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Supplementary Information for

Sphingolipid metabolism governs Purkinje cell patterned degeneration in Atxn1[82Q]/+ mice

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Supplementary Methods

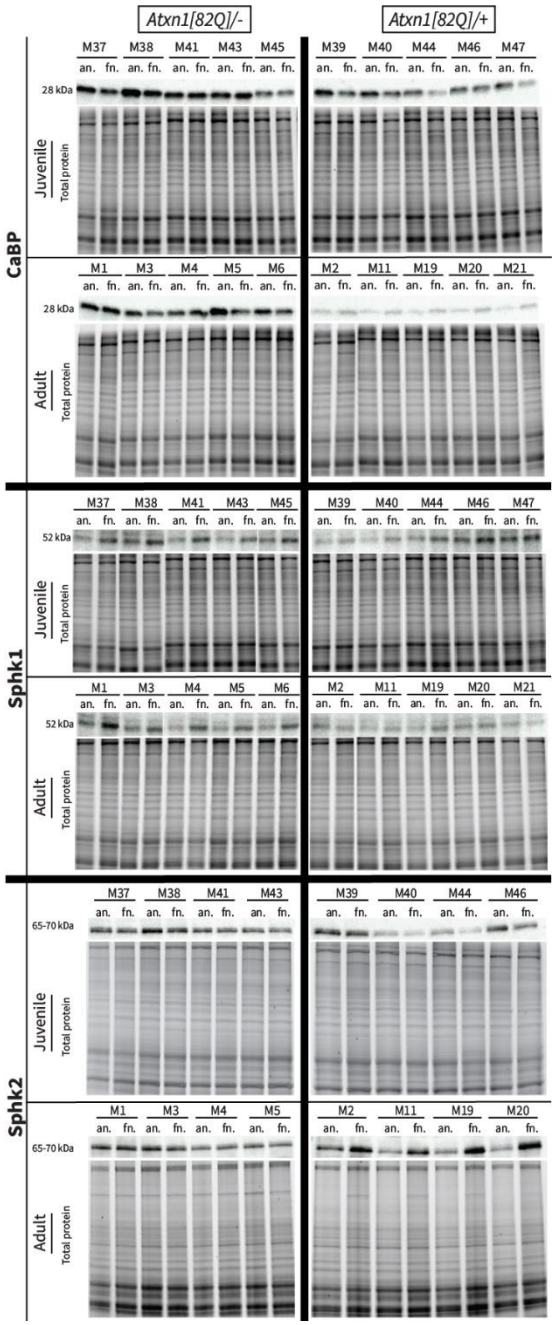

Fig. S1. *Western blotting for CaBP, Sphk1 and Sphk2 proteins.*

Western blotting gels for all biological replicates for CaBP (28kDa), Sphk1 (52kDa) and Sphk2 (65-70kDa) proteins, together with total protein migration used for normalization. Four to five mice (M) were used as biological replicates (M[number]) per age group, per genotype. *Atxn1[82Q]/-*=wild type (M/38/41/43 for juvenile and M 1/3/4/5/6 for adult); *Atxn1[82Q]/+*=transgenic mutant (M39/40/44/46/47 for juvenile and M2/11/19/20/21 for adult). Juvenile= P21-23, Adult= P143-153; anterior (an.), flocculo-nodular (fn.).

