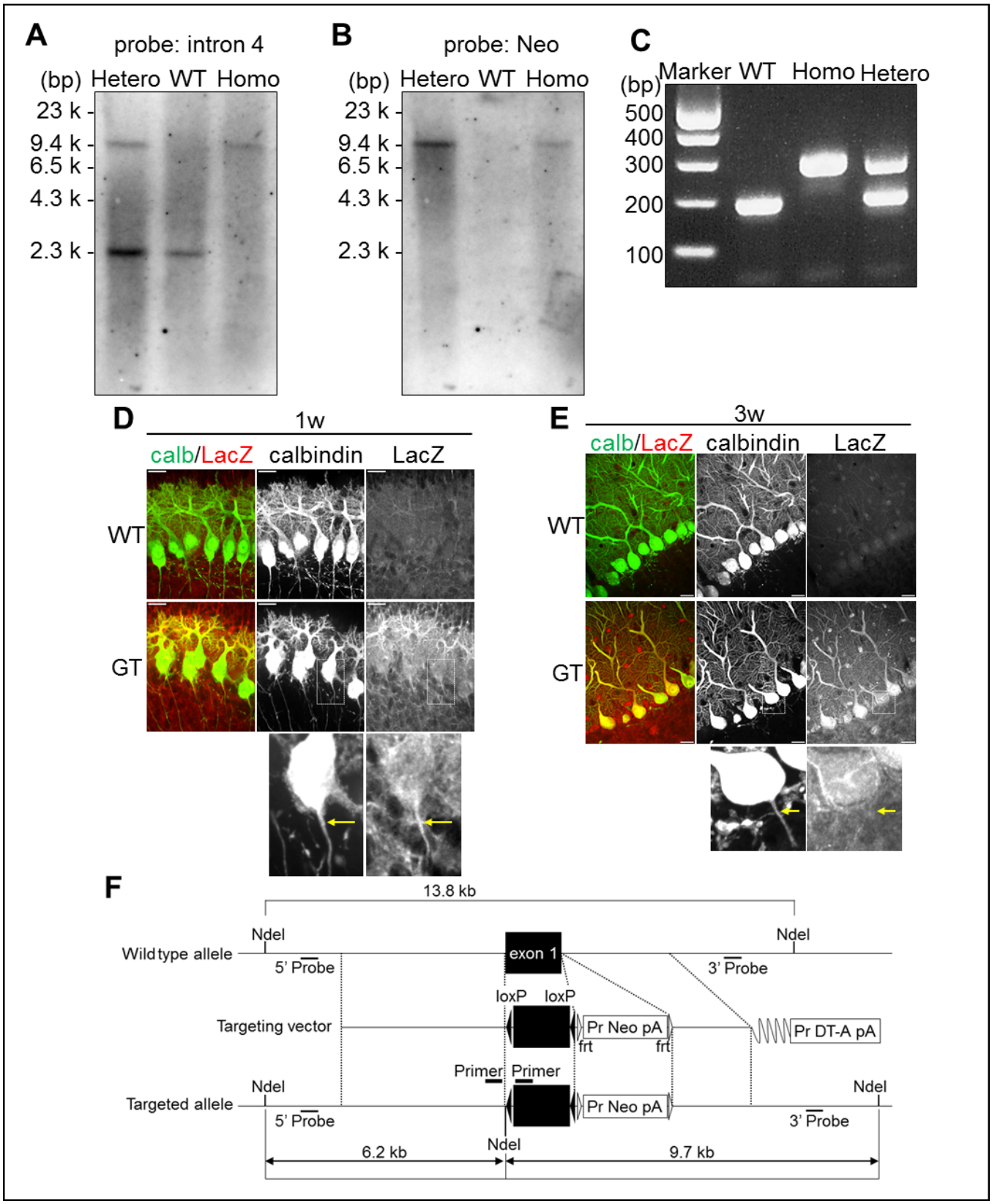
Appendix Contents



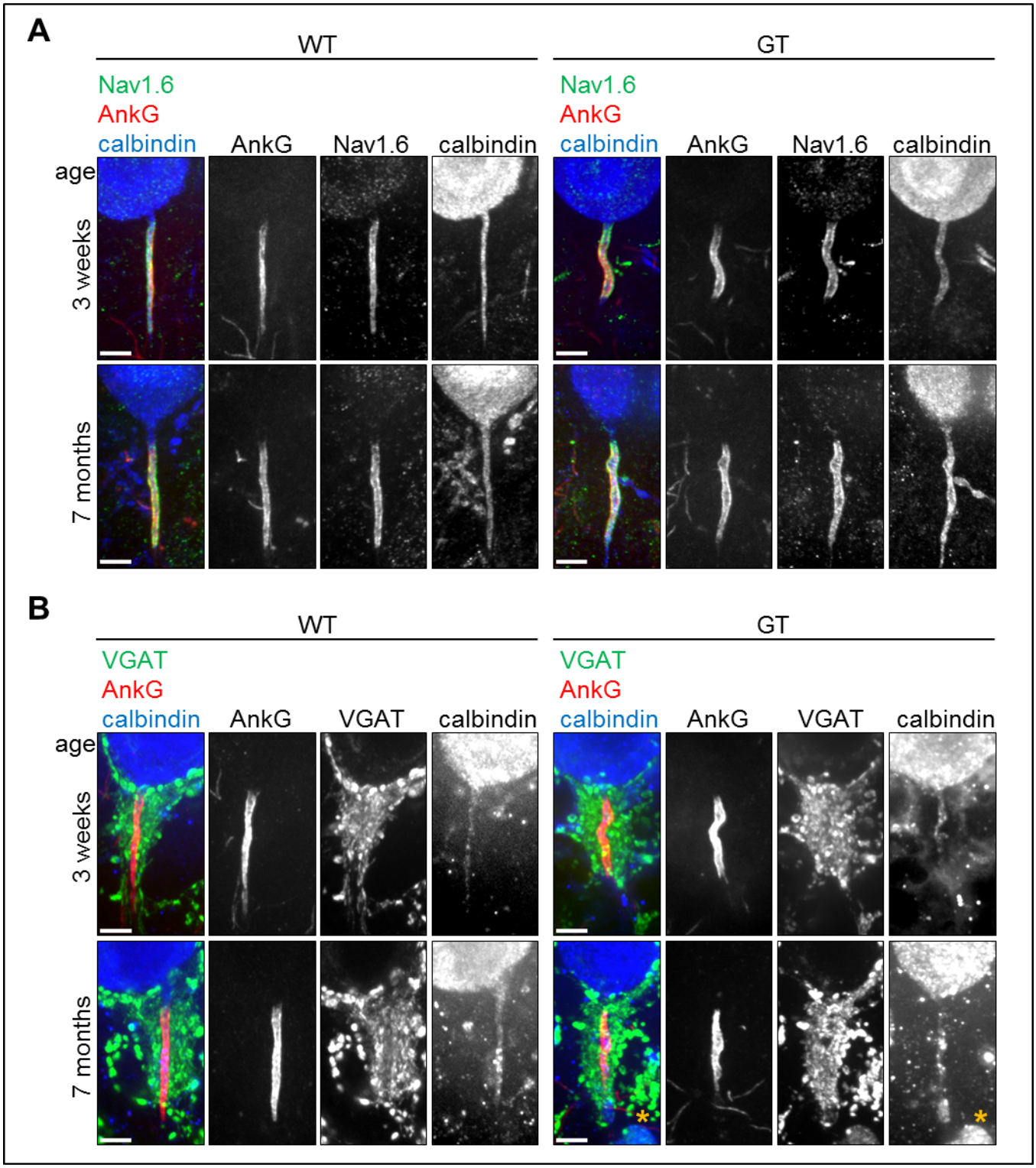
Appendix Figure S1. Analysis of the *Mtcl1* gene-trap locus. Related to Fig 1 and Fig 7.

(A and B) Southern blot analysis of genomic DNA from WT, heterozygous mutant, and homozygous mutant *Mtcl1* gene-trap mice, digested by *Hind*III and hybridized with probes for intron 4 (A) and neo (B).

(C) Representative genotyping PCR results.

(D and E) Expression of the beta-geo-fused N-terminal fragment of MTCL1 in Purkinje cells of GT mice. Cerebellar sections from WT and GT mice at 1 week (D) and 3 weeks (E) of age were immunostained for calbindin and LacZ. Boxed areas are magnified and shown in below. Arrows indicate proximal AIS region. Scale bars: 20 μm.

