Supporting Information

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SI Materials and Methods

Animals and Housing. Homozygous G-protein-coupled receptor 37-like 1 null mutant (*Gpr37l1*$^{-/-}$) male and female mice and their wild-type littermates were used. All mice were bred from heterozygous crossings (mixed genetic background 75% C57BL6/J; 25% 129S/SvEv). After weaning, mice were housed by litter of the same sex, three to five per cage, and maintained in a temperature-controlled room at 21 ± 2 °C, on a 12-h light–dark cycle (lights on at 7:00 AM), with food and water available ad libitum. C57BL/6J male mice were obtained from The Jackson Laboratory. All animals were born and bred in a specific pathogen-free facility and were subjected to experimental protocols (Consiglio Nazionale delle Ricerche, approved protocols 52/2009-B and 93/2012-B), as reviewed and approved by the Public Veterinary Health Department of the Italian Ministry of Health (Rome, Italy), according to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian laws and European Union's directives (86/609/EC).

PCR Primers Used for Genotyping Gpr37l1 Mutant Mice.

F1: GTGACAGTGTGTAATATTC

F2: CCTACGCCATCATGCTC

R1: CAAGCCAGTGGTCTTGTAC.

In Situ Hybridization. In situ mRNA detection (ISH) was performed as previously described (1). A 298-bp PCR fragment was amplified from *Gpr37l1* mouse cDNA using the following primers:

Forward: CTCATCTTTAGGTGGGCATAGAGCCAAG

Reverse: GCTCTCGGTCACCGGATACAGGGG

and subcloned into a pBluescript II KS vector to generate ^{35}S-CTP–labeled antisense and sense riboprobes from T7 and T3 promoters, respectively, using standard in vitro transcription reactions.

Production of the Gpr37l1 Targeting Vector and Generation of Mutant Mice. The *Cre* recombinase/*loxP* genetic recombination system (2) was used to produce a unique mutant strain carrying the *Gpr37l1* gene flanked by two *loxP* (*flox*) sites (*Gpr37l1*^{flox} allele; Mouse Genome Informatics (MGI) symbol: Gpr37l1$^{tm1.1Gtva}$, Gpr37l1 targeted mutation 1.1 Glauco Tocchini-Valentini; MGI accession no. 5439168). A single *loxP* site, derived from the pGEMloxP vector (3), was inserted into a unique EcoRI site (1,868 bp upstream of the translation start site), within a 7,883-bp genomic fragment containing the *Gpr37l1* promoter, the first exon, and part of the intron. A *loxP*-flanked neomycin resistance (neo) expression cassette, derived from the neoflox-8 vector (2), was inserted into the BglII site (nucleotide 4,183) within the *Gpr37l1* intron sequence. This construct was then subcloned in the CWKO vector (3). The resulting targeting vector was linearized by Acc65 I digestion and transfected into 129S/SvEv-XSV1 ES cells by electroporation, as described (2). The occurrence of correct gene targeting events was confirmed in 1 of 288 selected ES cell clones by Southern blot analysis with specific probes (Fig. S1). Clone M7 was transfected with the pCre-Pac in vitro expression vector (4) to generate a *Gpr37l1*^{flox} allele as a result of the deletion of the neomycin cassette by Cre-mediated recombination. Heterozygous mutant *Gpr37l1*$^{flox/+}$ mice were produced, injecting recombinant ES cell clones into blastocysts of the C57BL/6J mouse strain using standard procedures and crossing the resulting chimeric mice to C57BL/6J mice to check the germ-line transmission. The targeted mutant ES clones and *Gpr37l1*$^{flox/+}$ mice were produced by Xenogen Biosciences. Animals heterozygous for the disrupted *Gpr37l1* allele (*Gpr37l1*$^{+/-}$; MGI symbol: Gpr37l1$^{tm1.2Gtva}$, Gpr37l1 targeted mutation 1.2 Glauco Tocchini-Valentini; MGI accession no. 5439169) were obtained by subsequent crossing of *Gpr37l1*$^{flox/+}$ males with C57BL/6J-backcrossed (n = 10) Tg(CMV-cre)1Cgn transgenic females (CMV-cre; Mouse Genome Database accession no. MGI 2176179) that ubiquitously express the *Cre*-recombinase enzyme from the embryonic two-cell stage onwards, under the control of a human cytomegalovirus minimal promoter (5). Homozygous *Gpr37l1*$^{-/-}$ animals, not carrying the CMV-cre transgene, were obtained according to standard breeding schemes (6).

Immunofluorescence and Quantitative Analysis. Deparaffined midvermal sections from age-matched brains from mutant mice and their wild-type littermates were processed for immunofluorescence labeling according to standard protocols with antigen retrieval. Fluorescence micrographs were acquired with a TCS SP5 laser scanning confocal microscope or with a motorized LMD7000 microscope (Leica Microsystems) using the manufacturer's imaging software. Quantitative analysis of immunofluorescence signal were performed with ImageJ or processed with the Imaris 5.0.2 software (Bitplane). Experiments were performed in at least three animals per genotype and age group. Sections of similar size in similar regions were chosen and analyzed. The average count of positive cells was obtained from at least three different cerebellar fissures from each section and three nonadjacent sections from each mouse. All measurements were performed with the observer blind to the identity of the slides, and images from a representative experiment are shown.

