Sacs R272C missense homozygous mice develop an ataxia phenotype

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Abstract
Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS [MIM 270550]) is an early-onset neurodegenerative disorder caused by mutations in the SACS gene. Over 200 SACS mutations have been identified. Most mutations lead to a complete loss of a sacsin, a large 520 kD protein, although some missense mutations are associated with low levels of sacsin expression. We previously showed that Sacs knock-out mice demonstrate early-onset ataxic phenotype with neurofilament bundling in many neuronal populations. To determine if the preservation of some mutated sacsin protein resulted in the same cellular and behavioral alterations, we generated mice expressing an R272C missense mutation, a homozygote mutation found in some affected patients. Though SacsR272C mice express 21% of wild type brain sacsin and sacsin is found in many neurons, they display similar abnormalities to Sacs knock-out mice, including the development of an ataxic phenotype, reduced Purkinje cell firing rates, and somatodendritic neurofilament bundles in Purkinje cells and other neurons. Together our results support that Sacs missense mutation largely lead to loss of sacsin function.

Keywords: ARSACS, Purkinje cell, cerebellum, Sacsin, SACS, Ataxia, Mouse model

Introduction
Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS [MIM 270550]) was first described in the French Canadian population in 1978 [1]. Since then, ARSACS cases have been reported worldwide [2] (www.lovd.nl). The original French Canadian ARSACS clinical phenotype consists of a childhood onset progressive spastic ataxia accompanied by sensory-motor polyneuropathy and retinal thickening [3, 4]. French Canadian ARSACS patients become wheelchair-bound on average by the age of 41 and life expectancy is reduced to 61 years [5]. Pathological findings of post-mortem examination of two male ARSACS patient brains show atrophy of the anterior vermis associated with Purkinje cell death, while the cerebellar hemispheres are much less affected [6–8] ARSACS is the second most common form of recessive ataxia in the Netherlands and Northern UK [9].

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kinase-like ATPases) domain homologous to the nucleotide-binding domain (NBD) of the Hsp90 chaperone. The region combining the sr1 and sr2 corresponds to Anderson and colleagues’ SRR supradomain [11], which possesses ATPase activity. A missense pathogenic mutation, D168Y, within the sr1 completely abrogates the ability of this domain to hydrolyse ATP. The sr1 sequence in all three SIRPT domains are sites for a number of pathogenic homoyzgote missense mutations, or single mutations combined on the other allele with macrodeletion, frameshift and stop mutations: D168Y, or single mutations combined on the other allele with R1645Q and R2703C [9, 14].

The choice of the R272C missense mutation was based on reports of human homozygous cases in Canada and the actual presence of residual amount of mutated protein in patient lymphoblasts [2, 17]. Our hypothesis is that residual amount of mutated sacsin could retain some sacsin function and influence the severity of the phenotype. A novel knock-in (KI) mouse harbouring the c.816C>T (p.R272C) mutation in mouse Sacs exon 7 was generated by Ozgene using traditional gene targeting techniques (Additional file 1: Figure S1). This mutation lies at the end of the homologous HAT-Pase_C domain in the first sr1, as described by Romano et al. (Fig. 1a) [14]. The mutation was confirmed by Sanger sequencing of mouse tail genomic DNA (Fig. 1b), demonstrating the C to T mutation at position c.816 in heterozygous and homozygous animals. Sacs mRNA levels were quantified by qRT-PCR using RNA extracted from cortex and cerebellum (Fig. 1c). Sacs mRNA levels were comparable to controls in heterozygous and homozygous SacsR272C brain tissues (Fig. 1c). However, mutated sacsin protein levels were significantly reduced compared to controls. Homozygous animals had only a residual amount of 21% of mutated sacsin whereas heterozygous animals had 65% compared to normal sacsin protein levels (Fig. 1d,e). These results strongly suggest that R272C mutated sacsin is unstable and more rapidly degraded.

