Supporting Information

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SI Methods

Dogs. A total of 138 affected ASTs were recruited. Of these, 116 dogs were diagnosed as affected by a European or American board-certified veterinary neurologist. For each dog, a clinical history was collected and complete clinical and neurologic evaluations were performed. Routine hematology and serum biochemistry parameters were assessed. Cerebrospinal fluid (CSF) was obtained for analysis by cerebellomedullary cisternal puncture under anesthesia in 58 of the 138 dogs. MRI of the brain was performed as described previously (1). Images were acquired using a 1.5-T MRI unit (Siemens Medical Solutions). Affected status was confirmed in 23 of 116 dogs by histopathological analysis. Twenty-two of the 138 affected ASTs were diagnosed by their regular private-practice veterinarian following the same procedure; follow-up MRI examinations and CSF analyses could not be obtained for these dogs. Fourteen of these 22 affected dogs were confirmed by one of the authors (J.-L.T., N.J.O., or S. Blot), and 10 of these dogs were directly related to a confirmed affected dog.

Histology and Electron Microscopy. Organ samples were placed in 10% buffered formalin for 7 d and embedded in paraffin. Then 5-μm-thick representative sections from spleen parenchyma, lymph node, retina, olfactory bulb, frontal lobe, caudate nucleus, thalamus, occipital lobe, pons, cerebellum and medulla, were processed and stained with H&E, Luxol fast blue, Sudan black B, and PAS reagent.

Selected areas of the cerebellar cortex from a 4-y-old affected female were cut into small cubes, fixed in 2.5% glutaraldehyde for 6 h, washed in cacodylate buffer, postfixed in 2% osmium tetroxide for 2 h, dehydrated in graded alcohols, and routinely embedded in Epon. Ultrathin sections were mounted on bare copper grids, stained with uranyl acetate and lead citrate, and examined with an FEI/Philips EM 208S transmission electron microscope.

MSS2 and SNP Marker Genotyping. DNA was extracted from 3–5 mL of EDTA-stabilized blood samples obtained by venous puncture. A total of 247 canine autosomal microsatellite markers from the Minimal Screening Set 2 (MSS2) (2) were individually amplified for each dog from 30 ng of genomic DNA using a classical PCR protocol with AmpliTaqGOLD DNA polymerase (Applied Biosystems). Here, 1 μL of each fluorescent PCR product from each chromosome-specific panel was loaded onto a 3130 XL genetic analyzer (Applied Biosystems) and resolved with an internal size standard (GeneScan 500 LIZ; Applied Biosystems). Results were analyzed using GeneMapper version 3.7 (Applied Biosystems).

PCR primers for the *ABCA5*-SNP, rs9076656, rs24540437, rs8736681, rs24582426, rs24537663, rs24564834, and CA4-SNP are given in Table S2. Sequencing primers for these SNPs were 5′-GTTTTCAAATCTTACCTTCT-3′, 5′-GAGAGGAGTCCCTGAA-3′, 5′-TTCTAGGAGTTTTACTGTCA-3′, 5′-GATTCAAATACAGATGTTGG-3′, 5′-CATGGGCCCAATAAA-3′, 5′-TCTGAAGGGACATCTTG-3′, 5′-GGAGTATGGCTGGTTGA-3′, and 5′-GGAAGTGGTTCTTTGC-3′, respectively. PCR primers for the *ARSG*-SNP were 5′-biotinyl-CTCCTGGCCTGGCTTTCTGT-3′ and 5′-ATCCCCGTGACGTAGCCG-3′, and the sequencing primer was 5′-TTGTGCGTGACTCCG-3′.

RT-PCR. Poly(A)$^+$ RNA was extracted using the Ambion Poly(A) Purist MAG kit (Applied Biosystems). Here, 100 ng of Poly(A)$^+$ mRNA was reverse-transcribed using the SuperScript III RT kit (Invitrogen). The cDNA was then amplified (30 cycles) using Q-Bio Taq DNA polymerase (MP Biomedicals). PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Expression of the p.R99H Protein. The p.R99H mutation was introduced into pCMV6-*ARSG-Myc-FLAG* using the forward oligonucleotide 5′-GGCCGGCTTGGCCTTCACAATGGAGTCACAC-3′ and the reverse oligonucleotide 5′-GTGTGACTCCATTGTGAAGGCCAAGCCGGCC-3′ to construct the expression vector pCMV6-*R99H-ARSG-Myc-FALG*. Mutagenesis was assessed by sequencing using forward VP1.5 and reverse XL39 DNA vector sequencing primers supplied by OriGene.