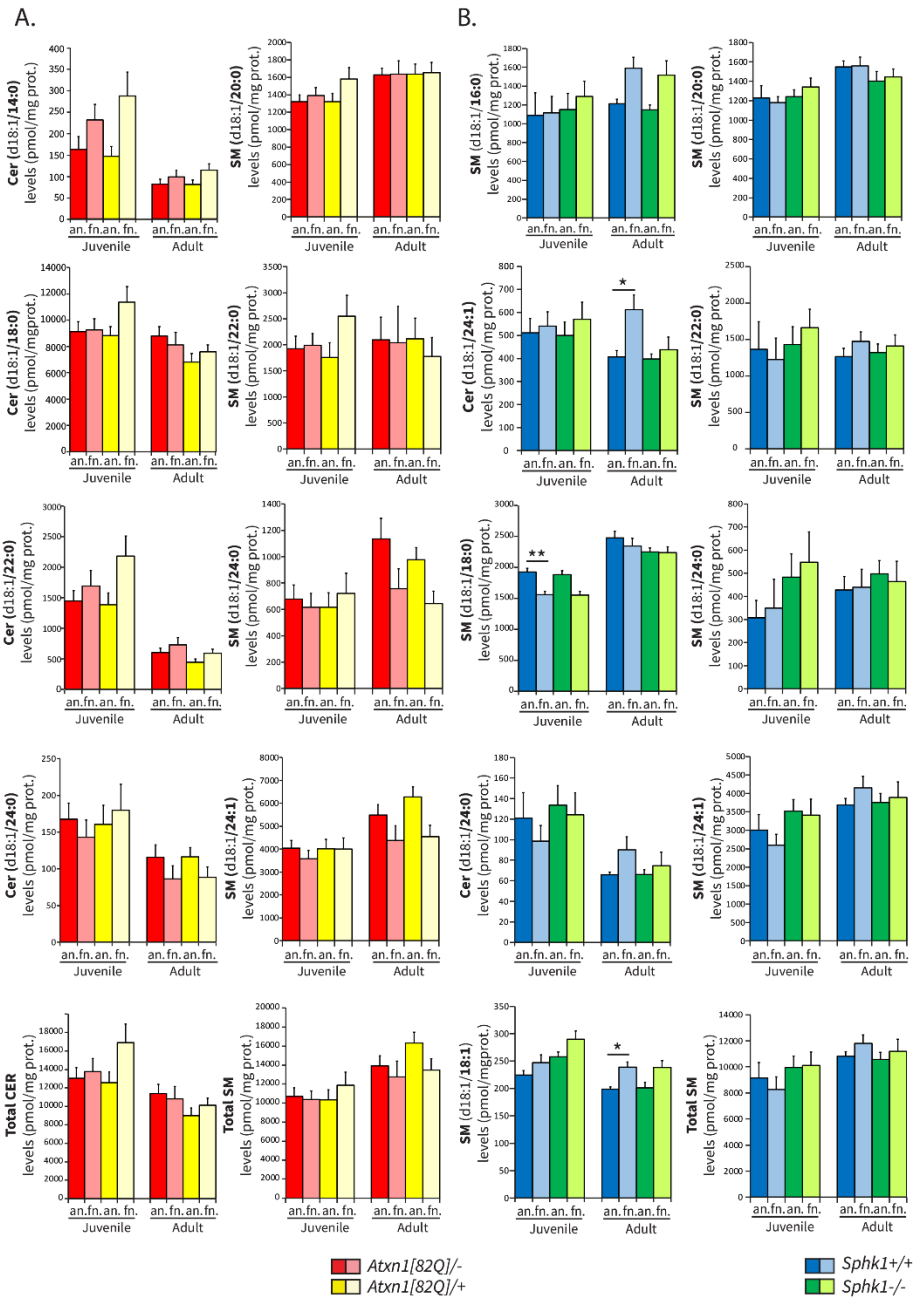

Fig. S2. *Extended list of sphingolipids measured in all experiments.*

Bar plot of HP-LC MS/MS quantification of supplementary selected sphingolipid levels in (A) wild type *Atxn1[82Q]/-* (FVB/N) and mutant *Atxn1[82Q]/+* and in (B) wild type *Sphk1+/+* (C57BL/6J) and mutant *Sphk1-/-*. Data are expressed as mean ± S.E.M. (N=10 per group); unit = pmol/mg of protein. Juvenile= P21-23, Adult= P143-153. Statistical analysis for LC-MS/MS lipid quantification were performed using a Levene's test for equality followed by a Student t-test, "paired" to compare anterior vs. flocculo-nodular fractions (black stars), and "unpaired" to compareSphk1+/+ vs. Sphk1-/- (purple stars), with Bonferroni correction for multiple comparisons. *p<0.05, **p<0.005, ***p<0.001.

