Materials and methods

Subjects

Seventy patients with ataxia, including 39 patients from 28 families with autosomal dominant cerebellar ataxia and 31 patients with sporadic ataxia and 264 patients with idiopathic PD, were enrolled in this study. Clinical diagnoses of ataxia and PD were made according to the published criteria. These 334 patients were recruited from the neurology clinics of Chang Gung Memorial Hospital. In addition, 198 unrelated subjects without neurodegenerative disorders were recruited as normal controls. All examinations were performed after obtaining informed consent from patients and control individuals.

Genomic DNA extraction

Genomic DNA extraction from whole blood is carried out using DNA Extraction Kit (Cat. No.200600, Stratagene). Three volume of 1x Solution 1 was mixed well with the blood sample and incubated on ice for 5 minutes. After centrifugation at 2,000 rpm for 10 minutes, the supernatant was removed and the pellet was resuspended in 2 ml of Solution 2. Then 10 µl of pronase is added to the suspension and incubated at 60˚C for several hours, or at 37˚C overnight for several days. The suspension was placed on ice for 10 minutes, followed by mixing with 0.8 ml of Solution 3 and stood on ice for 10 minutes. The protein precipitate was pelleted by centrifugation at 3,400 rpm for 15 minutes at 4˚C, and the supernatant containing nucleic acid was treated with 6 µl of RNase at 37˚C for 15 minutes. DNA precipitate was separated out by mixing with 2.5 ml of isopropanol, transferred to a fresh tube, and centrifuged at 14,000 rpm for 1 minute. The pellet was rinsed once

with 70% ethanol and air-dried. DNA was dissolved in adequate volume of ddH_2O and the concentration was determined using the spectrophotometer.

Polymerase chain reaction (PCR) and genotyping

Molecular analyses of tri- or pentanucleotide repeat loci of SCA1, SCA2, SCA3, SCA6, SCA8, SCA10, SCA12, SCA17, and DRPLA were carried out by polymerase chain reaction (PCR) with the primers and under the conditions listed in **Table 2**. Briefly, 100 ng of genomic DNA, 0.4 μM of each primer of which the forward primer was fluorescence labeled, 100-200 μM dNTPs, 0.8-1.5 mM $MgCl_2$, 10 mM of Tris pH 8.3, 50 mM KCl, 0.5 U Taq polymerase, and 10% dimethylsulfoxide were prepared in a final volume of 25 μl. Amplified products were analyzed in a linear polyacrylamide gel on an automated MegaBACE Analyzer and allele sizes were determined by comparing migration relative to molecular weight standards. DNA sequencing was performed to accurately assess repeat size and the presence of interruptions.

Gel extraction

After separation on the agarose gel, DNA fragment was recovered using Gel-M™ Extraction System (Cat. No.EG1001, Viogene). First, the gel slice was completely dissolved in 0.5 ml GEX Buffer at 60˚C for 5 to 10 minutes. Then the dissolved gel mixture was applied to the Gel-M™ column and centrifuged for 1 minute. The column was washed with 0.5 ml of WF Buffer followed with centrifugation for 1 minute, and washed with 0.7 ml of WS Buffer followed with centrifugation for 1 minute. The residual ethanol was removed by one more centrifugation for 1 minute. DNA was dissolved in 30 μl of ddH_2O for 5 minutes and eluted by centrifugation for 1 minute.

Statistical analyses

Possible differences between the normal and patient groups in normal repeat frequency distributions were assessed using a non-parametric Mann–Whitney *U*-test. Allele frequencies at each locus were estimated by the gene count method. Statistical analyses of differences in the frequency of large normal alleles (those corresponding to 5 to approximately 10% of the upper tails) for each locus were performed with the Fisher's exact test.

SCA8 cDNA cloning

Human cerebellar polyadenylated RNA (Clontech) was reverse transcribed into cDNA by using the SuperScript™ II reverse transcriptase (Invitrogen). Sense and antisense primers used for amplification of full length *SCA8* cDNA were (5'-ATCCTTCACCTGTTGCCT) and (5'-GCTTGTGAGGACTGAGAATG), respectively. The 1.3-kb full-length, $(CTA)_{11}(CTG)_{12}$ combined repeats (23R) containing cDNA (including exons D, C2, C1, B, and A) (SCA8-23R) (**Figure 2B**) (Nemes et al. 2000) was cloned into pGEM-T Easy vector (Promega) and sequenced. The *Nla*III-*Dra*I fragment containing $(CTA)_{11}(CTG)_{12}$ repeats was replaced with a 290bp fragment from the PCR clone of a patient with *SCA8* CTG_{88} expansion $[(CTA)_8CCACTACTGCTACTGCTA(CTG)_{64}CTA(CTG)_9]$ (SCA8-88R). The trinucleotide repeat number is further expanded to $[(CTA)_8CCACTACTGCTACTGCTA(CTG)_{67}CTA(CTG)_{65}CTA(CTG)_9]$ combined 157 repeats by ligation of *Fnu*4HI partially digested fragments (SCA8-157R) (項, 2005). To construct the CTA/CTG deleted *SCA8*, a *Dra*I site was introduced to the 5' end of repeats by site-directed mutagenesis [primer 5'-CCCTGGGTCCTTCATGTTAGAAAACCTGG-