Antibodies Used for Immunofluorescence Labeling. Brain lipid binding protein (Blbp, 1:100; Millipore); 5-bromo-2′-deoxyuridine (BrdU; 1:100, clone BU-1; Millipore); calbindin 1 (Calb1/CalbD-28K; 1:200, clone CB-955; Sigma-Aldrich); contactin 2 (Cntn2/Tag1; 1:100; R&D Systems); glial fibrillary acidic protein (Gfap; 1:50, clone 4A11; BD Pharmingen); glial high-affinity glutamate transporter (Glast; 1:100; Novus Biologicals); Gpr37l1 (1:100, clone 7–4A1; Mab Technologies); nestin (1:20; Developmental Studies Hybridoma Bank); cyclin-dependent kinase inhibitor 1B (Cdkn1B/p27-Kip1; 1:200, clone 57; BD Biosciences); proliferating cell nuclear antigen (Pcna; 1:150, clone PC10; Biosource); phosphohistone H3 complex (PH3; 1:200; Millipore); patched 1 (Ptch1; 1:50; Santa Cruz Biotechnology); vesicular glutamate transporter 1 (Vglut1; 1:1,000; Synaptic System); and vesicular glutamate transporter 2 (Vglut2; 1:100, clone 8G9.2; Millipore).

In Vivo Proliferation Assays. Postnatal day (P) 3, P5, or P7 *Gpr37l1*$^{-/-}$ and wild-type male littermates received a single BrdU injection (0.1 mg/g body weight) 2 h before euthanasia. Brains were dissected and tissue preparation and processing was performed as described above.

In Vivo Migration Assay. P7 male *Gpr37l1*$^{-/-}$ and wild-type littermates were injected intraperitoneally with BrdU (0.1 mg/g body weight) and killed 48 or 96 h postinjection.

Western Blot Analysis. The tissue samples were homogenized in lysis buffer (120 mM NaCl, 20 mM Hepes, 5 mM EDTA, 10% glycerol, 1% Triton X-100, Roche complete protease inhibitors), cleared by centrifugation, and the protein content of the supernatant

was quantified by Bio-Rad DC Protein Assay (Bio-Rad). Protein samples (50 μg) were separated by SDS/PAGE and analyzed by Western blot, according to standard protocols. Protein antigens were labeled with primary antibodies specific for: Gpr37 (1:1,000, clone 202) (7); Gpr37l1 (1:1,000, clone 7–4A1; Mab Technologies); sonic hedgehog (Shh; 1:500; Santa Cruz Biotechnology); patched 1 (Ptch1; 1:500, clone 413220; R&D Systems); smoothened (Smo; 1:1,000; MBL); neuroblastoma-derived myelocytomatosis viral-related oncogene (*N*-Myc; 1:500; Santa Cruz Biotechnology); glioma-associated oncogene family zinc finger 2 (Gli2; 1:500; Abcam); eukaryotic initiation factor 4E (eIF4; 1:1,000; Cell Signaling); and α-tubulin (1:1,000, clone DM 1A; Sigma). Horseradish peroxidase-conjugated secondary antibodies, specific for mouse, rat (Amersham), or rabbit (Cell Signaling) immunoglobulins were used, following the manufacturer's instructions. The blotted membranes were then processed for chemiluminescence detection with an ECL kit (Amersham) and exposed (Chemidoc XRS+ imager; Bio-Rad). The luminescent signal of immunoreactive bands was imaged and quantified with Image Lab software (Bio-Rad). The intensity of each band was normalized to the intensity of the corresponding α-tubulin band. The average values of each experimental group were expressed in arbitrary units, as a ratio to the mean values obtained from the wild-type control group at P5.

Immunoprecipitation. The solubilized tissue extracts were incubated either with the monoclonal antibodies specific for patched 1 (Ptch1; 1:100, clone 413220; R&D Systems) or Gpr37l1 (1:100, clone 7–4A1; Mab Technologies), in the presence of G-Sepharose beads (Sigma) following the manufacturer's protocols. Control precipitates were obtained by incubation with soluble extracts from *Gpr37l1*$^{-/-}$ littermates. Immune complexes were then washed five times and subjected to SDS/PAGE and Western blotting. Blots were probed with the Gpr37l1-, Ptch1-, Smo-specific antibodies according to the manufacturer's instructions.

Behavioral Tests and Data Analysis. Adult male mice were tested for motor coordination and learning at the age of 3, 6, and 12 mo in the rotarod, and at 4 and 7 mo in the pole test. Additional cohorts were tested at postnatal day P2, P5, P10, P15, and P20 to assess developmental milestones.