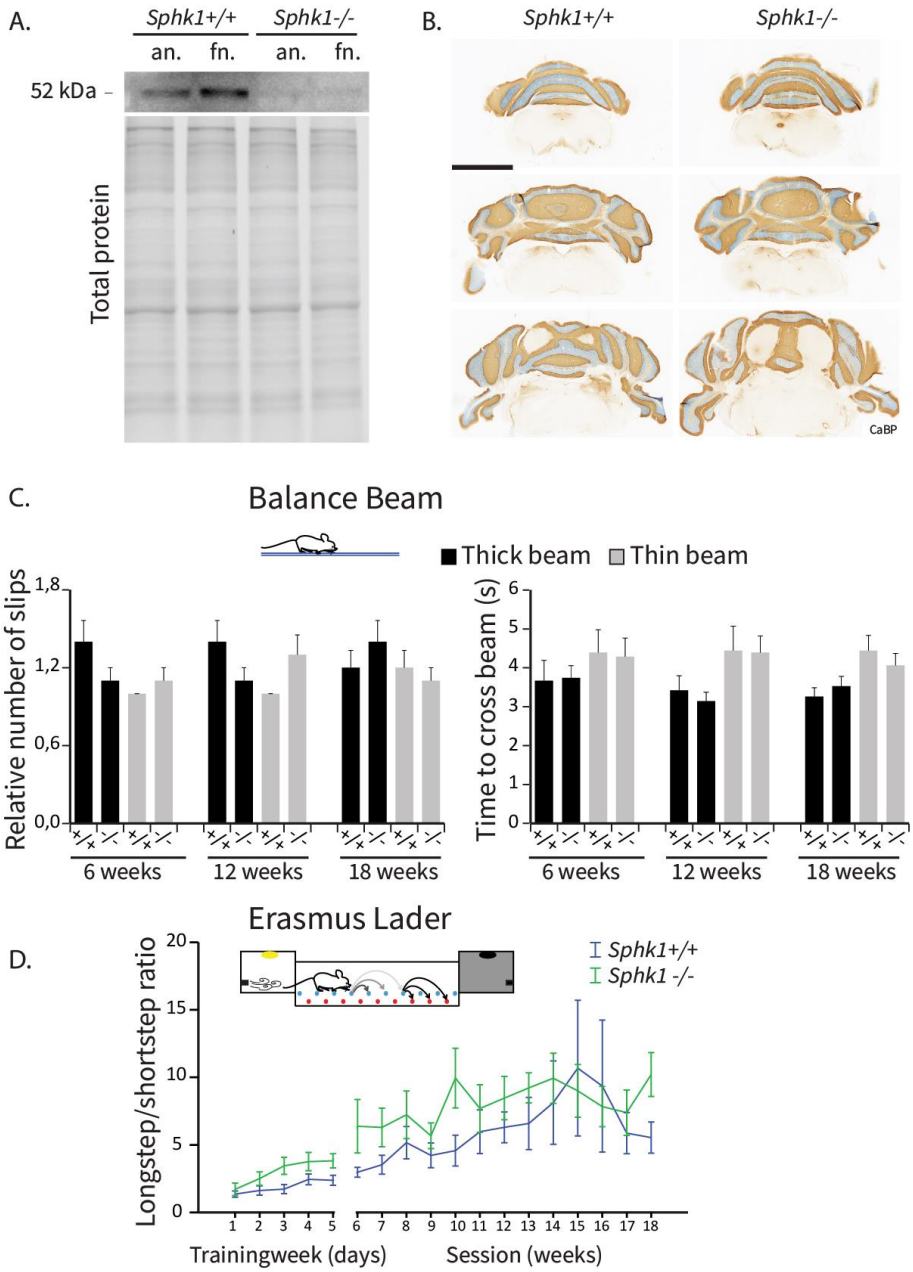

Fig. S3. *Sphk1-/- mice show normal anatomy and cerebellar function*

(A) Western blotting identification of Sphk1 protein in wild type *Sphk1+/+* and mutant *Sphk1-/-* mice, together with total protein migration. (B) CaBP (brown) immunostaining in the adult wild type *Sphk1+/+* mice (left panel) and the mutant *Sphk1-/-* mice (right panel). (C) Balance beam test for both wild type and mutant to measure relative number of slips (left panel) and time to cross the beams (right panel0, for both thin (=6mm, grey) and thick beam (=12mm, black). (D) Erasmus ladder test to evaluate the long-step/short-step ration, for both genotypes. Data are expressed as mean ± S.E.M. Balance beam data were analysed by a two-sided independent Students t-test. Erasmus Lader data were analysed by a repeated measures ANOVA *p<0.05, **p<0.005, ***p<0.001.

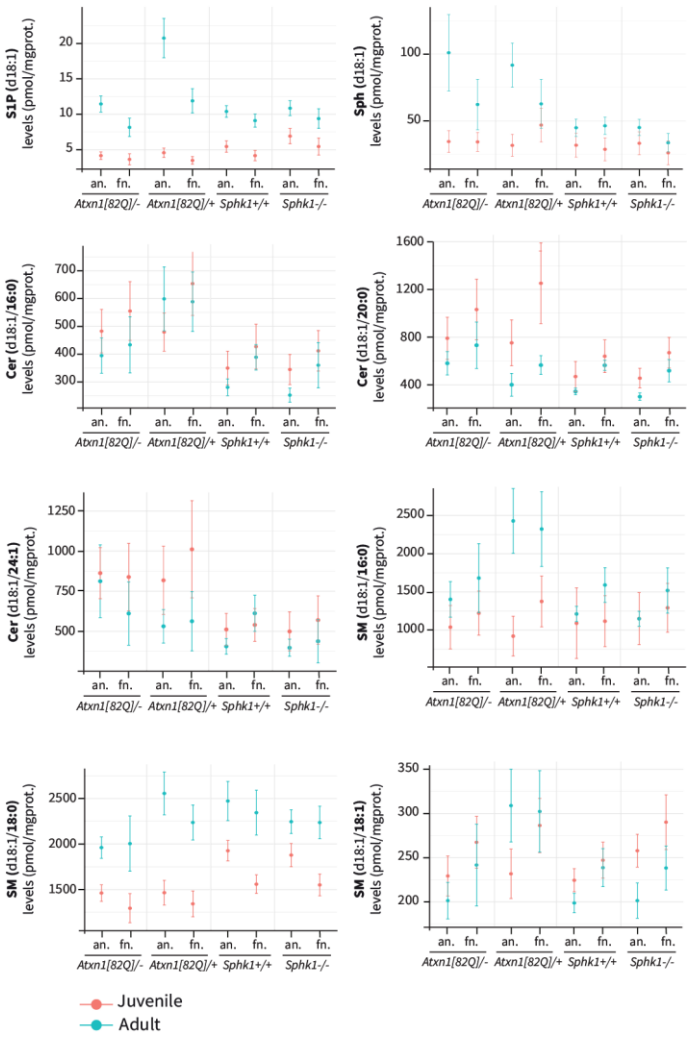

Fig. S4. *S1P(d18:1) and Sph(d18:1) are the only sphingolipids presenting a multi interaction effect of age*fraction*genotype.*

Classic mixed effects ANOVA with a random intercept on which we fitted separate error variance per subgroup. Plot of HP-LC MS/MS quantification of selected sphingolipid levels in all genotypes wild type *Atxn1[82Q]/-* and mutant *Atxn1[82Q]/+* (in FVB/N background); wild type *Sphk1+/+* and mutant *Sphk1-/-* (in C57BL/6J background). Data are expressed as mean with 95% confidence interval (± 2*S.E.M.) (n=10 per group). Unit = pmol/mg of protein; Juvenile= P21-23 (orange), Adult= P143-153 (blue); anterior (an.), flocculo-nodular (fn.). Three covariates were defined: genotypes (*Atxn1[82Q]/-*, *Atxn1[82Q]/+*, *Sphk1+/+* and *Sphk1-/-*), fractions (anterior and flocculo-nodular) and age groups (juvenile and adult), for a total of sixteen subgroups (4 genotypes x 2 fractions x 2 age groups). Animal number was established as a random effect to resolve the non-independency resulting from the extraction of both fractions from a single subject.