CTTTAAAA(CTA)$_8$C] as described below and the *Dra*I fragment containing CTA/CTG combined repeats was removed (SCA8-0R).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using QuikChange™ XL Site-Directed Mutagenesis Kits (Cat. No.200517, Stratagene). The reaction was set up in 25 μl of volume including 2.5 μl of 10 × reaction buffer, 10 ng of template DNA, 62.5 ng of both sense and antisense primers, 50 μM dNTPs, 1.5 μl of QuickSolution and 0.5 μl of *PfuTurbo* DNA polymerase. The reaction was temperature cycled with the condition: 1 cycle of denaturation at 95˚C for 1 minute, 18 cycles of reaction comprising denaturation at 95˚C for 50 seconds, annealing at 60˚C for 50 seconds, and elongation at 68˚C for 12 minutes, followed by elongation at 68˚C for 7 minutes. Following cycling, the reaction was cooled and 0.5 μl of *Dpn*I (10 U/μl) was added to digest the parental DNA template at 37˚C for 1 hour. Then 5 μl of the reaction was taken to transform the competent cells and the site-directed mutant DNA was extracted from the cultured clones and sequenced.

pEF-SCA8 constructs

To generate SCA8 expression constructs driven by the human polypeptide chain elongation factor 1α promoter (the EF promoter), *SCA8* cDNA bearing 0 to 157 CTA/CTG combined repeats was placed in the *Not*I restricted site of pEF-IRES/hrGFP vector (Chung et al. 2002) in which the *Kpn*I fragment containing 3' region of the IRES sequence and the humanized *Renilla* green fluorescent protein (hrGFP) gene were removed to result in pEF-SCA8-0R, 23R, 88R, and 157R constructs (**Figure 3A**). These constructs were sequenced and verified by restriction mapping.

KLHL1 cDNA cloning and pEF-KLHL1-EGFP construct

The 3.2 kb *KLHL1* cDNA was amplified also from human cerebellar polyadenylated RNA using sense (5'-CATGTCAGGCTCTGGGCGAA-AAG) and antisense (5'-TGGGCGATGAGAATATGAAGTCTG) primers. After cloning into pGEM-T Easy and sequencing, the 2.2 kb *KLHL1* coding sequences and the EGFP gene from pEGFP-N1 were fused in-frame and inserted into the *Not*I site of the modified pEF-IRES/hrGFP vector in which hrGFP had been removed by *Kpn*I restriction to generate pEF-KLHL1-EGFP (**Figure 3B**). The construct was verified by DNA sequencing and restriction mapping.

pCMV-$(CAG)_{36}$-IRES-EGFP construct

The 1.1 kb *TATA binding protein* (*TBP*) cDNA containing 36 CAA/CAG repeats [$(CAG)_3(CAA)_3(CAG)_9CAACAGCAA(CAG)_{16}CAACAG$] was amplified using sense (5'-CTGGTTTGCCAAGAAGAAAGTG) and antisense (5'-AGGCAAGGGTACATGAGAGCCA) primers (by 王政光). After cloning and sequencing, the 5' 254-bp cDNA fragment was placed between the *Eco*RI and *Pst*I sites of the pIRES2-EGFP vector (Clontech) (洪, 2005). The resulting pCMV-$(CAG)_{36}$-IRES-EGFP contains the 5' region of *TBP* cDNA upstream to the IRES and EGFP gene (**Figure 3C**). The construct was verified by DNA sequencing.

pCMV-ORF1-EGFP construct

The ORF1 translation termination sequence of *SCA8* cDNA (in pGEM-T Easy vector) was removed and a *Sma*I restricted site (underlined) added by PCR using primer 5'-GCGCCCGGGACACTTCAACTTCCTATACATACA.

The *Eco*RI (in MCS of pGEM-T Easy vector)-*Sma*I fragment containing *SCA8* ORF1 was in-frame fused to the *EGFP* gene in the pEGFP-N1 vector (Clontech) (between the *Eco*RI and *Bst*UI sites). The Kozak sequence of enhanced green fluorescence protein (EGFP) gene was removed by site-directed mutagenesis (primer 5'-CGGGCCCGGGATCCACCGGTCGCC$\underline{\Delta}$-GTGAGCAAGGGCGAGGAGCTG, $\underline{\Delta}$ = ACCATG) (by 蕭欣杰). The resulting pCMV-ORF1-EGFP construct (**Figure 4A**) was verified by DNA sequencing and restriction mapping.