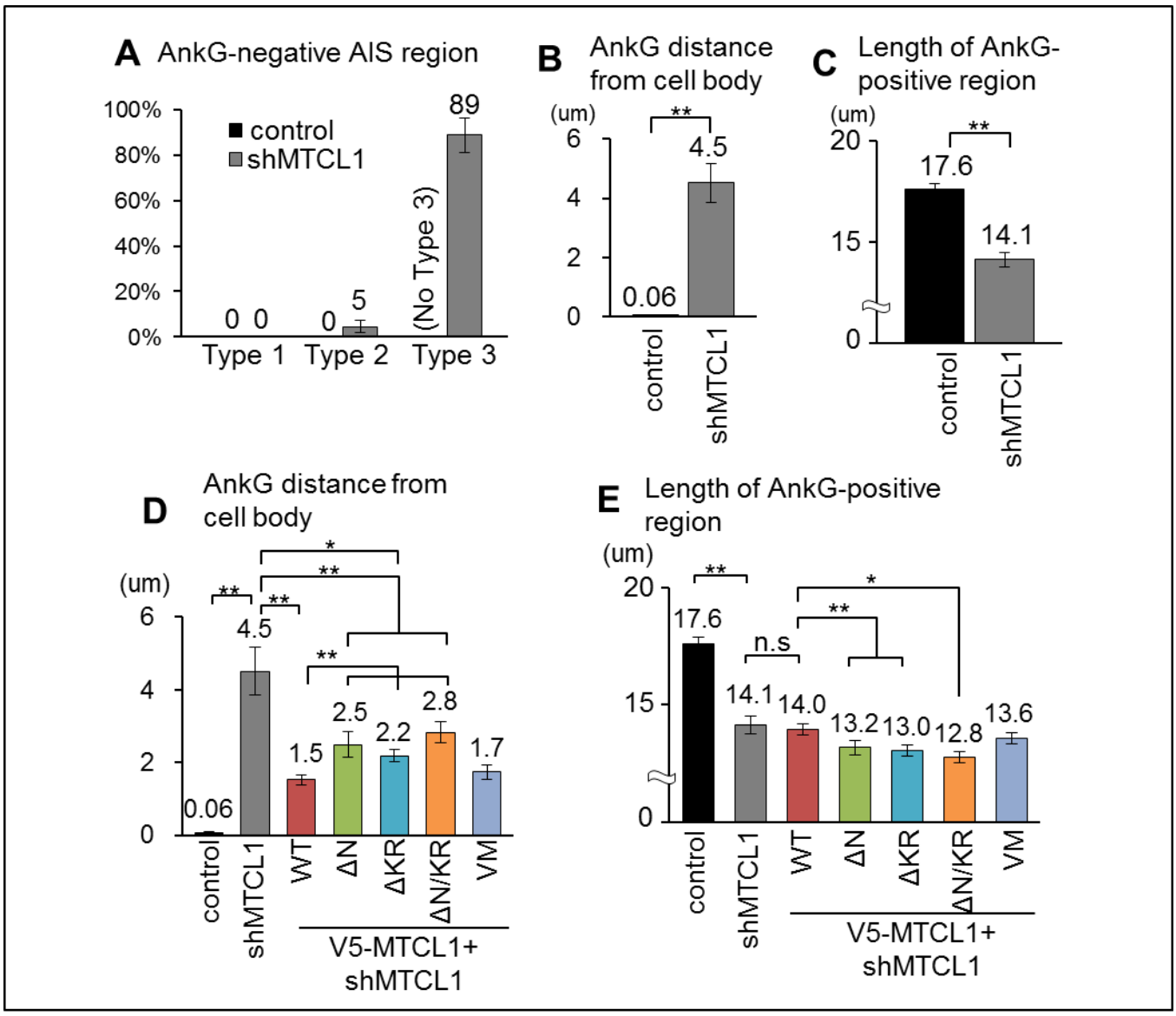
(F) Schematic of the *Mtcl1* flox allele in mouse. In Mtcl1 flox mice, exon1 of the *Mtcl1* locus was flanked with two loxP sites by homologous recombination. For Southern blotting, genomic DNA was digested with *NdeI* and hybridized with the probe depicted. Primers for *Mtcl1*-flox genotyping were also depicted.



Appendix Figure S2. Colocalization of Nav1.6, VGAT, and AnkG at the AIS. Related to Fig 3.

(A) Colocalization of Nav1.6 and AnkG at the AIS of Purkinje cells. Cerebellar sections from WT and GT mice at 3 weeks and 7 months of age were immunostained for calbindin, AnkG, and Nav1.6.

(B) Dense distribution of VGAT (pinceau synapse marker) around the AnkG-localized AIS of Purkinje cells. Cerebellar sections from WT and GT mice at 3 weeks and 7 months of age were immunostained for calbindin, AnkG, and VGAT. Asterisks indicate axonal swellings. Scale bars: 5 μm.