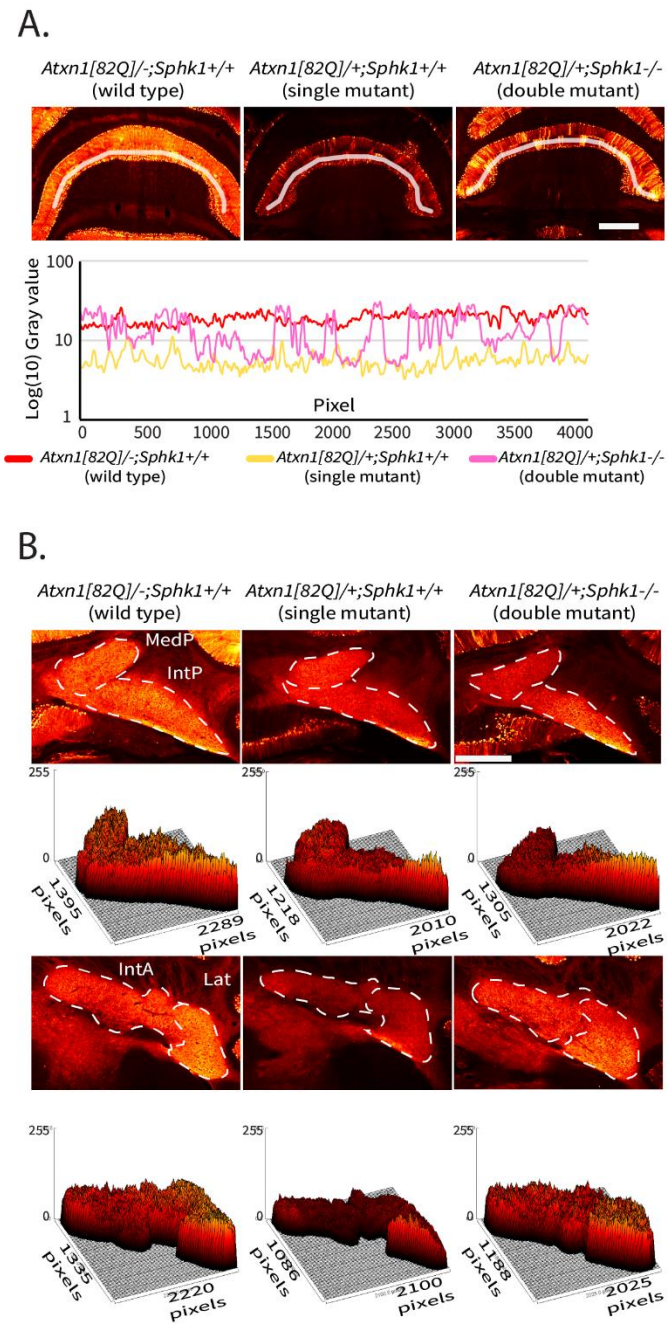

Fig. S5. *Sphk1 deletion in the Atxn1[82Q]/+ mice principally rescues the anterior cerebellum.*

(A) Plot profile of CaBP immunofluorescent intensity in wild type (red), single mutant (yellow) and double mutant (pink) mice. Measures were done in lobule III. Scale bar = 500 µm. (B) Surface plot profile of CaBP immunofluorescent intensity in the cerebellar nuclei. MedP: medial posterior, IntP: interposed posterior, IntA: interposed anterior, Lat: lateral. Scale bar = 500 µm.