Transfection of the expression vectors into HEK293T cells was performed using 800 μL of 250 mM CaCl2, 800 μL of HBS buffer [20 mM Hepes, 150 mM NaCl (pH 7.4)], and 16 μg of DNA for 4.10^6 cells. Transfected cells were seeded in DMEM supplemented with 10% FCS (PAA Laboratories) and cultured for 72 h in a 5% CO_2 atmosphere at 37 °C. Cultured HEK293T cells were trypsined, washed once with medium and twice with 0.9% NaCl, and harvested by centrifugation.

For ARSG Western blot analysis, a mouse monoclonal antibody directed against the Myc epitope was used as a primary antibody (anti-Myc4A6; Upstate Biotechnology). Signals were detected using a HRP-conjugated goat anti-mouse IgG secondary antibody (GE Healthcare) and ECL+ detection reagent (GE Healthcare). Calnexin detection was performed using a rabbit polyclonal antibody (Sigma-Aldrich) detected using a HRP-conjugated goat anti-rabbit IgG secondary antibody (GE Healthcare).

Enzymatic Assays. Transfected HEK293T cell and leukocyte pellets from affected (*A*/*A*) and healthy control (*G*/*G*) French ASTs were homogeneized in water and then frozen and thawed three times. The protein concentration in the cellular extract was determined using a Pierce BCA Protein Assay kit. Then 25 μL of cell lysate, adjusted to 2 mg/mL of protein, was incubated at 37 °C for 1 h or 2 h with 1–10 mM pNCS (Sigma-Aldrich, cat. no. N7251) in 0.5 M sodium acetate buffer (pH 4.6) containing 10% NaCl and 12.5–187.5 mM 3-(α-acetonylbenzyl)-4-hydroxycoumarin (Warfarin; Sigma-Aldrich, cat. no. A4571). The reaction was quenched by 50 μL of 50% trichloroacetic acid, and 200 μL of the clarified lysate was alkalinized by 800 μL of 1 M NaOH. Arylsulfatase activity was deduced from the absorption at 513 nm of pNCS reaction product color on a PowerWave KC4 spectrophotometer (BioTek).

Accession Codes. GenBank accession codes (www.ncbi.nlm.nih.gov/Genbank/): human (*Homo sapiens*) *ARSG* cDNA, NM_014960; dog (*Canis familiaris*) ARSG, NP_001041563; human (*Homo sapiens*) ARSG, NP_055775; mouse (*Mus musculus*) ARSG, NP_082986; chicken (*Gallus gallus*) ARSG, XP_425382; zebrafish (*Danio rerio*) GALNS (N-acetylgalactosamine-6-sulfatase), AAI-54124; worm (*Caenorhabditis elegans*) Sul-2 (Sulfatase domain protein family member), NP_505102; worm (*Ciona intestinalis*) ARS (predicted arylsulfatase), XP_002123283; human ARSA, NP_000478; human ARSB, NP_000037; human ARSC-STS, steroid sulfatase, NP_000342; human ARSD, NP_001660; human ARSE, NP_000038; human ARSF, NP_004033. Dog *ARSG* exon 2 containing the *ARSG*-SNP, FM246885. Dog *ABCA5* exon 14 and exon 17 containing one SNP each, FM211419 and FM211812, respectively. Ensembl accession code (www.ensembl.org): Fugu (*Takifugu rubripes*) ARSG, ENSTRUP00000042670.

1. Olby N, et al. (2004) Cerebellar cortical degeneration in adult American Staffordshire Terriers. *J Vet Intern Med* 18:201–208.
2. Clark LA, et al. (2004) Chromosome-specific microsatellite multiplex sets for linkage studies in the domestic dog. *Genomics* 84:550–554.
3. Frese MA, Schulz S, Dierks T (2008) Arylsulfatase G, a novel lysosomal sulfatase. *J Biol Chem* 283:11388–11395.
4. Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol* 16:1099–1106.