Rotarod test. This test is used to assess motor coordination and balance in rodents (8). Mice had to keep their balance on a rotating rod (3-cm diameter) set at an accelerating speed from 4 to 40 rpm in 300 s (model 47600; Ugo Basile).

To familiarize themselves with the apparatus, mice underwent a training session of three trials, 60 s each, in which the rod was kept stationary for the first trial and held at 4 rpm for the last two trials. The next day, for 4 consecutive days, mice were tested to evaluate motor learning (three trials per day with an intertrial interval of 30 min). A maximum of three mice were placed on the rod at the same time. The latency to fall from the rotating rod was recorded in each trial. If a mouse was passively rotating on the rod (i.e., clinging) the number of passive rotations was counted. For each day, data were expressed as mean final latency to fall minus 1 s for each passive rotation.

Pole test. This test of motor coordination is used to assay movement disorders in mice (9–11). The test apparatus consisted of a wooden vertical pole (46 cm height, 1 cm diameter) covered with tape to get a rough surface for grip. Mice received 1 d of training and were tested the next day. Each session consisted of five trials, with an intertrial interval of 5–10 min. Each mouse was placed head up at the top of the pole, and the time for turning downward (T turn), as well as the total time for climbing down, was measured. If a mouse was unable to perform the test, a time of 120 s was assigned (cutoff). The median value of all five trials in each session was used for the statistical analysis.

Assessment of Developmental Milestones. Eight litters of mice from heterozygous breeding (total 54 mice, 29 males, 25 females) were studied to assess developmental milestones such as physical landmarks, body measures, and sensory and motor reflexes (12, 13). Only litters of six to eight pups were included in the tests to ensure homogeneity in nutrition and growth rates. Pups were examined on postnatal days P2, P5, P10, P15, and P20, always at the same time of day. Immediately after behavioral testing at P2 or P5, pups were marked on the paw with animal tattoo ink (Ketchum Permanent Tattoo Inks, green paste, Ketchum Manufacturing) using a 30-gauge hypodermic needle, a procedure that causes only minor distress and does not need anesthesia. On each day of testing, pups were weighed and their nose-to-anus and tail lengths were measured (body measures).

Physical landmarks such as pinnae detachment, eye opening, incisor eruption, and fur development were observed and for each of them, a score of 0 (absence), 1 (initial, slight), 2 (incomplete), or 3 (adult-like) was given. To investigate sensory and motor development, pups were tested for the following reflexes and responses:

Negative geotaxis: The pup is placed head down on an inclined 45° wire mesh screen and the time to rotate face up is measured up to a maximum of 60 s.

Climbing: The pup is placed onto a wire mesh screen. It is then rotated to a vertical position. A score (0–3) is given if the mouse exhibits a climbing response within 60 s.

Bar holding: The pup is held gently by the trunk and allowed to cling to a horizontal thin bar with its forepaws, remaining suspended. The length of time it holds on is recorded up to a maximum of 10 s.

Wire hanging: This test was performed at P20 instead of bar holding. The pup is placed on a wire mesh screen maintained at ∼30 cm above a padded surface. After 10 s the screen is rotated at an angle of 90° and afterward at 180° and the length of time the pup hangs on in both positions is recorded up to a maximum of 20 s.

Cliff avoidance: The pup is placed on the edge of a table top with its nose and forepaws over the edge and observed for moving away from the cliff. A score from 0 to 3 is assigned.

Acoustic startle: A click box (Institute of Hearing Research-Medical Research Council; Harwell), which generates a brief 20-kHz tone at 90 dB is placed at a distance of ∼20–30 cm above the pup and its response to the tone is recorded (0–3 score).

Forelimb placing: The pup is held gently by the trunk and a wooden toothpick is placed in contact with the back of its forepaw. The reflex of raising and placing the paw on the surface of the toothpick is scored from 0 to 3 (data not shown).

Forelimb grasping: The paw is stroked with a blunt toothpick and the grasping reflex is scored from 0 to 3 (data not shown).

Level screen test: The pup is dragged horizontally across the screen by the tail and its holding response onto the screen is evaluated (score 0–3) (data not shown).

Surface righting: The pup is placed on its back and the time to turn over is measured up to a maximum of 60 s (data not shown).

During assessment, the observers were not aware of pup genotype because this was determined at P21, upon weaning.

Statistical Analysis. All data were analyzed by *t* test, simple factorial ANOVA, and repeated measures ANOVA with genotype, age, and treatment as between-subjects factors and time or trials as within-subjects factors using StatView 5.0 PowerPC software (SAS Institute). Post hoc analysis was performed where allowed. Level of significance was set at $P \leq 0.05$.