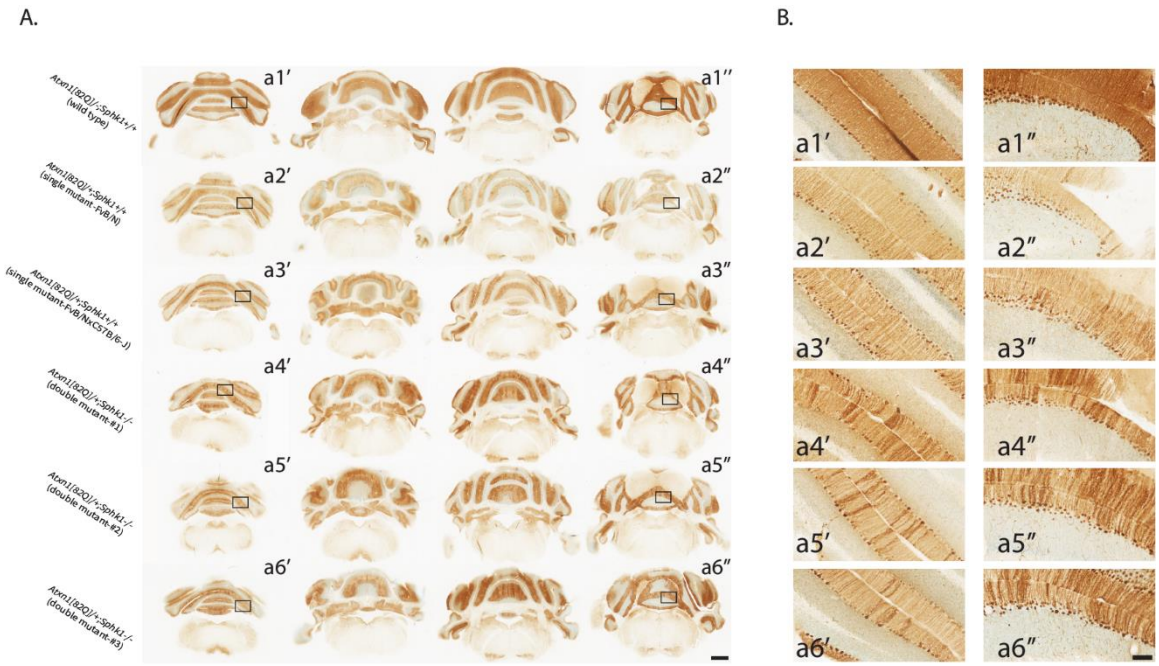

Fig. S6. *Sphk1 deletion in the Atxn1[82Q]/+ mice consistently rescues PC degeneration.*

(A) CaBP immunostaining in a wild type *Atxn1[82Q]/-*;*Sphk1+/+* and a single mutant *Atxn1[82Q]/+*;*Sphk1+/+* mouse in the original FVB/N background, a single mutant *Atxn1[82Q]/+*;*Sphk1+/+* mouse, (result of the crossbreeding (FVB/N;C57B/6-J)) and three *Atxn1[82Q]/+*;*Sphk1-/-* double mutant mice to show biological replication (#1/2/3) at P140. Scale bar = 1 mm (B) High magnification of dorsal lobule VIII-ventral lobule VII (a1', a2', a3', a4', a5', a6') and dorsal lobule II (a1'', a2'', a3'', a4'', a5'', a6''). Scale bar = 100 µm