pCMV-SCA8-EGFP constructs

The translation termination sequence of *SCA8* ORF3 (with 0R, 23R, 88R and 157R) was removed by *Afl*II restriction. A linker sequence (5'-CGACTCGCG) was added between the filled-in *Afl*II site and the *Bst*UI site of pEGFP-N1 vector to fuse *SCA8* ORF3 and Kozak-deleted EGFP gene in-frame. The resulting pCMV-SCA8-EGFP constructs (**Figure 4B**) were verified by DNA sequencing and restriction mapping.

pCMV-K-ORF3-EGFP constructs

To generate pCMV-K-ORF3-EGFP, *SCA8* cDNA sequences 1~1032 in pCMV-SCA8-23R-EGFP and pCMV-SCA8-157R-EGFP were removed and the bases in front of AUG start codon were modified to match the Kozak consensus sequence (CACC) (by Dr. 蘇銘燦). The resulting pCMV-K-ORF3-23R-EGFP and pCMV-K-ORF3-157R-EGFP constructs (**Figure 4C**) were verified by DNA sequencing and restriction mapping.

Preparation of electro-competent cells

The day before preparation, bacteria (TOP 10F', Cat. No.50-0059, Invitrogen) were cultured overnight in 100 ml of LB broth containing tetracycline. On the day, bacterial culture was aliquot into four 1-liter flasks, each containing 225 ml of LB broth, and inoculated with shaking at 37˚C until the OD_{600} reached 0.7 ~ 0.8. Cells were chilled on ice for 10 minutes and pelleted by centrifugation at 4,000 rpm at 4˚C for 5 minutes. The supernatant was removed and cells were resuspended and pooled in 250 ml of cold ddH_2O for pellet from 500 ml culture. Cells were centrifuged at 4,500 rpm at 4˚C for 5 minutes and washed with 250 ml of cold ddH_20 twice. The supernatant was poured off and cells were resuspended in 1 ml of ddH_2O containing 20% glycerol. Finally, cells were aliquot into eppendorf tubes in 40 µl amounts and stored at -80˚C.

Ligation

The adequate amount of insert DNA fragment was mixed with vector, 0.5 µl of 10 × Ligation Buffer, 0.5 µl of PEG 4000 Solution, and 1.25 U of T4 DNA ligase (Cat. No.EL0331, Fermentas) in a volume of 5 µl. The ligation reaction was incubated at room temperature for 1 hour or 16˚C overnight.

Electroporation

The ligation reaction was inactivated at 65˚C for 10 minutes and kept on ice after spinning down. Immediately prior electroporation, 1 µl ligation sample was mixed with 20 µl aliquot of electro-competent cells and then added to the 1 mm-gap cuvette (Cat. No.4307-000-569, Eppendorf). The electroporation was carried out using MicroPulser Electroporator (BIO-RAD) with 1.25 Kvolts under the manual program. Chilled LB broth (500 µl) which contains no antibiotics was added immediately to the cuvette. The

content of curvette was then transferred to the tube, incubated at 37˚C for at least 30 minutes, and plated on LB medium with appropriate antibiotic for incubation at 37˚C for 16-18 hours.

Minipreparation of plasmid DNA

A single bacterial colony was transferred into 1ml of LB broth containing the appropriate antibiotic. After overnight incubation at 37°C with vigorous shaking, the bacteria were harvested by 14,000 rpm centrifugation for 1 minute. The bacterial pellet was resuspended in 70 μl of Solution I (50 mM glucose – 25 mM Tris-HCl pH 8.0 – 10 mM EDTA pH8.0) by vigorous vortexing. Then 140 μl fresh Solution II (0.2 N NaOH – 1% SDS) was added into the bacterial suspension and the contents was mixed by inverting several times followed mixing with 105 μl Solution III (3 M potassium acetate). The cell debris and genomic DNA were precipitated at 14,000 rpm for 5 minutes, and the supernatant containing plasmid DNA was transferred to a fresh tube. The plasmid DNA pellet was precipitated after mixing with 0.8 volume of isopropanol and centrifuged at 14,000 rpm for 5 minutes. The DNA pellet was washed in 70% ethanol and dried in the air. The plasmid DNA was dissolved in 40 μl ddH_2O containing 10 μg/ml RNase and checked by 0.8% agarose gel electrophoresis.

