

Diversity of ARSACS Mutations in French-Canadians

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ABSTRACT: Background: The growing number of spastic ataxia of Charlevoix-Saguenay (*SACS*) gene mutations reported worldwide has broadened the clinical phenotype of autosomal recessive spastic ataxia of Charlevoix-Saguenay (*ARSACS*). The identification of Quebec *ARSACS* cases without two known *SACS* mutation led to the development of a multi-modal genomic strategy to uncover mutations in this large gene and explore phenotype variability. **Methods:** Search for *SACS* mutations by combining various methods on 20 cases with a classical French-Canadian *ARSACS* phenotype without two mutations and a group of 104 sporadic or recessive spastic ataxia cases of unknown cause. Western blot on lymphoblast protein from cases with different genotypes was probed to establish if they still expressed saccin. **Results:** A total of 12 mutations, including 7 novels, were uncovered in Quebec *ARSACS* cases. The screening of 104 spastic ataxia cases of unknown cause for 98 *SACS* mutations did not uncover carriers of two mutations. Compounds heterozygotes for one missense *SACS* mutation were found to minimally express saccin. **Conclusions:** The large number of *SACS* mutations present even in Quebec suggests that the size of the gene alone may explain the great genotypic diversity. This study does not support an expanding *ARSACS* phenotype in the French-Canadian population. Most mutations lead to loss of function, though phenotypic variability in other populations may reflect partial loss of function with preservation of some saccin expression. Our results also highlight the challenge of *SACS* mutation screening and the necessity to develop new generation sequencing methods to ensure low cost complete gene sequencing.

RÉSUMÉ: Diversité des mutations responsables de l'ARSACS chez les Canadiens-Français. Contexte : Le nombre croissant de mutations du gène de l'ataxie spastique de Charlevoix-Saguenay (*SACS*) rapportées à travers le monde a élargi le phénotype clinique de l'ataxie spastique de Charlevoix-Saguenay, une maladie autosomique récessive (*ARSACS*). L'identification de patients atteints d'*ARSACS* au Québec, qui ne sont pas porteurs de deux mutations *SACS* connues, a mené au développement d'une stratégie génomique multi-modale pour découvrir des mutations dans ce gène de grande taille et explorer la variabilité phénotypique. **Méthode :** Nous avons recherché des mutations du gène *SACS* en combinant différentes méthodes chez 20 patients porteurs du phénotype canadien-français classique de l'*ARSACS* mais non-porteurs de deux mutations connues, et un groupe de 104 cas sporadiques ou porteurs d'une ataxie spastique récessive de cause inconnue. Nous avons examiné le buvardage Western de protéines de lymphoblastes provenant de cas porteurs de différents génotypes afin de déterminer s'ils exprimaient encore la saccine. **Résultats :** Douze mutations, dont 7 nouvelles mutations, ont été identifiées chez les patients atteints d'*ARSACS* au Québec. La recherche de 98 mutations *SACS* chez 104 cas d'ataxie spastique de cause inconnue n'a pas identifié de porteurs de deux mutations connues. La saccine était peu exprimée chez des hétérozygotes composés pour une mutation *SACS* faux-sense. **Conclusions :** La taille du gène pourrait expliquer la grande diversité génotypique observée même au Québec où un grand nombre de mutations *SACS* sont présentes. Cette étude n'est pas en faveur d'une plus grande variabilité du phénotype de l'*ARSACS* dans la population canadienne-française. La plupart des mutations entraînent une perte de fonction et la variabilité phénotypique dans d'autres populations pourrait refléter une perte partielle de fonction avec préservation d'une certaine expression de la saccine. Nos résultats soulignent également le défi du dépistage des mutations *SACS* et la nécessité de développer des méthodes de séquençage de nouvelle génération afin de pouvoir offrir un séquençage complet du gène à faible coût.

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With more than 300 affected individuals living in Quebec, autosomal recessive spastic ataxia of Charlevoix-Saguenay (*ARSACS*) is the most common of all inherited recessive ataxias in the province¹. Though most families originate from the regions of Charlevoix and Saguenay-Lac-St-Jean (*SLSJ*), cases are found in other regions of Quebec. The high carrier rate for the c.8844delT spastic ataxia of Charlevoix-Saguenay (*SACS*) gene mutation explains the higher prevalence of *ARSACS* in French-Canadians². The carrier frequency is 1/22 in the *SLSJ* region, but is unknown in other regions of Québec³. Surprisingly,

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this founder mutation was never uncovered in other populations. Genetically confirmed cases of ARSACS have now been described in: Italy, Spain, Tunisia, France, Belgium, Hungary, Morocco, Turkey, Serbia, other provinces of Canada, Netherlands, United Kingdom, Algeria and Japan (Supplemental Table 1). Therefore, despite its name, ARSACS has a worldwide distribution but its prevalence in most countries is still unknown, except in the Netherlands where it may be as frequent as Friedreich Ataxia⁴.