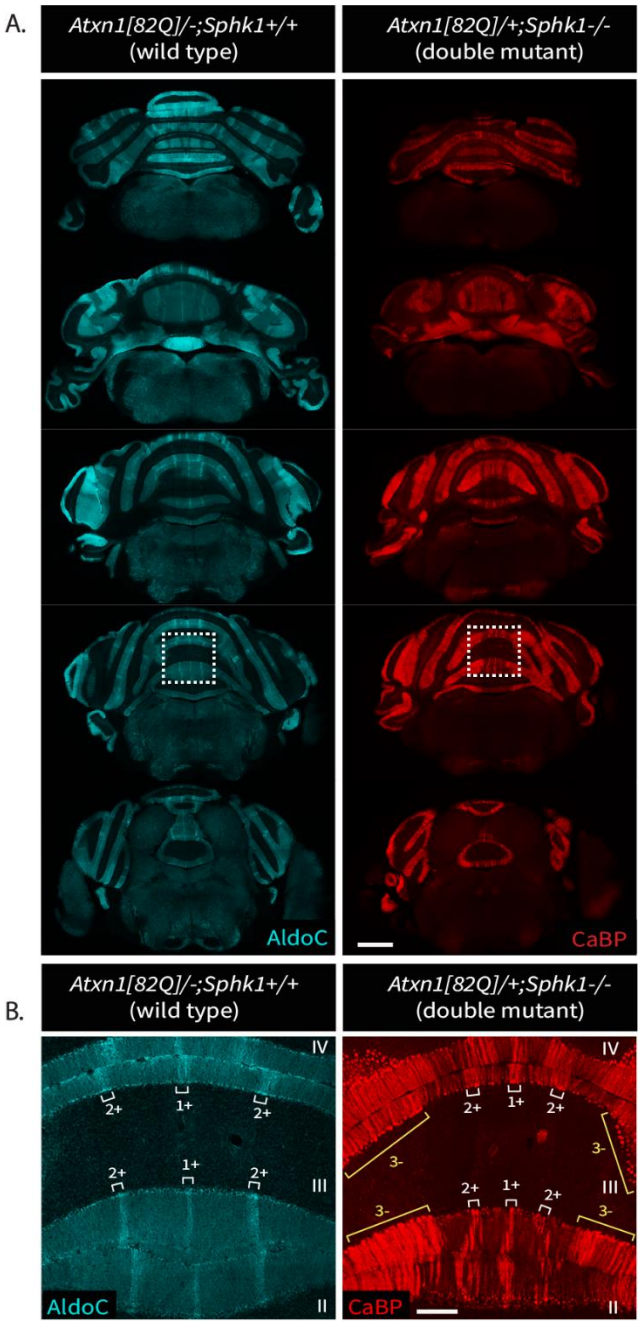

Fig. S7. *Distribution of rescued PC in the double mutant Atxn1[82Q]/+;Sphk1-/- mice partially correlates with AldoC expression pattern.*

(A) AldoC (cyan) immunostaining in wild type *Atxn1[82Q]/-;Sphk1+/+* mice (left), and CaBP (red) immunostaining in double mutant *Atxn1[82Q]/+;Sphk1-/-* mice (right), at P140. Scale bar = 1 mm (B) High magnification of vermal lobule II-III-IV. Scale bar = 200 µm.

Supplementary Methods

Western Blotting.

The animals were anesthetized with 0.2 mL pentobarbital (60 mg/mL) and perfused using sodium chloride to rinse out the blood. Cerebella were removed and isolated into the anterior fraction composed of vermal lobules I, II, and III, and the flocculonodular fraction composed of vermal lobules IX, X, Paraflocculus, and Flocculus. All tissues were frozen at −80 ◦C, avoiding freeze–thaw cycles. Cerebellar tissue was homogenized in lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate and protease inhibitor cocktail (11697498001 Merck)). Protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Fisher). Samples were centrifuged at 12,000 rpm at 4 ◦C for 10 min, and the supernatant was denaturated for 10 min at 95 ◦C. Proteins were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (CriterionTGX StainFreeGels; Bio-Rad) and transferred onto a nitrocellulose membrane with the Trans-Blot TurboBlotting System (Bio-Rad). Before transfer, gels were activated and imaged as a protein loading control using the Gel Doc EZ Imager (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in tris-buffered saline/Tween 20 (TBST, 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% Tween20) for 1 h and incubated with either anti-Sphk1 (mouse monoclonal, SC-365401; Santa Cruz Biotechnology), anti-CaBP (rabbit polyclonal, CB-38a; Swant), or anti-Sphk2 (rabbit polyclonal, 17096–1-AP; Proteintech) primary antibody diluted 1:1,000 in 5% BSA in TBST overnight at 4 ◦C. Protein levels were detected using either horseradish peroxidase (HRP) -conjugated goat anti-mouse antibody (P0447, 1:5,000; Dako) or goat anti-rabbit antibody(P0448, 1:10,000; Dako).

Imaging Acquisition and Analysis.