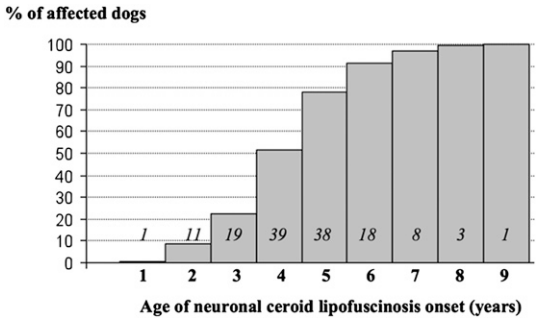

Fig. S1. Distribution of age at disease onset in the 138 French and US affected ASTs. The histogram represents cumulative percentages of ASTs diagnosed as affected dogs. These percentages were calculated using data collected from the 138 affected dogs. Dogs from both sexes were affected almost equally (males: $n = 74$; females: $n = 64$; $\chi^2 = 0.72$), showing identical penetrance. Among the 138 affected dogs, 116 were directly evaluated by board-certified neurologists from the Alfort School of Veterinary Medicine or the College of Veterinary Medicine, North Carolina State University. Of the 22 dogs that had been initially diagnosed by their regular veterinarian, 14 had their diagnosis confirmed by one of the authors (J.-L.T., N.J.O., or S. Blot). These dogs were mainly from France and the United States, with 3 dogs from Belgium and 1 dog from Germany. In most instances, the age of onset was inferred from the owner's report during the clinical evaluation and related to the time when the first sign of locomotor ataxia was noticed. The number of dogs identified with locomotor ataxia is indicated in italic font for each age. Expressivity of the disease was very variable. Some dogs had to be euthanized by 3 m after the diagnosis of NCL because they became totally unable to walk without falling repeatedly, whereas others survived for several years after the initial diagnosis.

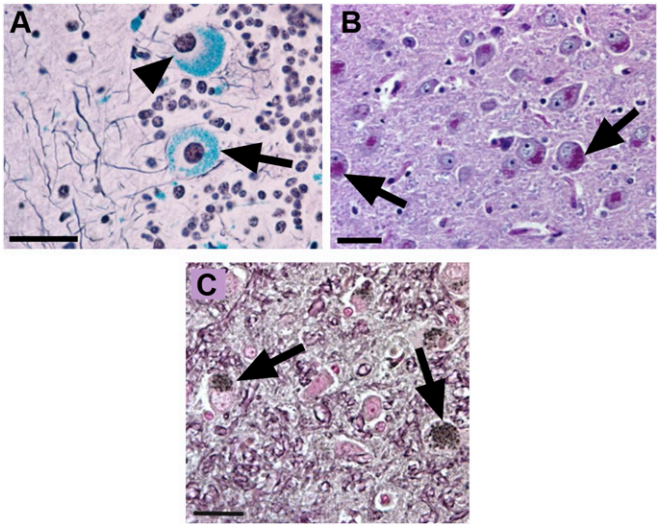

Fig. S2. Accumulation of cytoplasmic storage material in cerebellar Purkinje neurons and thalamic neurons in affected ASTs. Shown are transverse sections through the cerebellum (*A*) and thalamus (*B* and *C*) in 4- and 8-y-old affected ASTs, stained with Luxol fast blue (*A*), PAS reagent (*B*), and Sudan black B (*C*). In brain sections, Luxol fast blue stains myelin, myelinated axons, and lipoproteins; PAS stains carbohydrates (e.g., cerebrosides, glycogen); and Sudan black stains lipoproteins. (*A*) The Luxol fast blue–positive storage material is composed of multiple cytoplasmic granules (arrow), which induce displacement of the Nissl substance and nucleus (arrowhead) to the periphery of the neuron. (*B*) In thalamic neurons, PAS-positive storage material (arrows) induces displacement of the nucleus to the periphery of cells. (*C*) In these thalamic neurons, Sudan black–positive storage material (arrow) composed of multiple cytoplasmic granules within the cytoplasm alters neuron morphology. (Scale bar: 50 μm.)