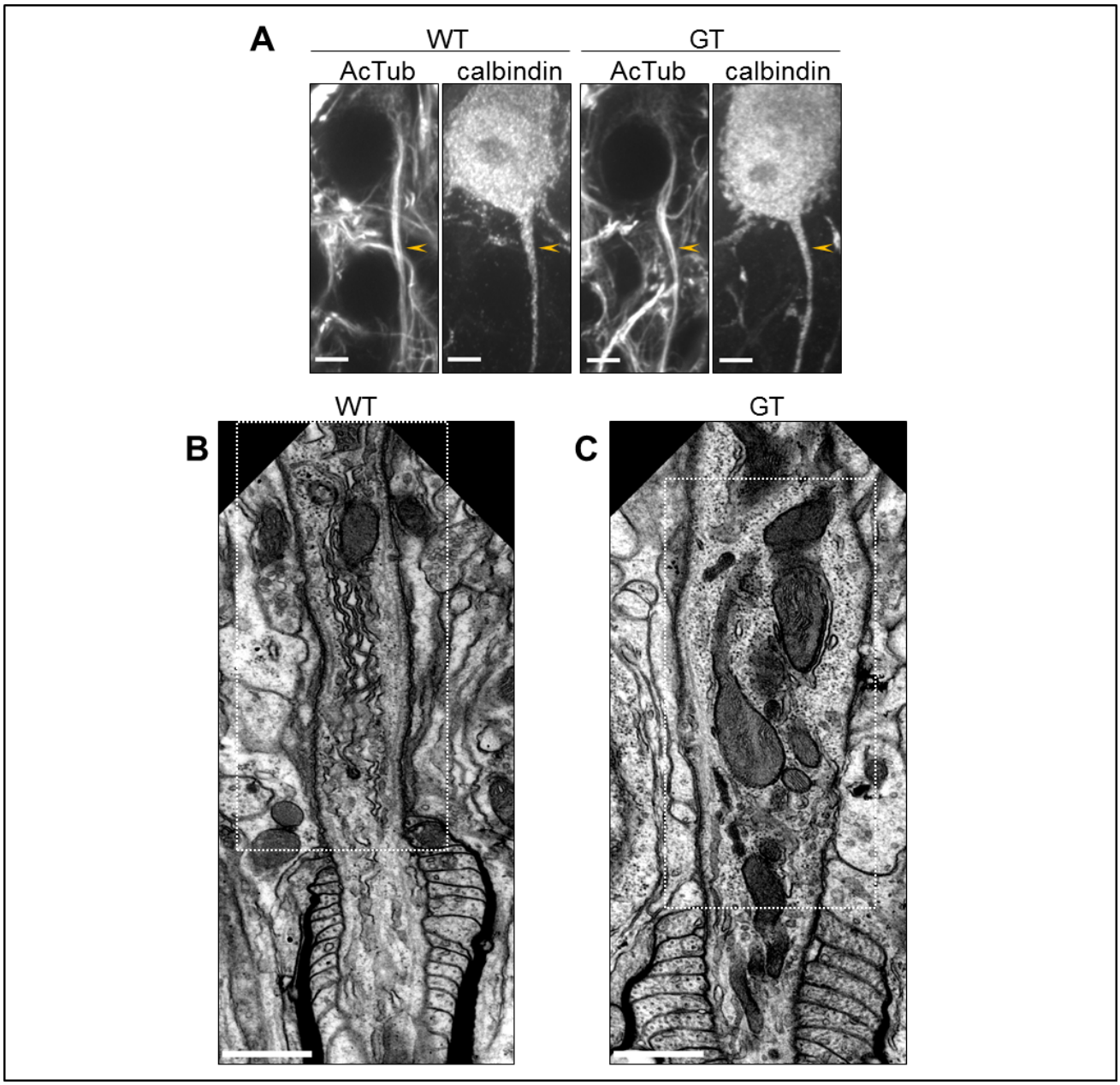


Appendix Figure S3. Quantification analysis of MTCL1 knockdown and rescue experiments. Related to Figs 4 and 5.

(A) Percentage of GFP-positive cells categorized based on AnkG-negative localization in each type of AIS region (as shown in Figure 5B) (control, *n* = 82; shMTCL1, *n* = 66). Data are represented as mean ± SEM. **p* < 0.001, compared with control (Fisher's exact test). See also Figure 4C.

(B and C) Measurement of length and distance of AnkG localized regions from the cell body in Type 1 and 2 AIS of transfected cells (control, *n* = 82; shMTCL1, *n* = 47). Data are represented as mean ± SEM. ***p* < 0.01, compared with control (Mann–Whitney *U*-test). See also Figure 4E.