Images were acquired with an upright Imager M2 (Zeiss) equipped with a 10× lens for fluorescence microscopy and a confocal microscope LSM700 (Zeiss) with upright 20× lenses and a Hamamatsu Nanozoomer 2 whole-slide imager equipped with 40× lenses for light microscopy. Images were treated with a Photoshop routine to perfect overall brightness and signal level homogeneously on the pictures, with all treatment being equally applied on the entire images. CaBP intensity measurements were performed with FIJI software. PC diameter measurements were performed manually on two to three sections per brain region for four regions per mouse (anterior hemispheres, anterior vermis, posterior, and flocculonodular) and three mice per genotype. Measurements were done by tracing a line parallel to the plane of the PC layer to obtain the axial diameter in ZEN software. Iba+ microglia counting was performed manually, in FIJI. In order to compare wild-type, single-mutant, and doublemutant mice, we performed an identical immunostaining process (antibody concentration, incubation time), identical image acquisition (gain, exposure time, resolution), and identical image adjustment for analysis (brightness and contrast values), all performed in parallel in a single session for the three genotypes with four animals per group.

Behavioral Tests.

Balance beam tests were performed using a thick (12-mm) or a thin (6-mm) beam, both with 80 cm of length, placed between an attachment pole and a cage 50 cm above a tabletop. Time to cross the beam and number of slips were quantified. A video camera was attached to the pole to record each measurement session. Mice were measured at 6, 12, and 18 wk of age. Each test consisted of one training session and two measurement sessions. Mice were placed on the beam, and the time was registered when the front paw crossed the starting stripe and stopped when the front paw crossed the end stripe. Mice had to travel each beam twice without falling, and these successful runs were averaged. Video recordings were used to analyze the slips and time to cross the beam (71). The Erasmus Ladder consists of a horizontal ladder composed of 37 rods, distributed between high and low positions, equipped with individual pressure sensors. Animals are trained to cross at a constant speed to reach either end of the ladder. We analyzed short steps and long steps along the ladder. In the paradigm we used, each mouse underwent a daily session for 5 consecutive days at 6 to 7 wk old. Hereafter, they performed one session every week for the following 12 wk. Every session consisted of 42 trials, nonperturbed, in which the mice had to walk from one box to the next after a light stimulus. First, mice had to wait between 8 and 12 s before the light cue. If mice left the box early, an air cue from the opposite box would drive them back, and the trial would start from the beginning. If the light cue did not induce a run, an air cue was given 3 s later to spur them on. Upon entering the goal box, the next trial would start. The first five sessions served to create a baseline. The definition of the steps concurs with the description made by Vinueza Veloz et al. (72). All behavioral experiments were performed blinded.

Statistical Analysis.

Statistical analyses for western blot protein quantification and LC-MS/MS lipids quantification were performed using Levene's test for equality followed by Student's t test, defined as paired for anterior and flocculonodular fractions comparisons (black asterisks describe values levels of statistical significance) and as unpaired when comparing fractions from different groups (purple asterisks describe values levels of statistical significance), with a Bonferroni correction for multiple comparisons. Greenhouse–Geisser correction was performed for an ANOVA when sphericity could not be assumed. Balance beam data were analyzed by a two-sided independent Student's t test. Erasmus Ladder data were analyzed by a repeated measures ANOVA. Average PC diameters were compared using a one-way ANOVA corrected for multiple comparisons (Dunn's correction). The percentage of PC diameters above the wild-type threshold were compared with a Kruskal–Wallis rank sum test for multiple independent samples. Analysis of Iba+ microglia counting was performed with one-way ANOVA and post hoc analysis with Bonferroni correction to compare all genotypes (black asterisks describe values levels of statistical significance). The linear mixed model is a classic mixed effects ANOVA with a random intercept on which we fitted separate error variance per subgroup. Three covariates were defined: genotypes (Atxn1[82Q]/−, Atxn1[82Q]/+, Sphk1+/+, and Sphk1−/−), fractions (anterior and flocculonodular), and age groups (juvenile and adult) for a total of 16 subgroups (four

genotypes × two fractions × two age groups). Animal number was established as a random effect to resolve the nonindependence resulting from the extraction of both fractions from a single subject.