Ataxia, motor deficit and muscle weakness of SacsR272C mice

Similar to the Sacs−/− mice, homozygous SacsR272C mice are born in a Mendelian ratio, breed normally and have a normal lifespan, with many mice surviving more than 2 years. We quantified motor performance of the animals on a series of tests measuring balance, motor coordination and muscle strength. A significant difference is observed between SacsR272C and control animals on the balance beam as early as 45 days of age. This is mostly evident in males, but also observed in females at later time-points starting at 120 days of age, where the SacsR272C−/− males do show significant difference in performance, though they tend to perform better at the 180 day time point (Fig. 2 b-f). The differences in balance beam performance observed between males and females are most likely due to animal size and differences in muscle strength independently of genotype, which is solicited during this motor coordination test. Performance on the rotarod test, used to assess
more general motor coordination, demonstrates no significant difference between the groups (Fig. 2 i, h). When assessing muscle strength using the inverted grid test, we detect significant and progressive muscle weakness in both female and male SacsR272C mice compared to age-matched controls starting at 120 days of age (Fig. 2. k, l). Altogether, these results demonstrate that SacsR272C mice display an early balance deficit and muscle weakness comparable though possibly a little milder to the one observed in the Sacs−/− mice.

**Purkinje cell loss in SacsR272C mice**

Our previous studies of Sacs−/− mice demonstrated a progressive Purkinje cell loss in cerebellar anterior lobules with little loss of Purkinje cell in the posterior lobules, mimicking the differential regional neurodegeneration observed in ARSACS patients [22]. In order to quantify Purkinje cell loss progression in our new mouse models, we stained sagittal cerebellar sections from animals at different time points (45, 90, 180 and 365 days) using Nissl stain to visualize neuronal nuclei. As it was observed for Sacs−/− mice [22], Nissl-stained sections from 45 day-old SacsR272C demonstrate normal cerebellar structure and lobulation compared to age-matched controls (Fig. 3a,b). SacsR272C mice do, however, present significant Purkinje cell loss in the anterior lobules starting at 90 days of age (Fig. 3e). Cerebellar sections from one-year-old SacsR272C mice, immunolabeled with calbindin antibody demonstrate regions of neuronal loss in the Purkinje cell layer (Fig. 3D and d1 yellow bracket). We also observed a significant decrease in Purkinje cell numbers in the posterior lobules in one-year-old SacsR272C mice (Fig. 3f). This result might reflect a deleterious effect of a decreased sascin function in distinct posterior cerebellar PC.
SacsR272C mice exhibit reduction Purkinje cell firing frequency

Next we wanted to determine whether alterations in Purkinje cell spiking output was observed in our SacsR272C knock-in mouse model as it has in the knock-out ARSACS model previously characterized [23], as well as several other forms of ataxia [24–33]. We performed cell-adjacent loose-cell attached recordings from visually-identified Purkinje cells to monitor their firing properties without disturbing their intracellular milieu (Fig. 4a, left), and recorded spontaneous action potentials in 90 day-old controls and SacsR272C mice (Fig. 4a, right). We observed a significant reduction in firing frequency in SacsR272C mice compared to control WT mice (WT: frequency = 65.5 Hz ± 3.6 Hz, N = 4, n = 31; SacsR272C: frequency = 50.4 Hz ± 3.8 Hz, N = 4, n = 28; significantly different, P = 0.0065; Fig. 4b). This ~ 25% reduction in P90 SacsR272C mice is similar to the reduction found at earlier ages in Sacs−/− mice, where a 15% reduction was observed at P20, and a 45% reduction at P40. Decreases in spike frequency has been observed in several forms of ataxia [24–33], which in some cases has also been accompanied by a reduction in spike regularity [31]. To determine if changes in firing precision were observed in our knock-in mouse model of ARSACS, we measured the coefficient of variation (CV) of Purkinje cell action potential intervals, since a reduction in firing regularity is associated with an increase in CV [31]. We found no significant changes in firing precision in SacsR272C mice (WT: CV = 0.11 ± 0.009; SacsR272C: CV = 0.10 ± 0.009; not significantly different, P = 0.23; Fig. 4c), consistent with our previous findings in Sacs−/− mice [23].