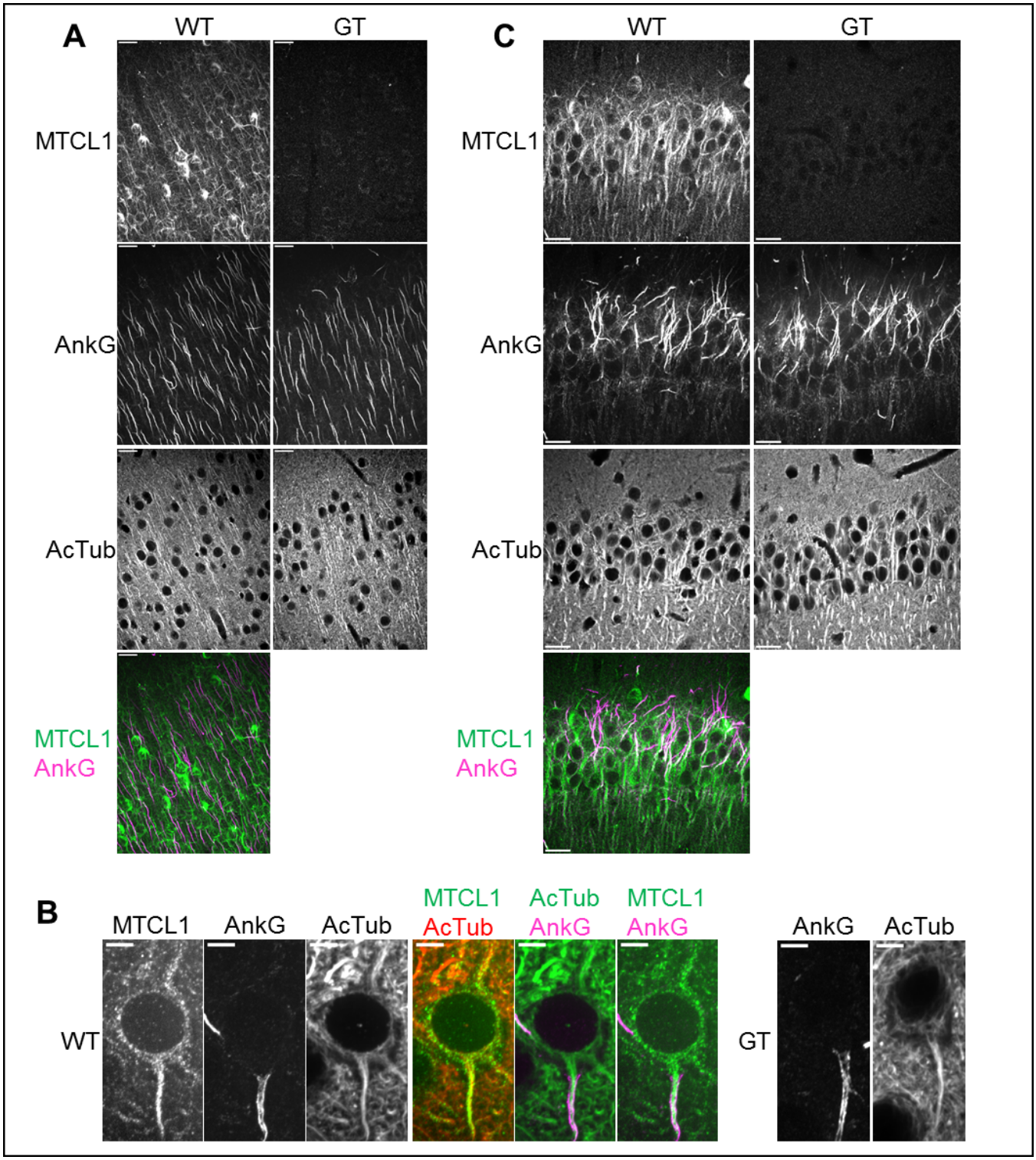
(D and E) Measurement of length and distance of AnkG localized regions from the cell body in Type 1 and 2 AIS of transfected cells (WT, *n* = 69; ΔN, *n* = 76; ΔKR, *n* = 64; ΔN/KR, *n* = 46; VM, *n* = 69). See also Figure 5D. Data are represented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with the corresponding bins (Mann–Whitney *U*-test).



Appendix Fig S4. Analysis of AIS microtubule bundles. Related to Figure 6.

(A) Cerebellar sections from WT and GT mice at 1 week of age were immunostained for acetylated tubulin (AcTub) and calbindin. See also Figure 6A.

(B) Electron micrographs of distal AIS regions in Purkinje cells from WT and GT mice at 4 weeks of age. Boxed areas are shown in Figure 6D.



Appendix Fig S5. MTCL1 expression in cortical and hippocampal neurons.

(A and C) Cortical (A) and hippocampal (C) sections from WT and GT mice at 3 weeks of age were immunostained for MTCL1, AnkG, and AcTub. Scale bars: 20μm.

(B) Representative examples of cortical neurons shown in (A). Scale bars: 5μm.

Appendix Supplementary Methods

Animals

Mtcl1 gene-trap mice: Mouse embryonic stem (ES) cells (ES cell line AT0556) containing a gene-trap vector (pGT01xr) in intron 4 of the *Mtcl1* gene were obtained from the Mutant Mouse Regional Resource Center (MMRRC, Davis, CA, USA). ES cells were microinjected into blastocysts of C57BL/6J mice and the resulting chimeric male mice crossed with C57BL/6J female mice to produce heterozygous F1 progeny. Tail DNA from the resulting agouti pups was examined by PCR for the neomycin resistance gene (*neo*) to confirm germ-line transmission of the gene-trap allele in the trap-vector. The *neo*-gene primers were: 5′-ATGGGATCGGCCATTGAACAAGATGG-3′ and 5′-ACGTCGAGCACAGCTGCGCAAGGAAC-3′. The gene-trap allele was backcrossed into the C57BL6/J strain by more than six generations for histological analysis, or 12 generations to observe gait abnormalities (Movie S1). Heterozygous gene-trap mice (hereafter, “GT mice”) were crossed with each other to produce wild-type (WT), heterozygous, and homozygous mice. To determine genotypes, three-primer PCR was performed using tail DNA. The forward primer (5′-AGGGAGTCAGATCTTGTTAAAGGTGG-3′) was designed from the upstream sequence of the insertion site. Two reverse primers were designed: from the downstream sequence of the insertion site in intron 4 (5′-GCAGCAGTGAGTCTTGGAGTCTTTGC-3′), and from the 3′ end of

the pGT0lxr vector sequence (5′-GTCTTGGGTTAGAGGGGTCTCTTTGT-3′). This three-primer PCR generated different-sized products from the WT (229 bp) and trapped (333 bp) alleles (see Fig. S1).