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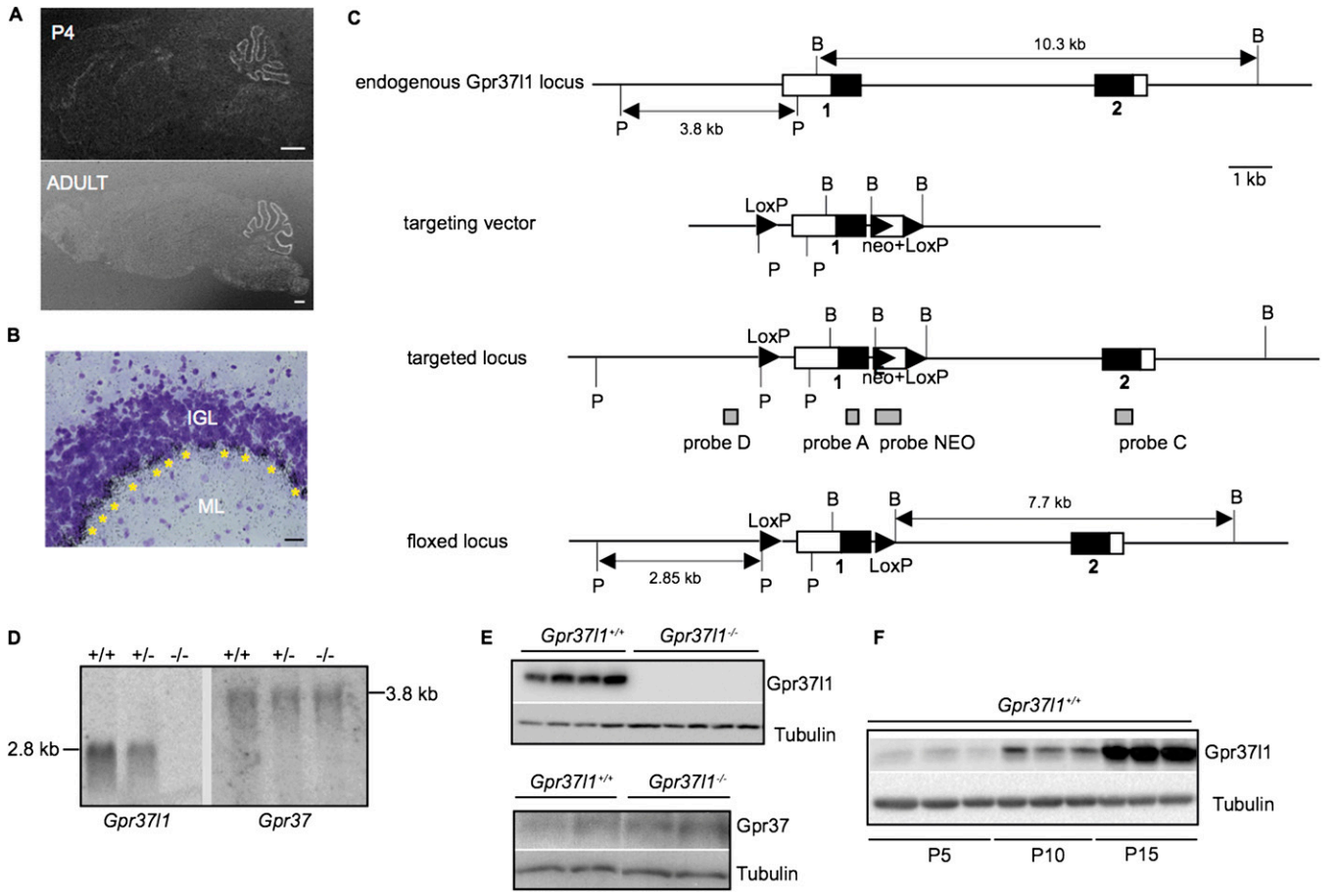

Fig. S1. Generation and characterization of *Gpr37l1*$^{-/-}$ mice. (*A*) In situ hybridization of *Gpr37l1* mRNA in cerebella of postnatal age P4 or adult wild-type mice (negative imaging). (*B*) In situ hybridization from an adult wild-type mouse cerebellum is shown in high magnification. Cell nuclei are stained with toluidine blue. (*C*) Schematic representation of the mouse *Gpr37l1* wild-type allele, targeting vector, targeted allele, and *loxP* flanked (floxed) *Gpr37l1* locus. Gray solid bars indicate the location of the probes used for Southern blot, along with the sizes of PvuII and BamHI restriction fragments. (*D*) Northern blot analysis of whole brain total RNA from adult *Gpr37l1*$^{+/+}$, *Gpr37l1*$^{+/-}$, and *Gpr37l1*$^{-/-}$ littermate male mice. The probes used were DNA fragments complementary to the complete cDNA sequence of the *Gpr37l1* or *Gpr37* genes. (*E*) Western blot analysis of whole cerebellar samples from adult *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermate male mice. Primary antibodies specific for Gpr37 were used, followed by horseradish peroxidase-conjugated secondary antibodies for chemiluminescence detection. (*F*) Western blot analysis of Gpr37l1 and α-tubulin proteins in whole cerebellar samples from P5, P10, or P15 *Gpr37l1*$^{+/+}$ male pups. (Scale bars in *A*, 500 μm and *B*, 25 μm.) Asterisks indicate Purkinje neuron somata.