Since differences in the onset of motor abnormalities have been observed in male and female mice in the past, we wondered whether the changes in firing we observe reflect differences in male and female mice. To examine this, we compared our findings in male and female mice.
Fig. 3 Progressive Purkinje cell loss in Sacs<sup>R272C</sup> mice. (a, b) Nissl stain of 45 day-old vermal cerebellar sections show normal cerebellar structure and lobulation in Sacs<sup>R272C</sup> mice (b) compared to control (a). (c-d) Calbindin immunolabeling on vermal sagittal brain sections from 300 day-old mice. Sacs<sup>R272C</sup> mice display cerebellar PC loss (d1, yellow bracket) compared to age-matched controls (c1). (e and f) Neuronal cell counts in the anterior (I to VI) and posterior (VII to X) lobules at different ages, demonstrate significant loss of Purkinje cells in Sacs<sup>R272C</sup> mice starting at 90 days of age in the anterior lobules (e). Some neuronal cell loss is observed in the posterior lobules at 365 days (f). Data represent means ± SEM, number of mice per group is indicated in each bars. ***P < 0.001, *P < 0.05 (two-way ANOVA with repeated-measures followed by Tukey’s post hoc comparison). Scale bar in d1 = 100 μm.

Fig. 4 Reduced Purkinje cell firing frequency in mice with R272C mutant sacsin. (a) Schematic representation of Purkinje cell loose-cell attached recording configuration (left) and sample traces for WT (top right, black) and Sacs<sup>R272C</sup> mice (bottom right, grey) Purkinje cell action potential recordings. (b) Purkinje cell firing rate is significantly reduced in Sacs<sup>R272C</sup> mice, while (c) the precision of firing, as reflected by CV, is unaffected. n.s. = P > 0.05; ** = P < 0.01.
and found that decreases in Purkinje cell firing frequency are observed in both sexes (male WT frequency = 49.0 Hz ± 2.5 Hz, n = 9; male \( \text{Sacs}^{R272C} \) frequency = 29.6 Hz ± 2.6 Hz, n = 8; significantly different, \( P < 0.0001 \); female WT frequency = 72.3 Hz ± 4.2 Hz, n = 22; female \( \text{Sacs}^{R272C} \) frequency = 56.0 Hz ± 4.2 Hz, n = 22; significantly different, \( P = 0.009 \), data not shown). Thus, we observe a reduction in Purkinje cell firing frequency without any change in firing precision in our \( \text{Sacs}^{R272C} \) mouse model, with low expression levels of mutated sacsin, that are broadly similar to changes previously reported in \( \text{Sacs}^{-/-} \) mice [23].

**Neurofilament (NF) accumulations in somatodendritic compartment in \( \text{Sacs}^{R272C} \) Purkinje cells**

Intermediate filament protein accumulations are a striking feature observed in neuronal populations in \( \text{Sacs}^{-/-} \) and ARSACS autopsied brain, ARSACS human-derived dermal fibroblasts as well as genetically engineered knock-out cell lines [22, 34] To explore if \( \text{Sacs}^{R272C} \) animals present the same characteristic IF bundling as KO animals, we performed immunolabeling using a pan-neurofilament heavy (NFH) antibody on sagittal brain sections from 300 day-old mice (Fig. 5). As expected, \( \text{Sacs}^{R272C} \) and \( \text{Sacs}^{-/-} \) display distinct NFH somatodendritic labeling in several CNS neuronal populations; such as cerebellar PC (Fig. 5b, c), neurons in layer II-III and V of the isocortex (Fig. 5e, f), CA1, CA2 and CA3 pyramidal neurons of the hippocampal formation (Fig. 5h, i) and neurons in the thalamus (Fig. 5k, l). NFH immunofluorescence show strong labeling in Purkinje cell dendrites as well as in cell bodies compared to very light immunolabeling in controls and heterozygous animals (Fig. 6a-c). NFH labeling in \( \text{Sacs}^{R272C} \) mice identifies mislocalization of NFH in Purkinje cell bodies and dendrites compared to controls where no NFH is detected in these cellular compartments (Fig. 6f). Western blots analysis revealed an increase in both, NFH protein levels, as well as most significantly, in the non-phosphorylated form of NFH, which has also been previously reported in the \( \text{Sacs}^{-/-} \) mice (Fig. 6d).