Midipreparation of plasmid DNA

Large-scale isolation of plasmid DNA was performed with Midi-V100™ Ultrapure Plasmid Extraction System (Viogene). A single bacterial colony was incubated in 65 ml of LB broth containing the appropriate antibiotic at 37˚C for 20 hours and then the bacteria cells were harvested by

centrifugation at 8000 g for 5 minutes. The cell pellet was resuspended in 4 ml of VP1 Buffer containing RNase. The cell suspension was mixed with 4 ml of VP2 Buffer and with 4 ml of VP3 Buffer, sequentially. The cell debris was centrifuged at 12,000 g for 15 minutes and the supernatant was applied to a Midi-V100™ column, which was pre-equilibrated with 10 ml of VP4 Buffer. The column was washed with 15 ml of VP5 Buffer and the plasmid DNA was eluted with 5 ml of VP6 Buffer. Then DNA was precipitated by mixing with 3.75 ml of isopropanol, allotted to 1.5 ml tube, and centrifuged at 14,000 rpm for 10 minutes. After removing the supernatant, DNA pellet was dissolved in 63 μl of ddH_2O for each tube and combined to a tube. To eliminate residual salt, DNA was precipitated by mixing with 20 μl of 5 M NaCl and 1000 μl of pure ethanol, followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was removed and DNA was allowed to dry in the air. Finally, DNA was dissolved in 400 μl of ddH_2O and the concentration was determined using the spectrophotometer.

Cell cultivation

Human embryonic kidney (HEK) 293 (ATCC No. CRL-1573) and IMR-32 (ATCC No.CCL-127) cell lines were maintained in DMEM supplement with 10% fetal calf serum (FCS), 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin, and 100 U/ml streptomycin, at 37°C in an atmosphere containing 5% CO_2.

Transfection

The rapid transfection process was carried out without plating cells the day before transfection. For transfection performed in the 12-well plate, 2 μg of

DNA and 2 ~ 4 μl of Lipofectamine™ 2000 reagent (LF2000) (Cat. No.11668-019, Invitrogen) were first diluted into 100 μl Opti-MEM® I Reduced Serum Medium (Cat. No.31985-062, Invitrogen), respectively, and incubated for 5 minutes at room temperature. Then DNA and LF2000 were combined in the well and incubated for 20 minutes at room temperature. At the meanwhile of DNA-LF2000 complexes formation, the cell suspension of appropriate number per well was prepared in 10% FCS-DMEM without antibiotics. The cell suspension was added to the DNA-LF2000, mixed gently by rocking back and forth, and incubated at 37˚C for at least 24 hours for further expression studies.

Fluorescence activated cell sorting (FACS) analysis

HEK293 cells cultivated in DMEM containing 10% FCS were transfected with the various cDNA constructs using a lipofection procedure. pEGFP-N1 was used as a negative control to show the specificity of SCA8 *trans* RNA interference. Forty-eight hours later, cells were harvested for fluorescence activated cell sorting (FACS) analysis. The amounts of GFP expressed were analyzed in a FACSCalibur flow cytometer (Becton-Dickinson), equipped with an argon laser operating at 530 nm. A forward scatter gate was established to exclude dead cells and cell debris from the analysis. 10^4 cells were analyzed in each sample.

Immunofluorescence analysis

The transfection process was performed as described above. Before transfection, circular coverslips of which the diameter was 15 mm were sterilized and coated with poly-L-lysine as noted below. Coverslips were first washed in 70% ethanol for 30 minutes twice, incubated in 0.1 N HCl

overnight, sterilized by autoclaving and stored in 95% ethanol. Coverslips were then placed in each well of 12-well culture plate and coated with poly-L-lysine (100 μg/ml, Sigma) for 1 hour at room temperature. After removing poly-L-lysine, coverslips were briefly washed with sterile ddH_2O twice, allowed to dry completely, and ready for use after sterilizing under UV light for at least 1 hour. After transfection, cells were allowed to grow on coverslips for at least 1 day depending on the confluence and/or the gene expression level. For immunofluorescence analysis, cells were rinsed briefly in PBS and fixed in 4% paraformaldehyde in PBS for 15 minute at room temperature. After washing twice with PBS, cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, followed by washing in PBS three times. The intracellular nonspecific binding of the antibodies was blocked with PBS containing 1% BSA for at least 1 hour. Cells were then incubated with the primary antibodies for 1 hour at room temperature or overnight at 4˚C, washed in PBS three times, and a second incubation with fluorescence-conjugated antibodies for another 1 hour at room temperature, followed by washing in PBS three times. The nuclei were stained using 0.05% DAPI (4'-6-diamidino-2-phenylindole) and the coverslip was mounted on glass slides in Vectashield (Cat. No.H-1400, Vector). The stained cells were observed with Leica TCS confocal laser scanning microscope.

Cell lysate preparation

Cells were harvested by centrifugation at 1,000 g for 5 minutes followed with cold PBS washing twice. The cell pellet was resuspended in 200 μl RIPA buffer (10 mM Tris pH7.5 – 150 mM NaCl – 5 mM EDTA pH8.0 – 0.1% SDS – 1% DOS – 1% NP-40) containing the protease inhibitor mixture,

sonicated 15 pulses three times, and sat on ice for 30 minutes. Protein extracts were centrifuged at maximum speed at 4°C for 30 min and the supernatant was transferred to a fresh tube. The protein concentration was determined using the Bio-Rad Protein Assay (Cat. No.500-0006, Bio-Rad).