Mtcl1 flox mice [1110012J17Rik(loxP), Accession No. CDB1237K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html]: Exon 1 of the *Mtcl1* locus was flanked with two loxP sites by homologous recombination in ES cells. Successful targeting was confirmed by southern blot analysis. Chimeric male mice were crossed with C57BL/6J female mice to produce heterozygous F1 progeny (flox/+). Tail DNA from the resulting pups was examined by PCR for *neo* to confirm germ-line transmission of the flox allele. F1 heterozygotes were crossed with CAG-FLPe (flippase) transgenic mice (B6-Tg(CAG-FLPe)36) (RIKEN Bioresource Center, Tsukuba, Japan) to remove the FLP recombinase target (frt)-flanked *neo* cassette. The flox allele was backcrossed by more than three generations to the C57BL6/J strain for histological analysis, or eight generations to observe gait abnormalities (Movie S2). To specifically deplete *Mtcl1*-gene expression in Purkinje cells using the Cre/loxP system, *Mtcl1* flox/+ mice were crossed with Purkinje cell protein 2 (*Pcp2-Cre*) transgenic mice (B6.129-Tg(Pcp2-cre)2Mpin/J) (The Jackson Laboratory, Bar Harbor, ME, USA). The primers for *Mtcl1*-flox genotyping were: 5′-GTGGTAGCCCCAGTACATCCAAAGGCAAAG-3′ and 5′-CTCGGCTGCTCCGGCGGAGAGAAGCTGCTG-3′. Different-sized products were generated

from the WT (219 bp) and flox (425 bp) alleles. The primers for *Pcp2-Cre* genotyping were: 5′-ACATGTTCAGGGATCGCCAGG-3′ and 5′-TAACCAGTGAAACAGCATTGC-3′.

Southern blot

Genomic DNA from *Mtcl1* gene-trap mouse tails were prepared using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and digested with *Hind*III. Probes were amplified from genomic DNA and digoxigenin (DIG)-labelled using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland). Probes against *neo* (neo-probe, 691 bp) detected GT fragments (10 kb), and a probe against *Mtcl1*-intron 4 sequence (AT-probe, 449 bp) detected 2.6 kb WT and 10 kb GT fragments. Fragment detection using CDP-star (Roche) was performed according to the manufacturer's protocol. Primers for the neo-probe were: 5′-GGATTGCACGCAGGTTCTCCGGCCGCTTGG-3′ and 5′-ATTCGCCGCCAAGCTCTTCAGCAATATCAC′; and for the AT-probe were: 5′-CACCAGGTCAATGAGGTTGAACAACTCTTA-3′ and 5'-GAAAAGGTAGCTGACTGTCTTAACGTGTCC-3'. Southern blotting was performed according to standard protocols.

Immunostaining of mouse cerebellar sections

Mice under deep isoflurane anesthesia were fixed by transcardial perfusion with 4% paraformaldehyde (PFA) in 0.1 M PB (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, pH 7.4). Brains were removed from the skull and post-fixed overnight at 4°C for paraffin sections, or for 3–5 h at room temperature (RT) for microslice sections. After fixing, brains were rinsed three times (for 10 min each) with 0.1 M PB.

For paraffin sections, brains were dehydrated through a graded ethanol series (30–100% ethanol in 0.9% NaCl). Dehydrated brains were cleared in methyl benzoate and xylene, embedded in paraffin, sectioned at 5–7 μm, and mounted on amino silane (APS)-coated glass slides (Matsunami Glass, Osaka, Japan). Sections were deparaffinized in xylene, rehydrated through a graded ethanol series, heated at 120°C for 5 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and rinsed in 0.9% NaCl. Following permeabilization with 0.1 % Triton-X-100 in TBST (150 mM NaCl, 20 mM Tris-HCl, and 0.05% Tween20, pH 8.0) for 30 min, slices were then blocked with 10% calf serum in TBST for 30 min at RT. Next, slices were incubated overnight at 4°C in primary antibodies diluted in TBST. Following three washes in TBST (for 10 min each), slices were incubated for 2 hours at RT or overnight at 4°C in appropriate secondary antibodies conjugated with Alexa Fluor-488, -555 or -647 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted in TBST. Alternatively, primary antibody binding was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with

3,3′-diaminobenzidine (DAB) peroxidase substrate.