Rearrangement of the intermediate filament network in sacsin-deficient cells is also observed in ARSACS patient dermal fibroblasts [34]. These cells display abnormal perinuclear accumulation of vimentin filaments [35]. To verify if the R272C mutation had similar effect on the IF network, we labeled vimentin in CRISPR/Cas9 genetically engineered \( \text{SACS}^{-/-} \) fibroblasts (\( \text{SACS}^{-/-} \)), in which there if no sacsin expression, and our fibroblast line derived from a patient with two distinct \( \text{SACS} \) mutations (Fig. 6h-j). On one allele, this patient bears the common French-Canadian c.8844del mutation (del) and on the other, the c.816C > T (R272C) mutation. As expected, our \( \text{SACS}^{-/-} \) fibroblasts demonstrate perinuclear accumulations of vimentin often forming a ball-like shape (Fig. 6j). The vimentin network in \( \text{SACS}^{-/-} \) fibroblasts is also perturbed, with bundles of IF filaments observed surrounding the nucleus (Fig. 6i). Both these phenotypes are distinctively different from the vimentin network observed in control patient fibroblasts, however the IF bundling is less important in the \( \text{SACS}^{-/-} \) fibroblasts compared to the one in the \( \text{SACS}^{-/-} \) (Fig. 6h-j).

**Mutated sacsin expression in the cerebellum and isocortex of \( \text{Sacs}^{R272C} \) mice**

To first determine sacsin expression in the brain, we performed immunolabeling using anti-sacsin antibody on sagittal brain sections from controls, as well as from \( \text{Sacs}^{-/-} \) mice serving as negative controls (Fig. 7). Immunolabeling revealed that sacsin is a neuronal protein with expression in most areas of the brain. The most extensive labeling was observed in the cell bodies, dendrites and axons of cerebellar Purkinje cells (Fig. 7a), certain neurons of the DCN (Fig. 7b) and several neurons in the pons and the medulla (Fig. 7c). Sacsin expression was also observed in cell bodies of olfactory bulb mitral cells (Fig. 7j), superior olivary complex neurons (Fig. 7f), as well as neurons in the cerebral cortex areas; visual and motor (Fig. 7g, i). In some areas, such as the thalamus (Fig. 7d), the hippocampus (Fig. 7k) and the isocortex sensory area (Fig. 7h), sacsin expression seem more restricted to neuronal processes with only very light labeling seen in the cell bodies. Sacsin immunolabeling could also be observed in fiber tracts in the cerebellum, the pons and medulla (Fig. 7c), as well as the corpus callosum (Fig. 7e). In summary, sacsin is widely expressed in the brain with some subcellular distinction from one neuronal population to the other.

Our \( \text{Sacs}^{R272C} \) mice do display an ataxic phenotype with a progressive PC cell loss, and seem to be less affected then the \( \text{Sacs}^{-/-} \) mice. To address whether this possible slight difference in phenotype was associated to the residual expression of mutated sacsin, we performed sacsin immunolabeling in sagittal brain sections from 300 day-old WT, \( \text{Sacs}^{R272C} \) and \( \text{Sacs}^{-/-} \) mice (Fig. 8). \( \text{R272C} \) mutant mice display an important reduction in sacsin immunolabeling in all areas of the brain, but labeling can still be observed in the cell bodies and dendrites of PC and cell bodies of DCN neurons (Fig. 8b). Indeed, sacsin immunohistochemical labeling quantification demonstrate a significant reduction of mutant sacsin immunolabeling in cell bodies, dendrites and axons of cerebellar Purkinje cells as well as in commissural fibers of the corpus callosum of \( \text{Sacs}^{R272C} \) mice (Fig. 9 a-f). The reduction in mutated sacsin labeling is consistent throughout Purkinje cell compartments, such as the cell body, dendrites and axons, where we observe a 40,
44 and 38% reduction respectively compared to the sacsin labeling in control mice (Fig. 9g). These results suggest that the observed phenotype is most likely attributed to an overall reduction in mutant sacsin protein levels leading to a loss of sacsin function.