Cytoplasmic and nuclear proteins preparation

Separation and preparation of cytoplasmic and nuclear extracts was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat. No.78833, Pierce). Transfected cells ($1 \sim 2 \times 10^6$) were harvested, resuspended in 100 μl of ice-cold CER I containing protease inhibitor, and incubated on ice for 10 minutes. Then 5.5 μl of ice-cold CER II was added to the cell suspension, mixing by vortexing for 5 seconds, and incubated on ice for 1 minute. After vortexing for another 5 seconds, cell extract was centrifuged at 4° C for 5 minutes at maximum speed, and the supernatant, which embraces the cytoplasmic fraction, was transferred to a fresh, pre-chilled tube and stored at -80˚C. The pellet was resuspended in 50 μl of ice-cold NER, vortexing for 15 seconds, and incubated on ice with vortexing for 15 seconds every 10 minutes, for a total of 40 minutes. The insoluble fraction was precipitated by centrifugation at 4˚C for 10 minutes at maximum speed and the supernatant was used for nuclear fraction. The protein concentration was determined using the Bio-Rad Protein Assay. For subcellular location determination, the protein extracts were reacted with antibodies against poly ADP-ribose polymerase (PARP) (sc-7150, Santa Cruz Biotechnology), the nuclear marker, and α-tubulin (sc-5286, Santa Cruz Biotechnology), the cytoplasmic marker, respectively.

Western blotting (*immunoblotting*)

Equal amount of cell lysates were denatured in 1 × sample buffer (50 mM Tris pH 6.8 – 2% SDS – 10% Glycerol – 2.5% β-mercaptoethanol – 0.005% bromophenolblue) at 95°C for 10 minutes and separated by SDS-PAGE according to the molecular weight and electrophoretic transferred to nitrocellulose membrane (Schleicher and Schuell) using XCell II™ Blot Module (Cat. No. EI9051, Invitrogen) in transfer buffer (25 mM Tris – 0.2 M glycine – 20% methanol) at 45 V for 1 hour. The membrane was blocked in PBS containing 10% non-fat milk for at least 1 hour at room temperature or overnight at 4˚C. The membrane was rinsed with washing buffer (10 mM Tris, pH 8.0 – 0.05% tween-20) and incubated with primary antibodies at room temperature for at least 1 hour. The membrane was washed three times with washing buffer for 5 minutes. Then the membrane was incubated with appropriate secondary antibodies conjugated horse radish peroxidase for 1 hour and washed three times with washing buffer. The immune complexes were detected with SuperSignal® West Femto Maximum Sensitivity Substrate (Cat. No.34096, Pierce).

RNA isolation

Total RNA was extracted using Trizol reagent (Cat. No. 15596-018, Invitrogen) according to the manufacturer's specifications. After PBS washing, 1ml of Trizol reagent was added to the culture dishes and cells were scraped from the culture dish. The cell suspension was incubated on ice for 5 minutes, mixed well with 1/5 volume of chloroform and incubated on ice for another 5 minutes. RNA was separated form DNA and proteins by centrifugation at 4˚C for 15 minutes. The colorless, upper aqueous phase was carefully removed to a fresh tube avoiding the material that collected at the interface, and mixed with 0.8 volume of isopropanol. The mixture was

sat at -20˚C for at least 1 hour and centrifuged at 4˚C for 15 minutes to precipitate RNA. The supernatant was discarded and RNA pellet was rinsed with 70% DEPC-H_2O. RNA was air dried and dissolved in adequate volume of DEPC-H_2O. The quality and quantity of RNA samples were determined by the agarose electrophoresis and the absorbance at 260 nm, respectively.

Establishment of SCA8 stably expressing Flp-In T-REx 293 cell lines

The generation of SCA8 stably expressing Flp-In T-REx 293 cell lines is based on the Flp-In™ T-REx™ system (Invitrogen). For constructing stably SCA8 expressing vectors, fSCA8-0, 23, 88, and 157R were restricted by *Not*I and inserted to *Not*I site of pcDNA5/FRT expressing vector. Then each of these expressing vectors and pOG44 were co-transfected into the Flp-In T-REx 293 cell with the molar ratio of 1:9. One day after transfection, the medium was replaced with fresh one. Next day, cells were split into fresh medium with the density not more than 25% confluent. Once cells attached to the dish, the culture medium was replaced with the selective medium containing 100 μg/ml of Hygromycin and 15 μg/ml of Blasticidin. After 1 month of Hygromycin selection with replacement of fresh selective medium every 4 days, genomic DNA was isolated from each cell line and verified by PCR with sense (5'-GCTTGTGAGGACTGAGAATG) and antisense (5'-CCCTGGGTCCTTCATGTTAG) primer pair. To induce expression of *SCA8* integrating into the FRT site, cells were treated with 1 μg/ml doxycycline (dox) for varying periods of time.