For microslice sections, brains were sectioned at 50-μm thickness using a Vibratome (VT1000S or VT1200S; Leica Microsystems, Wetzlar, Germany). Cerebellar vermis slices were collected into 0.1 M PB containing 0.1% sodium azide, stored at 4°C, and used for immunostaining within 1 month. Following permeabilization with 0.4% Triton-X-100 in 0.1 M PB with gentle shaking for 30 min, slices were then blocked with 10% horse serum or calf serum in TBST for 30 min. These steps were performed at RT. Slices were then incubated in primary antibodies diluted in TBST overnight at 4°C. Following three washes in TBST (for 10 min each), slices were incubated for 2 hours at RT or overnight at 4°C in appropriate secondary antibodies conjugated with Alexa Fluor-488, -555, or -647 and diluted in TBST. Slices immunostained with anti-GluD2, anti-VGLUT1, anti-VGLUT2, and anti-Nav1.6 antibodies were treated with 0.1 mg/ml pepsin (Dako) in 0.1 M PB for 5 min at RT before blocking to unmask epitopes.

DAB-stained sections were observed by bright field using a Keyence BZ-9000 microscope (KEYENCE, Osaka, Japan). Immunofluorescence sections were observed with an AxioImager Z1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CSU10 disc confocal system (Yokogawa, Tokyo, Japan), OrcaII CCD camera (Hamamatsu Photonics, Shizuoka, Japan), and 100 × 1.46 NA objective.

Mouse tissue lysate preparation and western blotting

Mouse tissues were homogenized in 250 µl ice-cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitor (P8340; Sigma-Aldrich) and incubated on ice for 5 min. Homogenates were then centrifuged at 10,000 × g for 5 minutes at 0°C, and the resulting supernatants collected as lysates. Lysate protein concentrations were estimated by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific), and then equal protein amounts (25 µg) separated by SDS-PAGE and analyzed by western blotting, according to standard protocols.

In utero electroporation (IUE)

Pregnant ICR mice (SLC, Shizuoka, Japan) at embryonic (E) day 11 were anesthetized by intraperitoneal injection of 6 µl per weight (g) of 2.5% avertin (2,2,2-tribromoethanol, 97% in tert-amylalcohol) in 0.9% NaCl. To prevent uterine muscle contraction during manipulation, 80–100 µl of 0.1 mg/ml ritodrine hydrochloride in PBS was evenly injected into the abdominal cavity. Next, 1–2 µl of plasmid DNA solution (2.5–5 mg/ml with 0.01% Fast Green) was injected into the fourth ventricle of the embryo using a mouth-controlled micropipette, and electrical pulses were delivered using the NEPA21 electroporator (voltage: 34–35V; pulse length: 30 msec;

pulse intervals: 970 msec; pulse repeat: 5) with a tweezer-type electrode (CUY650P3; NepaGene, Chiba, Japan).

Short hairpin RNA (shRNA) expression vectors

A shRNA plasmid targeting mouse *Mtcl1*, or control shRNA plasmid, were constructed using DNA oligos designed with a 19-mer sense sequence, nine-nucleotide loop, and 19-mer antisense sequence. Annealed oligos were cloned into *Bgl*II and *Sal*I sites of the pEB-Super-gfp vector. Oligo sequences for the shMTCL1 vector were: sense, 5′-gatccccGAATTCCATAGCAACGACTttcaagagaAGTCGTTGCTATGGAATTCtttttggaaaagcttatcgataccg-3′; and antisense, 5′-tcgacggtatcgataagcttttccaaaaaGAATTCCATAGCAACGACTtctcttgaaAGTCGTTGCTATGGAATTCggg-3′. Oligos for the control vector were: 5′-gatccccGAATATAGAAGAAGACTAGttcaagagaCTAGTCTTCTTCTATATTCtttttggaaaagcttatcgataccg-3′; and antisense: 5′-tcgacggtatcgataagcttttccaaaaaGAATATAGAAGAAGACTAGtctcttgaaCTAGTCTTCTTCTATATTCggg-3′.

Expression vectors

The GFP-human MTCL1-VM-C6 mutant used in Figure 8 was produced by site-directed mutagenesis PCR.

Transmission electron microscopy examination

Mice under deep isoflurane anesthesia were fixed by transcardial perfusion of 2% PFA, and 1.25% glutaraldehyde (GA) in 0.1 M sodium-cacodylate (pH 7.4). Cerebellar vermes were removed and sagittally sliced at 1-mm thickness, further fixed in the same fixative solution for 8–10 hat RT, and then post-fixed in a mixture of 1.25% GA, 1% PFA, 1% OsO_4, and 0.32% $K_3[Fe(CN)_6]$ in 30 mM HEPES (pH 7.4) for 1 h at RT. Fixed blocks were dehydrated through a graded ethanol series (50–100% ethanol), and embedded in an epoxy resin (consisting of Quetol651, nonenylsuccinic anhydride, methylnadic anhydride, and DMP-30). Polymerized blocks were ultra-thin sectioned at 70-nm thickness (EM UC7; Leica). Ultra-thin sections on a butvar film-supported grid were contrasted with EM stainer (Nisshin EM, Tokyo, Japan) and lead citrate, and examined with a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

HaloPlex sequencing in patients with spinocerebellar ataxia, cerebral cortical atrophy, and multiple system atrophy