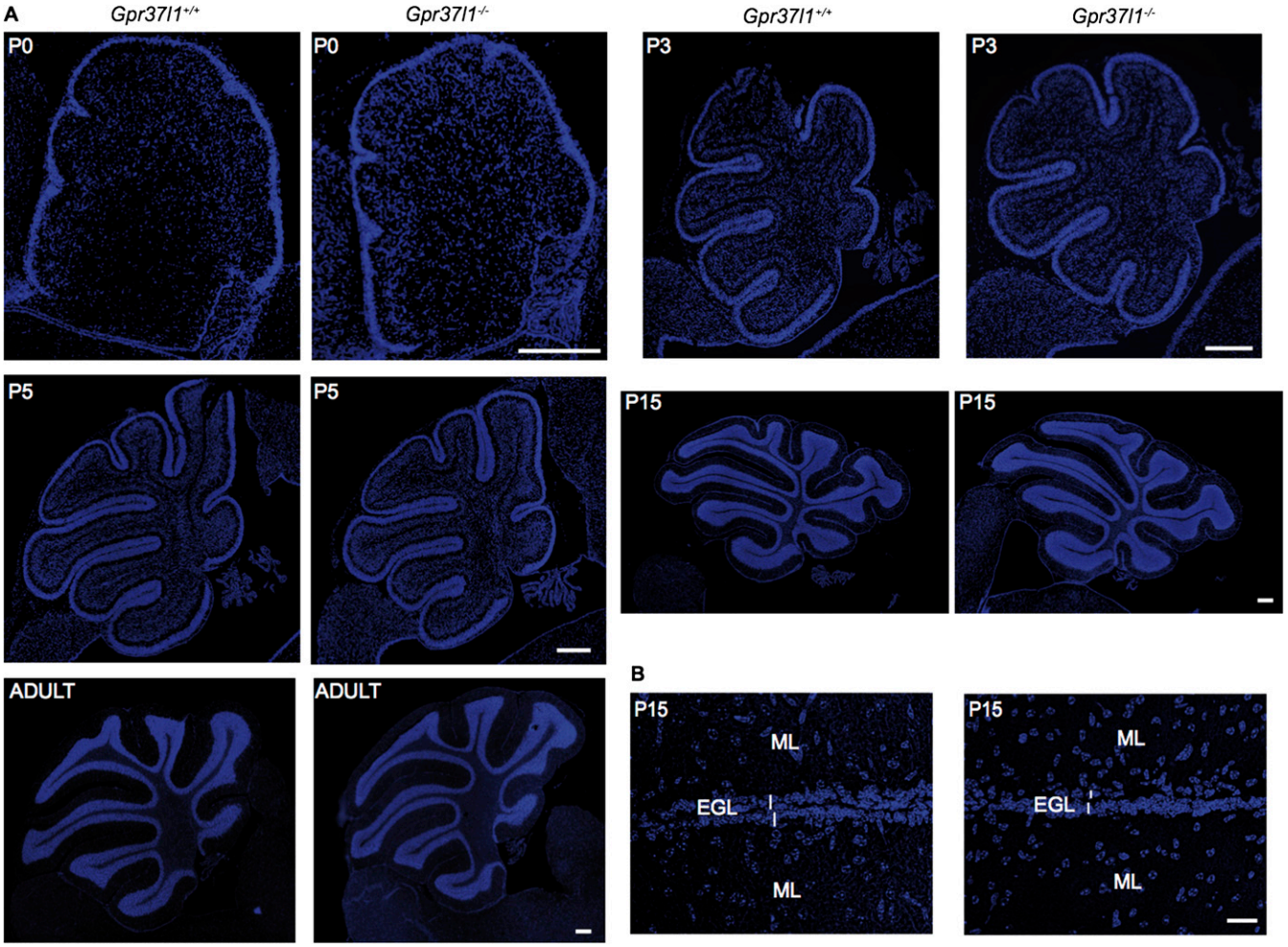

Fig. S2. Cerebellar morphology of developing and adult *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermate male mice. (*A*) Nuclear Hoechst staining of cerebellum at P0, P3, P5, P15, and adult age in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ mice. (*B*) High magnification of Hoechst staining of cerebellum at P15 in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ pups. (Scale bars in *A*, 250 μm and *B*, 25 μm.) EGL, external granular layer; ML, molecular layer.