Cell proliferation assessment (WST-1 assay)

The extent of cell proliferation was determined with PreMix WST-1 Cell Proliferation Assay System (Takara). After doxcycline induction and drugs

treating, the cells are added PreMix WST-1 in a final 1:10 dilution and incubated for 4 hours. The cell viability is determined by measuring the formazan dye formation, cleaved by the succinate-tetrazolium reductase which is active in the viable cells, at 450 nm using microplate reader.

Transgene construction and SCA8 transgenic mouse generation

Full length SCA8 cDNAs carrying 23 and 157 combined repeats were subcloned into the *Not*I site of pCEV expression vector in which *SCA8* expression is under the control of the cerebellar Purkinje cells specific pcp2/L7 promoter. The transgene constructs were purified as described above and verified. The transgene fragments containing pcp2/L7 promoter, SCA8 cDNA, and SV40 polyadenylation signal were excised with *Sph*I/*Bam*HI digestion and injected into FVB mouse pronuclei to generate transgenic mice (the service of transgenic core facility, Institute of Molecular Biology, Academia Sinica).

Transgenic mouse genotyping

The genomic DNAs from transgenic founders and offspring were isolated from tail biopsies and PCR genotyped using transgene-specific primers TG-F (5'-TATGGTGAGAGCAGAGATGG) and TG-R (5'-CATGTCAGGCTC-TGGGCGAAAAG). Briefly, 0.5-1.0 cm of tail biopsies were minced in 200 μl of tail solution (50 mM EDTA – 1% SDS – 50 mM Tris-HCl pH 8.0 – 100 mM NaCl – 0.35 mg/ml protease K) and incubated at 65˚C for 4 hours or at 37˚C overnight. The mixture then was mixed with 80 μl of 5 M potassium acetate and sat at 4˚C for 1 hour followed by centrifugation at 14,000 rpm at 4˚C for 30 minutes. The supernatant was transferred to the fresh tube and mixed well with 500 μl of pure ethanol. DNA was pelleted by

centrifugation at 14,000 rpm for 5 minutes and then washed with 70% ethanol. DNA pellet was air-dried, dissolved in 50 μl of ddH_2O. 0.5 μl of DNA was used as the template for PCR, and the amplified fragment spans form the pcp2/L7 promoter to the exon D of the human *SCA8*. The GAPDH fragment was PCR amplified with primers GAPDH-F (5'-CCCTTCATTGACCTCAACTA) and GAPDH-R (5'-CCAAAGTTGTCATGGATGAC), as the internal control.

Characterization of transgene copy numbers of transgenic mouse lines

The transgene copy numbers were determined using PCR. Briefly, copy standards were prepared by mixing non-transgenic tail DNA with a known amount of 1, 10, 50, and 100 copies of transgene DNA. The intensity of PCR amplified fragment with TG-F and TG-R primers was compared to copy standards to estimate the copy numbers.

RNA isolation from mouse brain tissues and reverse transcription-polymerase chain reaction (RT-PCR)

The brain tissues were dissected and 1 ml of Trizol reagent (Invitrogen) was added to isolate total RNA form half of the cerebellum. The procedure of isolation of RNA was as described above. After RNase-free DNase (Stratagene) treatment, 1 μg of RNA was reverse transcribed into cDNA using the SuperScript^{TM} III reverse transcriptase (Invitrogen). The RT-F (5'-CTTTTCGCCCAGAGCCTGACATG) and RT-R (5'-CTAACATGAAGGACCCAGGG) primers were used to amplify cDNA fragments to assess the expression of transgene transcripts.

Rotarod test

The rotarod is used to measure the ability of equilibrium and motor improvement with training. Mice are placed on the 38 cm-diameter rotarod (IITC Life Science) for three trials per day for four consecutive days with the rotarod undergoing a linear acceleration form 2 to 20 rpm over the first 5 min and maintaining at a maximum speed for another 5 min. The mice were allowed to rest for at least 60 min between trials to avoid fatigue and exhausting. Mice were scored for their latency to fall for each trial and averages of the three trials of the last day.

Immunohistochemical analysis

Avertin-anesthetized mice were perfused with 0.9% saline followed with 4% paraformaldehyde fixation. The brain tissue was dissected and incubated with 4% paraformaldehyde overnight. Then the tissue was transferred in order from 10% sucrose solution for 1 hour, 20% sucrose solution for 2 hour, to 30% sucrose solution fro 2 days. The brain was cryosectioned and sections were rinsed in 0.1 M phosphate saline buffer three times (10 min/wash). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 30 min. Sections were then washed in phosphate saline buffer three times (10 min/wash). Nonspecific epitopes were then blocked by incubation in 5% normal goat serum and 0.1% Triton X-100 in phosphate saline buffer for 2 h. Sections were incubated in primary antibodies overnight at room temperature and then washed three times in phosphate saline buffer for 10 min/wash. Secondary antibodies were applied to the sections by a linking reagent (DAKO) for 1h. Immunostainings were highlighted using substrate-chromogen solution and DAB oxidation. All sections were mounted on coated slides and cover-slipped for light microscopy.