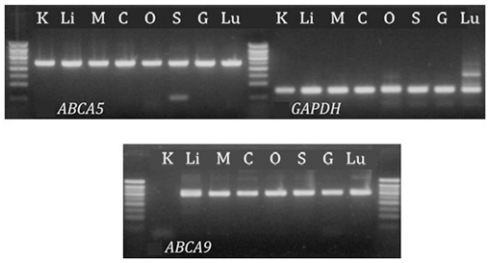

Fig. S3. Expression profile in canine tissues of two candidate genes. Expression of *ABCA5* and *ABCA9* was determined by semiquantitative RT-PCR. *ABCA5* was ubiquitously expressed and highly representative of the other candidate genes evaluated (Table S3). *GAPDH* was used as a qualitative and semiquantitative control of mRNA templates. *ABCA9* was expressed in all of the tissues tested except the kidney. K, kidney, Li, liver, M, skeletal muscle, C, cerebellar cortex, O, ovary, S, skin, G, gut, Lu, lung.

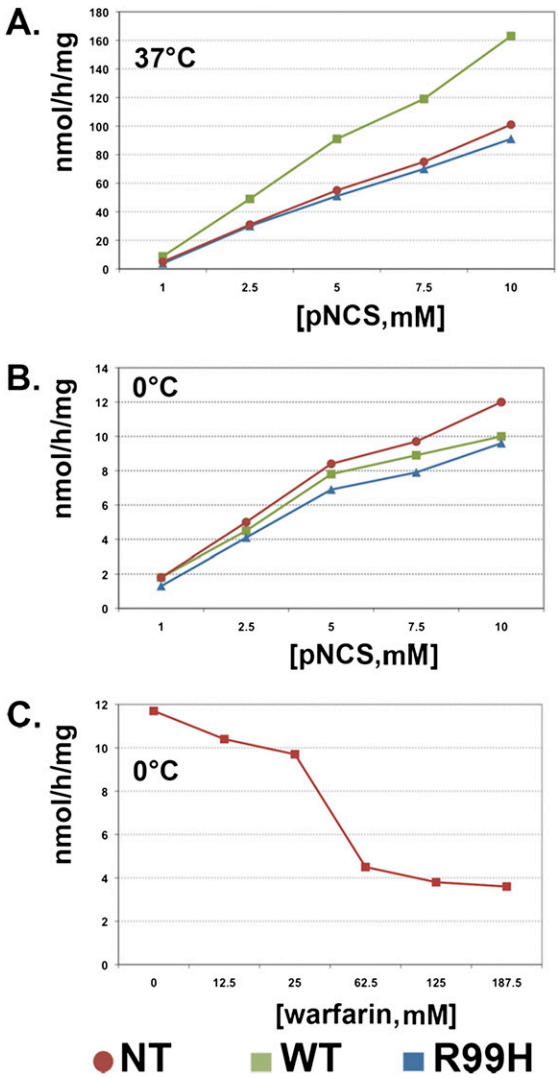

Fig. S4. Determination of the best conditions for assay of ARSG activity. HEK293T cells were not transfected (NT) and transfected with an expression vector allowing the overexpression of the WT human ARSG (WT) or a human mutated ARSG corresponding to the canine NCL mutation (p.R99H). All assays were carried out with cell extracts at pH 4.6, and 10% NaCl allowing the specific inhibition of ARSC-STS, ARSE, ARSF, and ARSB. (*A*) At 37 °C, increasing the concentration in pNCS substrate resulted in an increase of arylsulfatase activity (ARSA + ARSG). Overexpression of WT increased this global activity, whereas overexpression of p.R99H did not. (*B*) At 0 °C, arylsulfatase activity was not enhanced by WT proteins, showing that it is supported only by ARSA at this temperature. (*C*) At 0 °C, ARSA activity was inhibited by an increasing concentration of warfarin, whereas ARSG activity was not (3). Thus, ARSG activity can be specifically evaluated at 37 °C, pH 4.6, 10% NaCl, and 187.5 mM warfarin, using 10 mM pNCS.