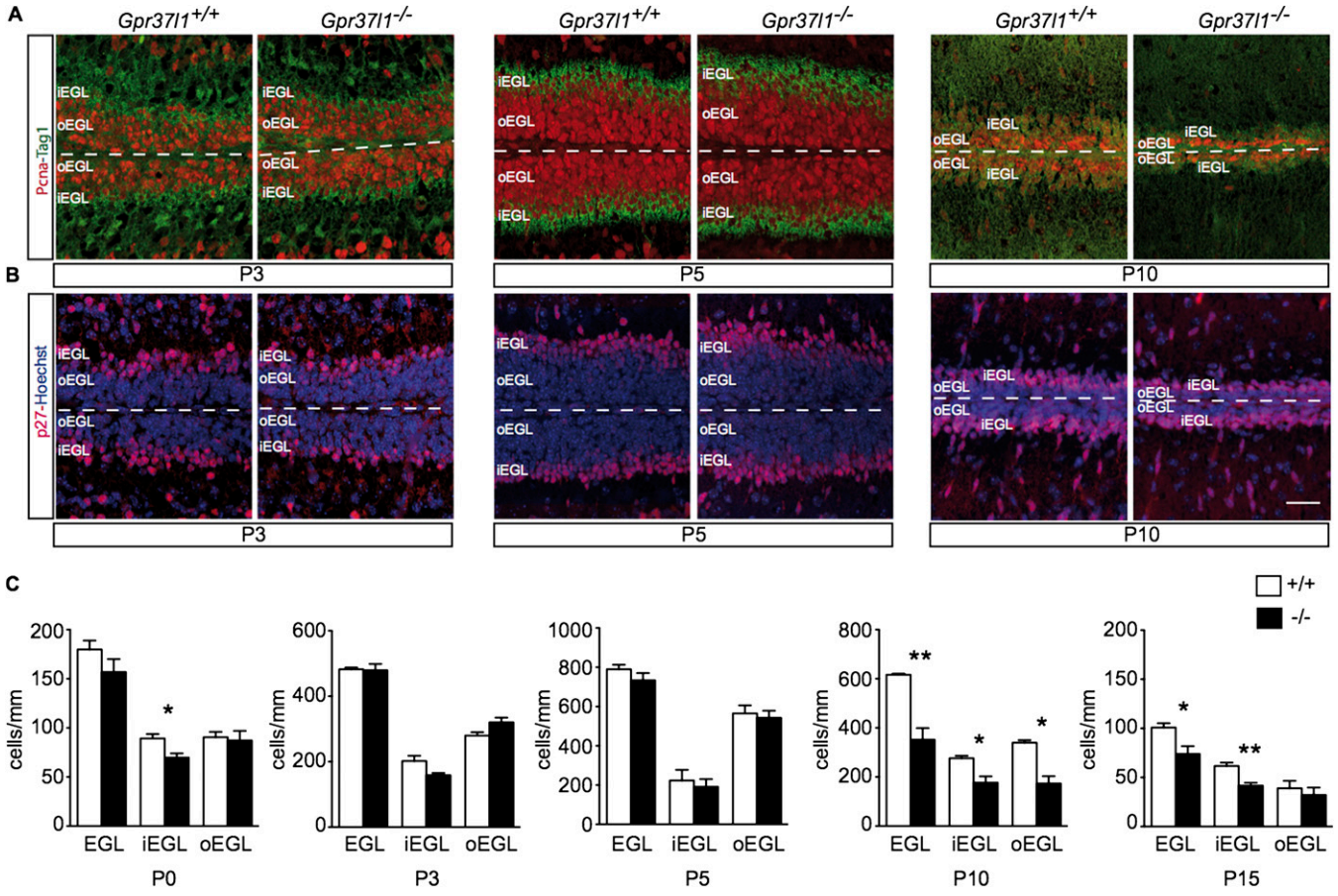

Fig. S3. Postnatal proliferation and differentiation of cerebellar granule neuron precursors in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ male mice. (*A*) Double-immunofluorescence labeling of Pcna and Tag1, which distinguish, respectively, the proliferating and postmitotic differentiating neurons of outer EGL (oEGL) and internal EGL (iEGL), at P3, P5, and P10 in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermates. (*B*) Immunofluorescence labeling with p27-Kip1 and Hoechst staining of the EGL at P3, P5, and P10 in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermates. Two EGL zones are identified: the oEGL and iEGL, with a lower and higher proportion, respectively, of p27-Kip1–expressing cells. (*C*) Quantification of the average number of total EGL cells (blue, Hoechst), and p27-Kip1–positive (iEGL) or p27-Kip1–negative (oEGL) cells normalized by the EGL lenght in P0, P3, P5, P10, and P15 *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermates (mean ± SEM, $n = 3$–4 per group). $^{*}P < 0.05$,$^{**}P \leq 0.005$ $^{+/+}$ vs. $^{-/-}$, unpaired *t* test. (Scale bar, 25 μm.) EGL, external granular layer; IGL, internal granular layer; ML, molecular layer.