**Discussion**

Here we report that expression of mutated R272C sacsin protein in mice leads to a similar and potentially milder phenotype than that previously characterized in the Sacs−/− animals [23]. However, a comparative parallel study of age-matched SacsR272C and Sacs−/− mice would require too many resources for the limited insight we expect such a study would provide, considering the large spectrum of clinical severity observed in human patients, even between those carrying the same mutations. The R272C mutation was previously shown to affect proper protein fold and/or protein stability of the sr1 domain [20]. Although we did not investigate R272C mutant sacsin protein folding, we did identify a significant decrease in mutant protein levels on Western blot and immunohistochemical labelings from animal brains. SacsR272C mice exhibit significant balance deficit and muscle weakness detectable as early as 45 days of age. These balance difficulties preceded extensive neuronal loss, suggesting that the ataxic phenotype most likely corresponds to Purkinje cell dysfunction prior to degeneration. The progressive Purkinje cell loss was largely localized to the...
most anterior cerebellar lobules, just as observed in ARSACS patients. Our sacsin immunolabeling demonstrate that sacsin expression is detected in cerebellar PC across all vermal cerebellar lobules and therefore cannot account for a greater vulnerability of anterior PC in ARSACS. Other cellular or physiological properties of these cells must account for this greater vulnerability in ARSACS. We recently reported changes in synaptic input and intrinsic firing of cerebellar PC, as well as synaptic output to the DCN in Sacs<sup>R272C</sup> mice prior to their motor coordination deficit [23]. These changes were only observed in anterior cerebellar lobules, but not in non-degenerating posterior lobules. These results support the idea that cerebellar PC across the cerebellum have distinct properties that could render certain populations more vulnerable to the absence or to deficient sacsin function. In our Sacs<sup>R272C</sup> mouse model, we also observed Purkinje cell firing rate deficits in anterior lobules that are similar to those detected in our Sacs<sup>−/−</sup> mouse model [23], supporting the hypothesis that Purkinje cell firing deficits contribute to motor coordination deficits [23, 36].

The IF cytoskeletal rearrangement observed in numerous neuronal populations in the brains of Sacs<sup>R272C</sup> and Sacs<sup>−/−</sup> mice, in Sacs<sup>−/−</sup> primary neuronal cultures, in ARSACS patient-derived fibroblasts and in genetically engineered Sacs<sup>−/−</sup> cell lines is the most striking cellular change observed to date in the ARSACS pathology [22, 34]. This phenotype appears to occur prior to motor coordination deficit and other cellular features of ARSACS,
such as mitochondrial elongation and impaired transport, at least in Sacs−/− mice [22]. We do not fully understand what causes the accumulations of non-phosphorylated NFH proteins in the somatodendritic compartment of Purkinje cells, but one explanation is that sacsin directly acts on NF assembly and/or turnover. Our recent results suggest a direct interaction of distinct sacsin domains in the regulation of IF assembly and dynamics, with certain domains, namely the SIRPT1 and DNAj domains, being capable of dismantling NF bundles in cultured Sacs−/− neurons [37]. These results argue that sacsin could serve as an important IF protein co-chaperone.

Another possible explanation for the accumulation of NFH in the somatodendritic compartment of SacsR272C and Sacs−/− neurons could be a mis-targeting or mis-sorting of proteins. Neurons are highly polarized cells exhibiting axonal and somatodendritic domains...
with distinct complements of cytoplasmic organelles and cytoskeletal proteins. Polarized sorting is thought to depend mainly on selective association of these cytoskeletal organelles or proteins with different microtubule motors in the pre-axonal exclusion zone (PAEZ), a specialized area within the axon hillock and the axon initial segment (AIS) [38]. Defects in this polarization could impede axonal proteins from entering the axon and stall them in the somatodendritic compartment causing neuronal defect. For example, Purkinje cell-specific knock-down of microtubule cross-linking factor 1 (Mtcl1) causes AIS disorganization by impairing ankyrin G localization, and loss of axonal polarity [39]. In mice, genetic disruption of Mtcl1 results in abnormal motor coordination associated with Purkinje cell degeneration, arguing that Purkinje cells are susceptible to such deregulation of neuronal polarization [39]. Furthermore, a point mutation in the C-terminal microtubule-binding domain of MTCL1 has been found to segregate in a Japanese dominant spinocerebellar ataxia family [39]. The accumulation of NFH protein in the somatodendritic compartment of several neuronal populations in the Sacs<sup>R272C</sup> and Sacs<sup>−/−</sup> mice raises the possibility that sorting of dendritic and axonal proteins might be perturbed in ARSACS. Understanding the potential role of sacsin in the establishment and/or maintenance of neuronal polarity will be an important area of future study. Further studies will also be needed to elucidate whether