Table S1. Segregation analysis of NCL in French ASTs

	Affected pups*		Healthy pups*	
Litters with at least one affected pup*	Observed	Expected	Observed	Expected
Two healthy parents	7	4.5	11	13.5
χ^2 test	1.85; no statistical difference			
One affected parent	12	13	14	13
χ^2 test	0.15; no statistical difference			
Litters with no affected pups*				
One affected parent	27/27 healthy pups in a total of four litters			

*Pups who became affected adults.

Data were collected from 73 related dogs. The first type of analyzed litters with at least one affected dog resulted from the mating of two healthy parents, with genotypes thus inferred to be "healthy carriers." No statistically significant difference was seen between the observed numbers of affected (n = 7) and healthy (n = 11) dogs and the expected numbers of each class (25% = 4.5; 75% = 13.5 ; χ^2 = 1.85). The second type of analyzed litters with at least one affected dog resulted from the mating of one affected parent and one healthy parent, for which the genotype was also inferred to be a healthy carrier. No statistically significant difference was seen between the observed numbers of affected (n = 12) and healthy (n = 14) dogs and the expected numbers of each class (50% = 13 ; χ^2 = 0.15). The final type of analyzed litters with no affected dog resulted from the mating of one affected parent and one healthy parent, for which the genotype was inferred to be a "healthy noncarrier." Twenty-seven pups from four different litters were observed, making a autosomal dominant inheritance pattern highly unlikely. Thus, we assume that the autosomal recessive mode of inheritance of NCL in French pedigrees is accurate, confirming previous conclusions obtained from US lines (1).

Table S2. Additional markers from CFA09

Marker name	Uni STS ID	Position (Mb)	Primers (forward and reverse)	PCR product size, bp	PIC in AST control dogs
RENacp1	860551	14.63	5′-6-FAM-ACCCCCTGTGCACCTCATCACTTA-3′ 5′-AGGTCACTGTCTGTACACGTAGTG-3′	86	0.746
RENg4935	860550	16.12	5′-6-FAM-GGGATCCCTCTTTCTTTTTCTTTT-3′ 5′-CTGGAACTCTTTCAAGAAGGAAGAC-3′	242	0.324
RENrgs9.3	860549	17.94	5′-6-FAM-AGTTAGACTGCCTTCTGATGAAGTG-3′ 5′-6-FAM-TGTCTATCGATTCTTCCCAACTAAC-3′	151	0.541
RENrgs9.2	860548	17.96	5′-6-FAM-GCACGACTCCAGGAATATAGTAGAA-3′ 5′-TGGGACTTAAACGCTAAATTGTATG-3′	171	0.476
rs9076656	—	17.99	5′-biotinyl-GATCTCATCCCTCTTCTGCTTACA-3′ 5′-ATAGTAGGGAGCAGTGGAGAGGA-3′	59	0.171
REN198p23	264454	18.09	5′-6-FAM-TTGTACATTATCTGTTCTACCTCGG-3′ 5′-TCTTCAGCAGGCCTTTTCTC-3′	132	0.248
FH3596	263877	18.11	5′-6-FAM-ACATCAGGTGAAGAGCTTGC-3′ 5′-GAAGTTGGCTGGGGAAGG-3′	285	0.587
rs24540437	—	18.13	5′-TGCGTGAATTTGTAGGTTTTGT-3′ 5′-biotinyl-AAGGGCTACTGTGAAGCATTTT-3′	123	0.331
DTR9Alf1	860545	18.18	5′-6-FAM-TTCCAGGGGCACTTTCTACTT-3′ 5′-TCTCCCTCTGCCTATGTCTCA-3′	205	0.765
rs8736681	—	18.18	5′-ACGGGTTAGCTATTATCACAACTG-3′ 5′-biotinyl-GTTCCAGAGACAAATCCCTGTTA-3′	102	0.375
DDR9Alf2	860546	18.23	5′-6-FAM-GGGCGTTGAACAGATCAAATA-3′ 5′-TCATCTCCACACCAGGAGACT-3′	235	0.754
rs24582426	—	18.23	5′-biotinyl-AGGCCACAGGTAGGCATGTC-3′ 5′-GCGGTGGCAGAGAAGTTAGC-3′	278	0.372
DDR9Alf3	860547	18.24	5′-6-FAM-GGGCTCTGAGTCTGGTCTTTT-3′ 5′-ACGTATGTGCGTATCCCGTAT-3′	173	0.556
rs24537663	—	18.25	5′-CCTCTATGCCCAACACTTCG-3′ 5′-biotinyl-GTACAGAGAACACCCGGATGA-3′	162	0.370
rs24564834	—	18.40	5′-GCAAGGAGTATGGCTGGTTGA-3′ 5′-biotinyl-GCTCGGTTAATGGTCTTAGAAGC-3′	71	0.375
ABCA5-SNP	GenBank accession # FM211813	18.84	5′-biotinyl-TTCCATCCCTTTCACAGTCTTT-3′ 5′-ACGATGGTTTTCAAATCTTACCT-3′	60	0.353
REN144l19	264317	24.83	5′-6-FAM-TGTCATCCTGCATCCAATGT-3′ 5′-CAATTTACTTTTGGGCGTCA-3′	216	0.544
REN206j15	264466	27.45	5′-6-FAM-CCCCCAACAATCAAATGTTTA-3′ 5′-AATGCAGCTATATGGGCCAC-3′	227	0.452
FH4059	264000	30.54	5′-6-FAM-GGATCTGTGTTTCTTCGTTAGC-3′ 5′-TTGATTAAAGAGCAGCTTAGCC-3′	396	0.726
CA4-SNP	GenBank accession # FM253749	39.65	5′-CTCTTCTTTCGGGTGGACCT-3′ 5′-biotynyl-CAGCAGACAGTAGGGAAACTGAT-3′	79	0.374