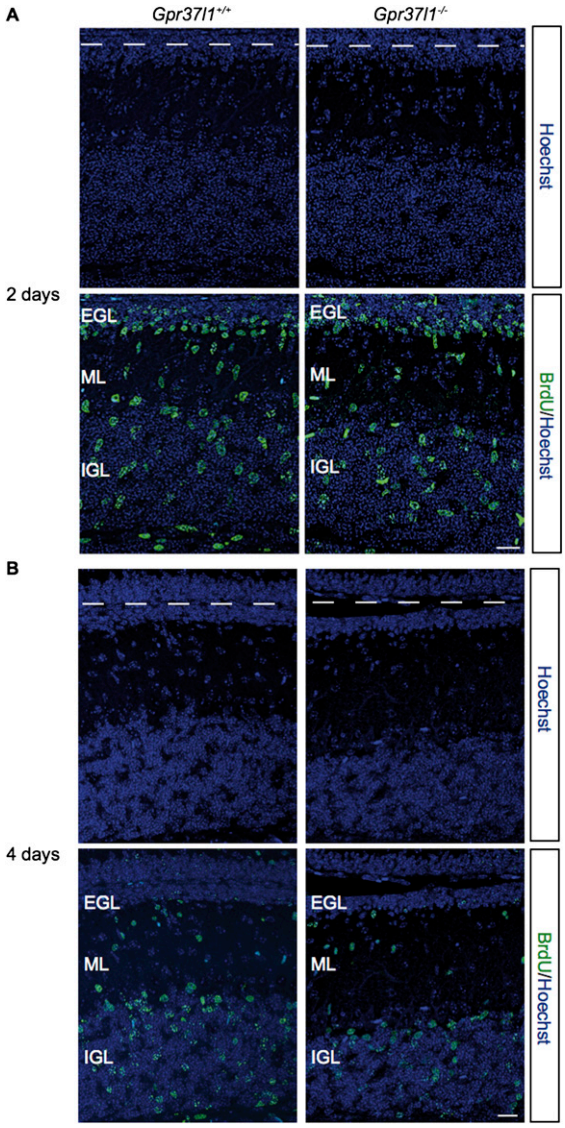

Fig. S4. Migration of cerebellar granule neurons in *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermate male mice. (*A* and *B*) Immunofluorescence labeling with BrdU and Hoechst staining of cerebella of *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ pups injected with BrdU at P7 and killed 48 or 96 h postinjection. (Scale bar, 20 μm.) EGL, external granular layer; IGL, internal granular layer; ML, molecular layer.