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**Fig. 8** Mutant sacsin expression in Sacs<sup>R272C</sup> mice. (a-f) Immunohistochemistry using antibody against sacsin on sagittal brain sections from 300 day-old Sacs<sup>WT</sup>, Sacs<sup>R272C</sup> and Sacs<sup>−/−</sup> mice demonstrate that some neuronal population express low levels of mutated sacsin. R272C mutant mice display an important reduction in sacsin immunolabeling in all areas of the brain, but labeling can still be observed in the cell bodies and dendrites of cerebellar PC (arrows in b1) and cell bodies of DCN neurons (arrows in b2). Scale bar in c2 = 200 μm, scale bar in F = 100 μm.
the bundles of IF cytoskeletal proteins in ARSACS are pathophysiological and directly lead to cellular death or simply by-products. Although it is easy to conceptualize that large bundles of cytoskeletal proteins in the neuronal cell soma and dendritic branches would physically hinder organelle and cargo protein transport in ARSAC [40], future studies are needed to confirm its pathological role.

Our results demonstrate that expression of low levels of mutant R272C sacsin in mice leads to motor coordination deficit and muscle weakness reminiscent of the human ARSACS pathology, with similar cellular deficit previously observed in the Sacs<sup>−/−</sup> mouse model. The mutant R272C mouse demonstrate that missense SACS mutations are likely to interfere with sacsin function despite some low mutant protein levels, supporting that a loss of function most likely underlines its pathophysiology.

Materials & methods

**Sacs<sup>R272C</sup> mice generation and analysis**

Sacs<sup>R272C</sup> mice were generated by Ozgene (Bentley, Australia) on a C57BL/6J background. Targeting vector was constructed by first cloning the gene segment which includes exons 6 through 8 into PelleR B00001F7_G01 Ozgene proprietary plasmid containing a PGK-neo cassette flanked by two FRT sites, followed by site-directed mutagenesis for introduction of the R272C mutation at the beginning of exon 7. Targeting vector was completed by incorporation of 6.3Kb 5′ and 3′ homology arms. Mice were genotyped by PCR using primers: 5′-AGCAACCTGCATATTGACAGAA-3′ and 5′-GGTTTCTGGTTTGAGGCAAT-3′. Total RNA from mouse cerebella and cortex was extracted with the miRNeasy kit (Qiagen) and treated with DNAse I (Qiagen) according to the manufacturer’s instructions. RNA quality was assessed on an Agilent 2100 Bioanalyzer and RNA Integrity Numbers (RIN) were routinely above 9. For qRT-PCR, 1 μg of RNA was reversed transcribed using the High Capacity cDNA Reverse Transcriptase (ThermoFisher). The following primers were used to amplify Sacs: 5′-CGCTGAGACCAGCTTTCC-3′ and 5′-CCATCTTGATCCAATCAGGTATC-3′. Real-time PCR was performed in technical duplicates using FastStart Universal SYBR Green Master (ROX) (Roche) on a ViiA™ 7 Real-Time PCR System (Applied Biosystems). The ΔΔCt method was used to calculate relative Sacs mRNA expression, with normalization to the endogenous genes Ppia and Hprt1. Sacs<sup>R272C</sup> mice were maintained in the C57Bl/6J background and bred and maintained under standard conditions consistent with the Canadian Council on Animal Care and approved by the University Animal Care and MNI Animal Care committees.

**Fibroblast cell lines**

Control human-derived fibroblasts were obtained from the Repository for Mutant Human Cell Strains of the Montreal Children’s Hospital. SACS<sup>del/R272C</sup> human-derived fibroblasts were obtained using the previously described protocol [41]. Briefly, patient skin punch biopsies were minced in small pieces and put in 6-well plates in complete DMEM/20% FBS (Wisent) media. Media was changed every 2–3 days. Cells were trypsinized and...
passaged once they reached confluence. Fibroblasts were then frozen at 1 × 10^6 cells/ml per vial. Primary cultures were kept at low passage (p4–8). Cells were cultured in regular medium, DMEM (Wisent) with 10% FBS (Wisent) at 37°C under 5% CO_2 humidified atmosphere. Primary human-derived fibroblasts were immortalized at low passage as previously described [42]. SACS<sup>em1KO</sup> CRISPR/Cas9 cell line was generated following manufacturer guidelines using saccin double nickase plasmid (sc-404,592-NIC, SCBT). Briefly, cells were nucleofected with 2μg of vectors and positive clones were selected using 1μg.ml<sup>−1</sup> puromycin (ThermoFisher Scientific). Absence of saccin was verified by Western blotting. Genomic DNA was extracted from clones of interest and Sanger sequenced using the following primers (Fwd: CACAGTAATCATGCAAAGTCTCTA TGCCCTG, Rev.: ACAGAGAACTGGTGTTAGAGTG ACTTC). Our SACS<sup>em1KO</sup> Crispr/Cas9 cell line presents a 44pb duplication in exon 8 of the SACS gene (c.1668_1711dup) leading to insertion of a stop codon and total absence of protein (data not shown). Absence of off-target recombination was verified in silico (crispr.mit.edu). Studies using human cell lines were approved by the institutional review board of the Montreal Neurological Institute (edtu). Studies using human cell lines were approved by the institutional review board of the Montreal Neurological Institute (edtu). Studies using human cell lines were approved by the institutional review board of the Montreal Neurological Institute (edtu).