Forward primers used to amplify microsatellites were 5′-end-labeled with 6-carboxyfluorescein (6-FAM). Positions are indicated from the centromere. The initial 61 °C annealing temperature was decreased by 1 °C at every cycle until a touchdown annealing temperature of 51 °C was reached. Nine of these markers were identified in our laboratory (in bold type). Among the 20 markers, 14 confirmed that the *NCL* locus mapped onto canine chromosome 9 (Fig. 2*A*) and 6 SNPs from the Illumina array were used to fine-map the locus (Fig. 2*B*). Polymorphic information content (PIC) was calculated using the CERVUS 3.0.3 software (4).

Table S3. Candidate genes and transcriptional units located in the 678-kb shared haplotype

Position in Mb	Gene	Name	Function	Expression in human and mouse (from databases)	Expression in dog (from RT-PCR)	Polymorphisms detected in our ASTs
18.18	*ARSG*	Arylsulfatase G	Sulfatase activity. Lysosomal enzyme	Ubiquitous	Ubiquitous	c.296G > A (exon 2) nonsynonymous, (ARSG-SNP) associated with NCL.
18.24	*WIPI1*	WD repeat domain, phosphoinositide interacting 1	WD40 repeat protein of 49 kDa interacting with phosphoinositides	Ubiquitous high expression in heart	Ubiquitous	—
18.31	*PRKAR1A*	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue- specific extinguisher 1)	Protein kinase. Tumor-suppressor gene. When mutated in humans, *PRKAR1A* is responsible for the Carney complex	Ubiquitous	Ubiquitous	—
18.33	*FAM20A*	Family with sequence similarity 20, member A	Murine *FAM20A* is a secreted protein expressed in hematopoietic cells	Ubiquitous	Ubiquitous	—
18.43	*LOC610988*	Similar to 60S ribosomal L23a	Hypothetical ribosomal protein	Unknown	Not tested	—
18.44	*LOC610995*	None	Unknown	Unknown	Not tested	—
18.55	*ABCA8*	ATP-binding cassette, subfamily A (ABC1), member 8	ABC transporter	Ubiquitous, high expression in olfactory bulb (human) and liver (mouse)	Ubiquitous except kidney	—
18.57	*U6*	U6 spliceosomal RNA	Hypothetical spliceosomal RNA	Unknown	Not tested	—
18.64	*ABCA9*	ATP-binding cassette, subfamily A (ABC1), member 9	ABC transporter	Ubiquitous	Ubiquitous except kidney	—
18.72	*ABCA6*	ATP-binding cassette, subfamily A (ABC1), member 6	ABC transporter	Ubiquitous	Ubiquitous	—
18.85	*ABCA5*	ATP-binding cassette, subfamily A (ABC1), member 5	ABC transporter	Ubiquitous	Ubiquitous	1- c.1905A>T (exon 14) nonsynonymous, not associated with NCL. 2- c.2331C>A (exon 17) nonsynonymous not associated with NCL. 3- c.4764T>C (exon 36) synonymous (*ABCA5*-SNP), associated with NCL.
18.97	*MAP2K6*	Mitogen-activated protein kinase kinase 6	Protein kinase. Activates p38 MAP kinase	Ubiquitous	Ubiquitous	No SNP