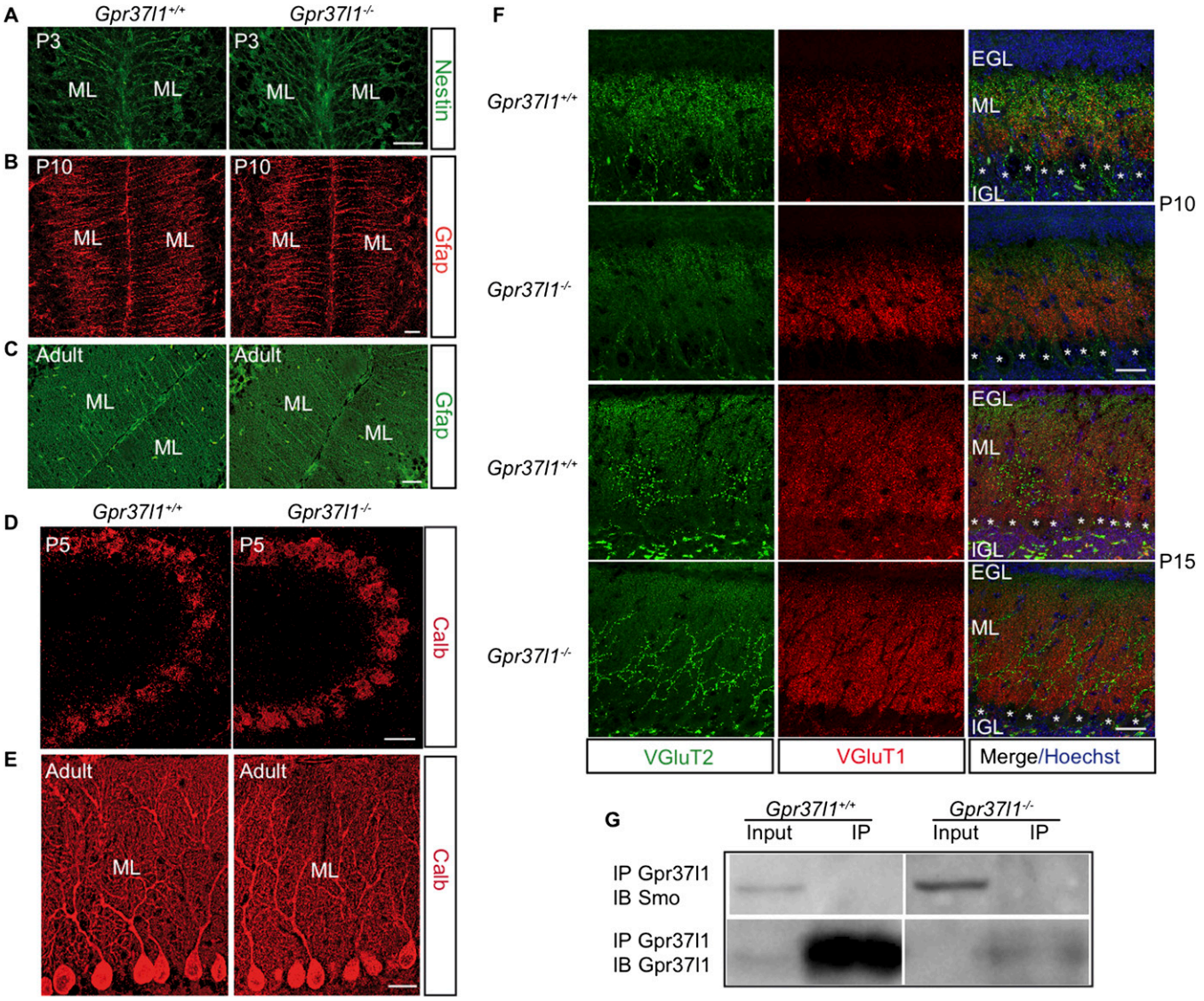

Fig. S5. Immunofluorescence of Bergmann glia and Purkinje neurons and Smo coimmunoprecipitation analysis in cerebellar samples of *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ male littermate mice. (*A*) Immunofluorescence staining of nestin in P3 cerebella from *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ pups. (*B* and *C*) Immunofluorescence staining of Gfap in P10 or adult cerebella from *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ pups. (*D* and *E*) Immunofluorescence staining of Calb in P5 or adult cerebella from *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ pups. (*F*) Immunofluorescence staining of VGluT2 (green) and VGluT1 (red) in P10 and P15 cerebella of *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermates. (Scale bars, 25 μm.) EGL, external granular layer; IGL, internal granular layer; ML, molecular layer. Asterisks indicate Purkinje neuron somata. (*G*) Absence of Gpr37l1 and Smo coimmunoprecipitation in cerebellar extracts from P10 *Gpr37l1*$^{+/+}$ and *Gpr37l1*$^{-/-}$ littermate pups. Immunoprecipitations (IP) were performed with the Gpr37l1 monoclonal antibody followed by immunoblotting with Smo- or Gpr37l1-specific antibodies. A representative result from three distinct experiments is shown.

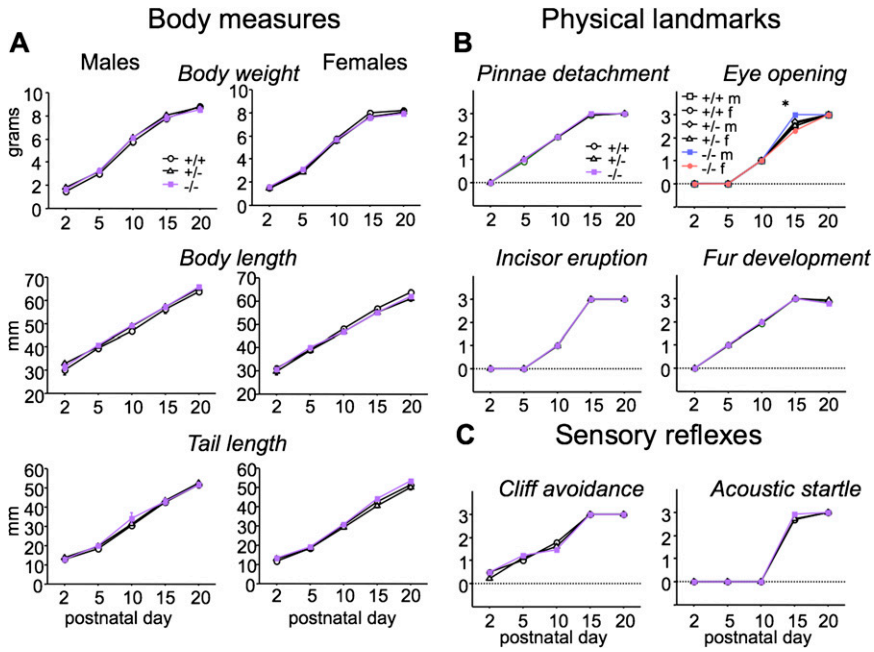

Fig. S6. Developmental milestones in *Gpr37l1*$^{+/+}$, *Gpr37l1*$^{+/-}$, and *Gpr37l1*$^{-/-}$ male and female mouse pups. (*A*) Body measures: body weight, body length, and tail length. (*B*) Physical landmarks: pinnae detachment, eye opening, incisor eruption, and fur development. Data of male and female pups were pooled or displayed separately as indicated. *$P < 0.05$ male $^{+/+}$ vs. $^{-/-}$, *t* test. (*C*) Sensory reflexes: cliff avoidance and acoustic startle. Wild type ($^{+/+}$), $n = 8$; *Gpr37l1*$^{+/-}$ ($^{+/-}$), $n = 15$; *Gpr37l1*$^{-/-}$ ($^{-/-}$), $n = 6$.