RNA cleanup

Isolated RNA was cleaned up using RNeasy Mini Kit (Cat. No.74104, Qiagen). As described in the manufacture, the volume of RNA sample was first adjusted to 100 μl with DEPC-H_2O, mixed with 350 μl Buffer RLT containing 1% β-mercaptoethanol, followed with 250 μl of ethanol. The mixture was then applied to the RNeasy mini column and centrifuged for 15 seconds at ≥8,000g. The column was transferred into a new collection tube, added 500 μl Buffer RPE, and centrifuged for 15 seconds at ≥8,000g. Another 500 μl Buffer RPE was added to the column and centrifuged for 2 minutes at ≥8,000g. And another centrifugation for 1 minute was carried out to eliminate residual ethanol. Finally, 20 μl of DEPC-H_2O was added the column which has been transferred to a new tube, stayed at room temperature for 3 minutes, and centrifuged at ≥8,000 g for 1 minute. To obtain a more yield, another 15 μl of DEPC-H_2O can be added to perform the second elution. The quality and quantity of cleaned-up RNA samples were determined by the agarose electrophoresis and the absorbance at 260 nm, respectively.

Microarray analysis

We are indebted to the conduction of microarray assay of Genomic Medicine Research Core Laboratory in Chang Gung Memorial Hospital. The cleaned-up RNA was first reverse transcribed to the first-strand cDNA followed by the second-strand cDNA synthesis. Then the double-stranded cDNA was purified and serves as a template in the subsequent *in vitro* transcription reaction coupled with biotin labeling. The biotinylated cRNA targets were then purified, fragmented, and hybridized to MOE430A

(Affymetrix). The array was incubated for 16 hours at 45˚C, then automatically washed and stained. The array image was scanned and the acquired signal intensities were normalized. One-way ANOVA and fold-change analysis were performed to determine which genes were differentially expressed between transgenic and control littermates. The cut-off values of up-regulated and down-regulated transcripts were set as 1.5 and 0.7 fold changes, respectively.

Real-time RT-PCR (Quantitative RT- PCR)

6.25 μg of total RNA, isolated from either SCA8 transgenic or wild-type littermates as mentioned above, was treated with RNase-free DNase (Stratagene) in 25 μl of reaction volume and then 1 μg of DNase-treated RNA was used as a template to synthesize the first-strand cDNA using High Capacity cDNA Reverse Transcription Kit (Cat. No.4368814, Applied Biosystems) with random primers. Specific primers for each studied gene and for mouse endogenous control, Gapdh, were listed in **Table 7** and obtained from Applied Biosystems. Real-time quantitative PCR experiments were performed in the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Amplification was performed on a cDNA amount equivalent to 50 ng total RNA with 1x TaqMan® universal PCR Master mix (Cat. No.4304437, Applied Biosystems) and 1x specific Applied Biosystems Assay-on-Demand (AOD). The PCR condition was: initiation step at 50˚C for 2 minutes, denaturation at 95˚C for 10 minutes, then 40 cycles of denaturing at 95˚C for 15 seconds and combined annealing and extension at 60˚C for 1 minute. Experimental samples and no-template controls were all run in duplicate. Results from duplicate reactions were averaged and used as the value for each biological replicate.

Protein samples preparation for proteomic analysis

Half of dissected cerebella from transgenic mice and wild type littermates were homogenized in 200 μl of Lysis solution (8 M urea – 4% CHAPS) and sat on ice for 20 minutes. Protein extracts were centrifuged at maximum speed at 4°C for 30 min and the supernatant was transferred to a fresh tube. To separate proteins in the crude extracts from contaminating substances, the protein extracts were applied to the 2-D Clean-Up Kit (Cat. No.80-6484-51, Amersham Biosciences). According to the instruction, the supernatant was mixed well with 3 volumes of precipitant and incubated on ice for 15 minutes. Then the mixture of protein and precipitant was mixed with co-precipitant followed by the centrifugation at 4˚C for 10 minutes. The supernatant was removed as much as possible and 40 μl co-precipitant was layered on the pellet. After sitting on ice for 5 minutes, the mixture was centrifuged for 5 minutes and the supernatant was discarded. The pellet was piled with 25 μl of ddH_2O and dispersed by vortexing. Then 1 ml of chilled wash buffer and 5 μl of wash additive was added to the protein pellet. The mixture was incubated at -20˚C for at least 30 minutes to 1 week with occasional vortex for 20-30 seconds. Protein was pelleted by centrifugation at at maximum speed for 5 minutes. The supernatant was discarded and the pellet was allowed to air dry for no more than 5 minutes. Finally, the protein pellet was resuspended in 200 μl of Lysis solution with sonication. The quantity of cleaned-up protein sample was determined using 2-D Quant Kit (Cat. No.80-6483-56, Amersham Biosciences)

Minimal labeling with CyDye Fluors for 2-D difference gel electrophoresis

(DIGE)

For 2-D DIGE, protein samples were labeled with Cy™2, Cy3 and Cy5 fluors (Cat. No.25-8010-65, Amersham Biosciences) after protein quantization. 50 μg of protein samples were mixed with 400 pmol CyDye and incubated on ice for 30 minutes in the dark. Then the labeling reaction was quenched by adding 1μl of 10 mM lysine and leaving on ice for 10 minutes in the dark.