**Immunolabeling**

For preparation of tissue sections, mice were anesthetized with mouse anesthetic cocktail (ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine (10 mg/ml)), perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde. Brains were dissected and post-fixed for 2 h at 4°C in the same fixative. Tissues were then equilibrated in 30% sucrose/PBS until sectioning. Sagittal sections (35μm) were cut using a freezing sledge microscope. Free-floating sections were processed for immunofluorescence as previously described [22]. Antibodies were used as polyclonal anti-calbindin-D-28k (Sigma, C2724), monocular anti-neurofilament-H (NFH) (Millipore, MAB5266), polyclonal anti-MAP2 (Abcam, ab5392).

For immunohistochemistry, mouse brains were dissected out, immersed in 4% paraformaldehyde and post-fixed for 48 h at 4°C in the same fixative. Tissues were then processed for paraffin embedding and sectioned at 4μm in the parasagittal plan. For saccin immunohistochemistry, sections were subjected to heat-mediated antigen retrieval in demasking solution (10 mM Tris-HCL, 1 mM EDTA, 0.05% Tween-20) at 95°C for 35 min. Sections were allowed to cool down at room temperature for 30 min followed by inactivation of endogenous peroxidase. Sections were then incubated in blocking buffer (phosphate buffer 0.1 M; 10% normal goat serum; 0.25% TX-100) 1 h. Endogenous biotins were blocked with the avidin & biotin blocking kit (Vector Labs, SP-2001) according to the manufacturer’s protocol. Sections were then incubated with anti-saccin (Abcam; ab181190) or anti-neurofilament-H (NFH) antibody (Millipore, MAB5266) diluted in phosphate buffer 0.1 M; 1% normal goat serum; 0.25% TX-100 overnight at 4°C. Sections were then incubated with appropriate biotinylated secondary antibodies (Vectors Labs) followed by VECTASTAIN ABC reagent for 1 h, washed, and reacted with VECTOR DAB substrate. Sections were dehydrated in a graded series of ethanol dilutions, cleared in xylene, counterstained with cresyl-violet or not and coverslipped using Protocol mounting medium (Fisher Scientific). Immunolabeling was performed simultaneously in at least three aged-matched animal per group. Mean grey values were collected using ImageJ in three different fixed-sized regions of interest per mouse for Purkinje cell dendrites and axons, as well as for the corpus callosum. For Purkinje cell bodies, mean grey values were collected from 8 to 10 cell bodies with a fixed-sized region of interest.

Immunolabeling of human-derived fibroblasts was performed as followed. Cells plated onto 12 mm round glass coverslips were fixed in ice-cold methanol 7 min at -20°C. Cells were then washed with phosphate buffered-saline (PBS) three times. Cells were incubated 30 min in PBS; 5% normal goat serum. Cells were then incubated in the presence of primary monoclonal anti-vimentin antibody (1/4000, clone V9, SIGMA-Aldrich) diluted in PBS; 1% normal goat serum for 2 h at room temperature. Secondary anti-mouse Alexa-Fluor 555 antibody (ThermoFisher Scientific) was applied for 45 min.

Imaging was performed using Zeiss Axiovert M2 microscope or an Olympus IX81 inverted microscope with appropriate lasers using an Andor/Yokogawa spinning disk system (CSU-X), with a sCMOS camera using a 20×, 60× or a 100× objective lenses (NA1.4).