The positions are given from the centromere of the acrocentric CFA09 chromosome. Genes that are fully sequenced are indicated in bold type. The molecular function of these transcriptional units in biological processes and pathways was obtained from the Panther classification system (www.pantherdb.org/) and the GeneCards database (www.genecards.org/). All annotated genes are ubiquitously expressed in human and mouse tissues (data from the Gene Expression Atlas database and *Sym*Atlas from the Genomics Institute of the Novartis Research Foundation; http://biogps.gnf.org/?referer=symatlas#goto=welcome). Using a panel of ~10 organs collected from a 4-y-old female Labrador Retriever (brain, skeletal muscle, heart, gut, liver, lung, esophagus, kidney, pancreas, skin, and ovary), we confirmed that the nine annotated genes are also ubiquitously expressed in canine tissues, with the exceptions of *ABCA8* and *ABCA9* that could not be detected in kidneys. Semiquantitative expression levels also were assessed in a panel of tissues from a 7-y-old affected AST, and no differences were seen. In addition to the previously described synonymous SNP in exon 36 of the *ABCA5* (*ABCA5*-SNP) highly associated with the *NCL* locus, two nonsynonymous SNPs were detected in exons 14 and 17 and were found not to be associated with the disease. No sequence difference was found for *MAP2K6*.

Table S4. Dogs, sorted by breed, that are *G/G* at the c.296G>A *ARSG*-SNP locus

Breed	Number of dogs
Staffordshire Bull Terrier	22
American Bulldog	18
Bull Terrier	139
Toy Bull Terrier	33
Bull Mastiff	23
Mastiff	31
English Bulldog	82
Dogo Argentino	18
German Boxer	159
American Cocker Spaniel	2
Australian Cattle Dog	3
Barzoi	5
Beagle	5
Beauceron	2
Belgian Shepherd Dog	6
Bermese Mountain Dog	3
Bichon	1
Bordeaux Dogge	1
Brittany Spaniel	2
Chow-Chow	2
Collie	1
Coton de Tulear	2
Dalmatian	5
Daschund	5
Drahthaar	1
English Cocker Spaniel	2
English Setter	2
Fox Terrier	4
French Bulldog	4
German Shepherd	7
German Short-Haired Pointer	2
Golden Retriever	7
Great Dane	2
Husky	1
Irish Setter	2
Jack Russell Terrier	4
Labrador Retriever	8
Leonberger	5
Lhassa Apso	1
Malamute	1
Newfoundland	1
Poodle	5
Pyrenean Mountain Dog	3
Rottweiler	5
Saluki	1
Samoyede	2
Schnauzer	1
Shar Pei	6
Shih Tzu	2
Tatra Shepherd Dog	1
Tibet dogge	1
Tibetan Terrier	2
Whippet	1
Yorkshire Terrier	3
Mongrel	3

Bull and terrier breeds suspected to be related to the AST breed are in bold type. The panel includes 525 dogs not affected by NCL from 9 AST-related breeds, 45 other breeds, and 3 French mongrels.

Movie S1. Static (astasia) and dynamic (abasia) ataxia in a 5-y-old affected AST. The first detectable symptoms include stiffening, nystagmus (abnormal vertical, horizontal or rotary movements of eyes) and a tendency to fall on elevation or shaking of the head. In later stages (Movie S1) affected dogs suffer from hypermetria, have difficulty in initiating movements, stumble when cornering, are prone to coarse intention tremor (for example before they intend to walk down a stair) and fall frequently. They always keep normal conscious proprioception, motor strength and spinal reflexes. Most affected dogs were euthanized within 1 to 4 y, once they became unable to walk without falling.

Movie S1