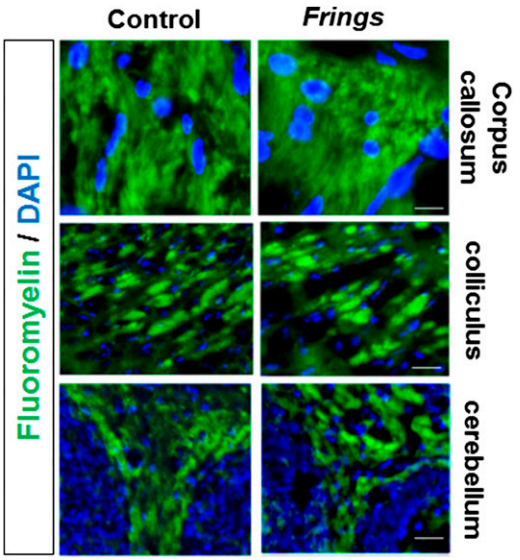

Fig. S5. Fluoro-myelin staining analyses on cryosections of control and *Frings* mice showed no gross difference in the myelin of the corpus callosum, cerebellum white matter tracts, and colliculus.

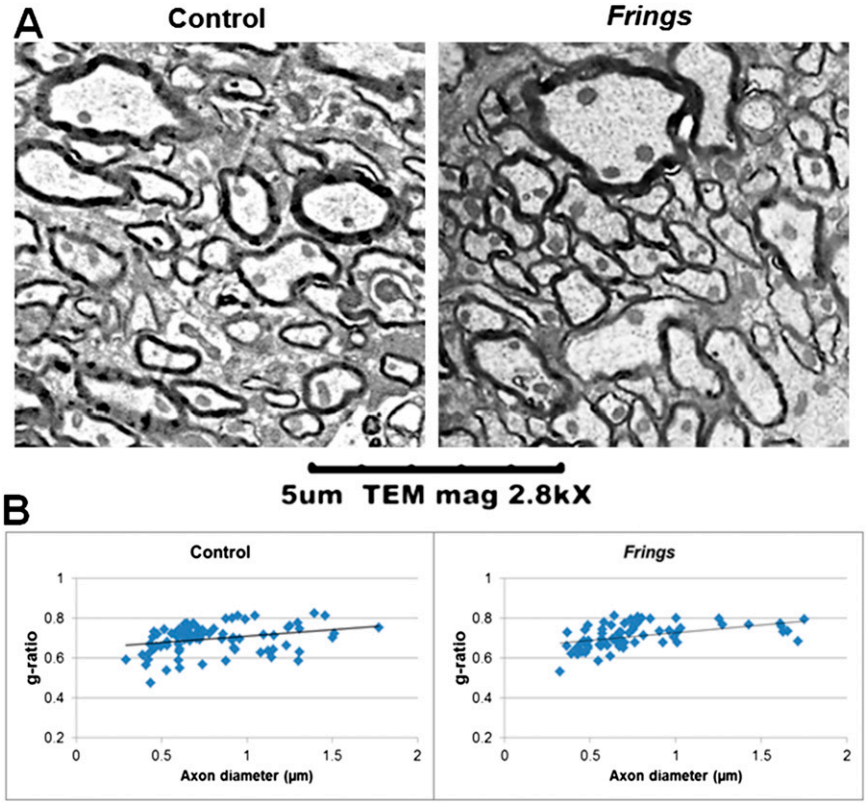

Fig. S6. (*A*) Electron microscopy analysis using 1-mo-old control and *Frings* mice showed that there is no gross change in myelin structure of superior colliculus. (*B*) There was no obvious change in the *g*-ratios between *Frings* and control.