IPG strip rehydration and first-dimension isoelectric focusing (IEF)

For the first dimension, the protein sample was mixed with rehydation buffer(8 M Urea – 2% CHAPS – 40 mM DTT – 0.5% IPG buffer –0.002% bromophenol blue) to make up for the volume 250 μl (for 13-cm strips). Then the sample mixture was loaded in the strip holders evenly and a pH 3-10, 13-cm Immobiline™ DryStrip (Amersham Biosciences) was faced down to absorb the protein. Strips were rehydrated at 20˚C for 12 hours on IPGphor II (Amersham Biosciences). Then protein samples were separated according to each isoelectric point (pI) with the running program: Step 500 V for 4 hours, Grad 1000 V for 1 hour, Grad 8000V for 3 hours, and Step 8000V for 38000 Vhours.

Second-dimension SDS-PAGE

After IEF steps, strips were equilibrated in SDS Equilibration buffer (2% SDS – 50 mM Tris-HCl pH 8.8 – 6 M Urea – 30% Glycerol –0.002% bromophenol blue) containing 1% (wt/vol) dithiothreitol (DTT) for 15 minutes with shaking followed by the second equilibration in SDS Equilibration buffer containing 2.5% (wt/vol) iodoacetamide for 15 minutes

with shaking. The strip then was transferred to the top of an 18 × 16 cm, 1.5 cm thick, 12.5% polyacrylamide gel and sealed with melting agarose (0.5% dissolved in 1x SDS running buffer). Gels were run at 30 mA for the first 15 minutes followed by 60 mA for 4.5~5 hours until the bromophenol blue dye reached the bottom.

Gel staining with SYPRO Ruby

Gels were rinsed with ddH_2O and then fixed in 40% methanol and 10% acetic acid overnignt. Then they were washed with ddH_2O for 10 minutes 3 times, followed by staining with SYPRO Ruby protein gel stain (Cat. No.S12000, Molecular Probe) overnight. Before visualizing the gel image, the gel was washed with 10% methanol and 7% acetic acid for 30 minutes and rinsed with ddH_2O for 5 minutes twice. The gels were imaged on the Molecular Dynamics Typhoon 9210 (Amersham Biosciences) using optimal excitation/emission wavelength.

Image analysis

ImageMaster™ 2D Platinum Software (Amersham Biosciences) was used for analyzing the DIGE images. Briefly, the DIGE images underwent spot detection and quantitation with an average of 2200 spots per gel detected. Next, the matched images were analyzed and based on the Student's *t*-test feature, only those statistically significant spots ($p < 0.05$) were accepted. The volume ratio of matched spots with greater than 1.3 was considered significantly differentially expressed.

In-gel digestion

Differentially expressed protein spots were picked with Ettan™ Spot Picker (GE Healthcare) from the SYPRO Ruby stained gel which had been matched to DIGE gels. The picked gel spots were rinsed with 100 μl of ddH_2O, and then washed with 100 μl of 50 mM ammonium bicarbonate (NH_4HCO_3)/acetonitrile (ACN) (1:1, v/v) for 15 minutes. Next, the solvent was removed and the gel particle was covered with 50 μl of ACN with vortexing for 1 minute. ACN was then removed, and replaced with 50μl of 50 mM NH_4HCO_3 for 5 minutes. ACN was added and the mixture was incubated for 15 minutes. The solvent was removed and replaced with 50 μl of ACN with vortexing for 1 minute, and then ACN was removed. The gel piece was vacuum dried for 30 minutes. The gel piece was incubated with 8 μl of freshly diluted trypsin (2.5 ng/μl in NH_4HCO_3) at 37˚C overnight. Next day, the gel piece covered with at least 3 μl of solvent was sonified for 10 minutes and the supernatant was recovered. The gel was then covered with 3 μl of 50% ACN with 1% trifluoracetic acid and sonified for another 10 minutes. The supernatant was recovered and pooled together.

Mass Spectrometry

Trypsinized protein fragments were identified using the MALDI-TOF MS (conduction of Genomic Medicine Research Core Laboratory in Chang Gung Memorial Hospital).The MALDI spectra used for protein identification from trypsinized fragments were searched against the National Center for Biotechnology Information (NCBI) protein databases using the MASCOT search engine (www.matrixscience.com).