**Preparation of cerebellar tissue lysates and western blotting**

We used our published protocols for the preparation of cerebellar protein extracts and western blot analysis [43]. Immunoblots were probed with polyclonal anti-saccin (Abcam, ab181207, 1:2000) and monoclonal anti-vinculin (SIGMA-Aldrich, V9131, 1:1000).

**Behavioral test**

Mice were tested for motor balance, motor coordination and muscle strength using the balance beam, rotarod and inverted grid tests. Female and male cohorts were tested at 45, 90 and 180 days of age (females n ≥ 7 per groups, males n ≥ 6 per groups) (females n ≥ 9 per groups, males n ≥ 7 per groups). Behavioural testing was performed as previously described in Lariviere et al. [22].
Purkinje cell counts

Purkinje cell counts were performed as previously reported [43].

Acute slice preparation

Animals between the ages of P90–100 were deeply anaesthetized with isoflurane, rapidly sacrificed, and brains were removed into ice-cold low-Ca2+ artificial cerebrospinal fluid (ACSF) that was bubbled with an O2/CO2 (95% / 5%) mixture as previously described [23, 44]. Sagittal cerebellar vermis slices (250 µm) were cut using a VT1200S microtome (Leica Microsystems, Germany). Slicing ACSF contained (in mM): NaCl, 125; KCl, 2.5; MgCl2, 4; NaH2PO4, 1.25; KCl, 2.5; MgCl2, 4; NaH2PO4, 1.25; NaHCO3, 26; CaCl2, 2; dextrose, 25; with a final osmolality of ~ 320 mOsm and pH 7.4. Slices were then transferred to ACSF that contained 1 mM MgCl2 and 2 mM CaCl2 (incubation and recording ACSF), were incubated at 37 °C and then cooled to room temperature where they were incubated in bubbled ACSF for up to an additional 6 h.

Electrophysiology

Loose-cell attached recordings were made with a glass electrode pulled with a P-1000 puller (Sutter Instruments, Novato, CA, USA) filled with ACSF to record action potentials without dialyzing the intracellular solution and thereby altering intrinsic firing rates. Data was collected and analyzed off-line using custom acquisition and analysis routines with Igor Pro software (WaveMetrics, Portland, OR, USA). Statistical comparisons were made using Igor Pro or JMP (SAS, Cary, NC, USA) software. Data are represented as mean ± standard error of the mean (SEM), N = animal number, n = cell number.

Statistical analysis

Data for the behavioral phenotyping are shown as the mean ± standard error of the mean (SEM). Beam and rotarod analyses were done using GraphPad Prism7 software and significance level was set at 0.05. Two-way ANOVA with repeated-measures was performed to assess the effect of time and genotype followed by Tukey's post hoc pairwise comparisons. For all other statistical analyses, comparisons were made using unpaired Student t-test with significance level of 0.05.

Additional file

7. Targeting vector was completed by incorporation of 6.3kb 5′ and 3′ homology arms. Localization of forward and reverse primers for genotyping are identified on the knock-in allele. (B) PCR analysis of tail genomic DNA extracted from SacR272C, heterozygous (Het) and control (WT) mice. PCR amplicons migrate to 220 bp for the wild-type allele, whereas the R272C allele migrates to 330 bp. (TIF 4742 kb)

Abbreviations

AIS: Axon initial segment; ARSACS: Autosomal recessive spastic ataxia of Charlevoix-Saguenay; CV: Coefficient of variation; DNA: Deoxyribonucleic acid; NF: Neurofilament; NFH: Neurofilament-heavy; PCR: Polymerase chain reaction; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RNA: Ribonucleic acid; SacR272C: Mice homozygous for sacsin R272C mutation

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

RL, RAM, AJW and BB conceived the study and designed the experiments. RL performed qRT-PCR. NS performed PC quantification. RL and NS performed electrophysiology and analyzed results. BTM performed electrophysiology experiments and analyzed results. RL, AJW and BB wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experiments were performed according to good practice of handling laboratory animals consistent with the Canadian Council on Animal Care and approved by the University Animal Care and MNI Animal Care committees. Studies using human cell lines were approved by the institutional review board of the Montreal Neurological Institute and with McGill University Research Ethics Board Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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