Genomic DNA was extracted from peripheral blood leukocytes. Targeted resequencing

(including *MTCL1*) was performed using the HaloPlex target enrichment system (Agilent Technologies, Santa Clara, CA, USA). For target enrichment of *MTCL1*, 18 comprehensive target regions were chosen based on RefSeq and Ensembl sequences, including 14 coding exons from NM_015210.3 with region extension of 50 bp from the 3′ end and 50 bp from the 5′ end. The total region size was 8,029 bp, encompassing 99.76% coverage of the entire *MTCL1* coding region. Further, 37 other targets or genes were also captured using the same system, resulting in a total sequence-able design size of 569 kb. HaloPlex target enrichment was performed according to the manufacturer's protocol. Genomic DNA from two samples was mixed at an equal amount, and 225 ng mixed genomic DNA used as the input. After equimolar sample pooling, sequencing was performed on a HiSeq2500 (Illumina, SanDiego, CA, USA) with 101-bp reads in one lane. Mean coverage for *MTCL1* was 243 × (fold) for each mixed sample (i.e., 121.5 × for each sample). Approximately 90.5% of the coding region was covered 10 × or more. Image analysis and base calling were performed by sequence control software for real-time analysis and CASAVA software v1.8 (Illumina). Reads were aligned to GRCh37 using Novoalign (http://www.novocraft.com/). After excluding common variants registered in the common dbSNP135 database (minor allele frequency ≥ 0.01), variants were called using the Genome Analysis Toolkit (http://www.broadinstitute.org/gatk/) and annotated using ANNOVAR (http://www.openbioinformatics.org/annovar/). Variants observed in five or more (out of 575)

in-house Japanese controls were excluded from further analysis. Calls with a minor allele frequency ≥ 0.25 were identified as possible heterozygous variants for one of the mixed samples. Sanger sequencing was performed for confirmation of the detected rare variant.

Whole-exome sequencing analysis on an individual with a *MTCL1* variant

Genomic DNA was captured using a SureSelect Human All Exon v5 kit (Agilent Technologies) and sequenced on a HiSeq2500 with 101 bp paired-end reads (Illumina). Downstream analyses were the same as above, as well as removal of PCR duplicates using Picard (http://picard.sourceforge.net/). Whole exome sequencing mean coverage depth against the RefSeq coding sequence for this individual was 93.8 ×, with 92.6% of total coding sequence bases covered by 20 reads or more.

PolyPhen-2, SIFT, and MutationTaster are computational prediction algorithms of protein stability changes due to missense mutations. PolyPhen-2 scores close to 1 are likely to be pathogenic. HumVar-trained PolyPhen-2 is a preferred model for diagnosing the Mendelian disease. This prediction was based on PolyPhen-2 v2.2.2r398. SIFT scores less than 0.05 are likely to be pathogenic. In MutationTaster, Bayes classifier is employed to predict the disease potential of an alteration. The probability value is provided with a prediction in which a value close to 1 indicates a high 'security' of the prediction.

Clinical presentation of patients II-2 and III-1

Patient II-2

Patient II-2 was born to non-consanguineous parents. His father and two of his siblings are also affected (Fig. 8A). Dysarthria was first recognized at 40 years of age. Cerebellar ataxia was observed. Spinocerebellar ataxia (SCA)1, 2, 3, 6, 7, 12, and 17, and dentatorubral-pallidoluysian atrophy (DRPLA) were excluded by genetic analyses. He died at 75 years of age.

Patient III-1

Patient III-1 was 54 years old at the time of the study. He is the oldest son of patient II-2. At 31 years of age, he presented with tremor, dysarthria, and gait disturbance, all of which gradually worsened, and he lost his ambulation. At 53 years of age, muscle weakness in his right upper extremity was noticed, leading to a diagnosis of cervical spinal canal stenosis by magnetic resonance imaging (MRI). He was surgically treated. Neurological examination at 54 years of age found that he had ataxia in his upper and lower extremities as well as ataxic speech. The deep tendon reflex was decreased in his upper extremities, but increased in his lower extremities with a negative Babinski sign. He also had pollakiuria and constipation. He could not stand still by himself. Brain MRI revealed cerebellar atrophy.

Cell culture, transfection, and immunofluorescence

HeLa-K cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), and under 5% CO_2 at 37°C. For transfection, HeLa-K cells were seeded onto coverslips at a density of 3×10^4 cells/cm. Plasmid transfections were performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific). After 20 hours, cells were fixed with 2% paraformaldehyde followed by permeabilization with 0.5% Triton-X-100. Fixed cells were blocked with 10% calf serum in PBST (8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) for 1 h at RT, and incubated with primary antibodies diluted in TBST containing 0.1% BSA for 45 min at RT. Following three washes in PBST, samples were incubated for 45 min at RT in appropriate secondary antibodies conjugated with Alexa Fluor-555 or -647 and diluted in TBST.