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) was first described in a cohort of French-Canadians (FC) that presented in childhood with a progressive spastic ataxia accompanied by a sensory-motor polyneuropathy and retinal changes that were referred to as “hyper myelinated fibers”^{5,6}. The two *SACS* mutations c.8844delT (6594delT) and c.7504C>T (5254C>T) were found to explain 92.6 and 3.7% respectively of carrier chromosomes². Therefore, since 2000 it was clear that other *SACS* mutations in this population needed to be identified. Prior to this publication, 100 *SACS* mutations were uncovered in 14 countries (Supplemental Table 1 - on-line only). Together, the growing number of mutation-proven cases suggests that ARSACS is one of the most common recessive spastic ataxia worldwide. The search for *SACS* mutations in different cohorts has also broadened the clinical spectrum of ARSACS. The retinal changes are clearly not present in all cases⁷, though they may be under observed⁸. Cases with important cognitive impairment have also been described, but overall it is not a common feature of ARSACS⁴. Four cases with onset in the twenties have also been diagnosed molecularly⁹. The identification over years of a group of Quebec ARSACS cases with a single or no known *SACS* mutation led us to a systematic search for new mutations in the large *SACS* gene in these classical FC ARSACS cases and sporadic or recessive spastic ataxia cases of unknown cause. This study reports the development and use of various technologies to uncover a broader spectrum of *SACS* mutations in ARSACS in this population and the preservation of saccin expression in some carriers of missense mutations.

MATERIALS AND METHODS

Clinical ascertainment and consent

Through specialized ataxia clinics and treating neurologists, we identified a cohort of 20 ataxia cases that exhibited a “classic FC ARSACS phenotype”, that could not be differentiated from other FC ARSACS cases clinically. This group included 15 carriers of one c.8844delT mutation and five cases with none of the two common FC mutations. Twenty-five homozygote carriers of the common c.8844delT (6594delT) were typed to establish if they carried a large genomic deletion. We also studied a group of 104 FC spastic ataxia cases with unknown genetic diagnosis to search for *SACS* mutations. Detailed genealogical information, medical histories, neurological examinations, electromyogram (EMG) and nerve conduction studies were retrieved from medical charts on all participants. This project was approved by institutional Ethics Committee of the Centre de recherche du CHUM. Informed consent was obtained from all patients. All non-ataxic healthy or disease controls signed an informed consent.

Screening for new *SACS* mutations

Screening for the two common *SACS* mutations in Quebec was performed on all ARSACS cases by the Service de Génétique Médicale, CHU Sainte-Justine, Montreal, Quebec. Genomic DNA and RNA were extracted from peripheral blood lymphocytes or from saliva using the Oragene DNA or RNA extraction kit (DNA Genotek, Kanata, Canada) using standard methods. The nine *SACS* coding exons and flanking intronic sequences of the *SACS* gene [NM_014363.4] were amplified from genomic DNA using 42 primer pairs designed with UCSC ExonPrimer (<http://ihg.gsf.de>). RT-PCRs on RNA from white blood cells or saliva were performed using 34 primer pairs to cover the entire coding sequence. Oligonucleotide primers were synthesized by LifeTechnologies (www.lifetechnologies.com). Genomic and cDNA PCR products were forward and reverse sequenced at the McGill University and Genome Quebec Innovation Centre using an ABI 3730xl DNA Analyzer (ABI; Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using SeqMan 4.03 (DNASTar, Wisconsin, USA) and Chromas 1.62 (Technelysium Pty Ltd, Australia).

Custom Sequenom iPlex screening for 96 *SACS* variants

To rapidly assess the frequency of known *SACS* mutations in sporadic or recessive spastic ataxia cases, we designed Sequenom iPlex Gold custom panels (Sequenom, San Diego, CA, USA) in collaboration with the McGill University and Genome Quebec Innovation Centre. Three panels were designed for a total of 87 *SACS* mutations reported at the time in the literature or uncovered by us in the FC ARSACS cohort and nine previously identified variants predicted to lead to an amino acid change or to a premature STOP codon (Supplemental Table 2 - on-line only). The iPLEX® Gold assay consists in a multiplex PCR followed by a single base primer extension reaction. Following the PCR the remaining nucleotides are deactivated by Shrimp Alkaline Phosphatase (SAP) treatment. The single base primer extension step is performed, and the primer extension products are detected using MALDI TOF Mass Spectrometry. Data analysis and genotype calling was performed using Typer 4.0 Analyzer software (Sequenom, San Diego, CA, USA).

Copy number variation analysis

In order to detect deletions or duplications in *SACS*, DNA samples from ten ARSACS patients with a single mutation were sent to McGill University and Genome Quebec Innovation Center (Montreal, Canada) for a copy number analysis scan using Illumina Human 1M Duo that includes 108 markers in the *SACS* gene. Analyses of changes in gene copy number were performed using Copy Number Variation (CNV) Analysis with BeadStudio v3.1 (Illumina, San Diego, CA, USA) and PennCNV program as previously described¹⁰. To further assess small deletion or duplication in *SACS*, we used 24 pre-designed ABI®-based Copy Number Assays for human CNV screening; eleven were located in *SACS* coding regions, and one in each coding sequence of the surrounding genes (FGF9, SCGC, TNFRSF19, MIPEP, PCOTH, RP11-45B20.2, SPATA13 and PARP4) (Supplemental Table 3 - on-line only). Each reaction was performed in quadruplicate on 384-well PCR plates with the ABI® Copy Number Reference Assay (RNaseP). CopyCaller™

(Applied Biosystems, Carlsbad, CA, USA) was used for data analysis.

Western blot analysis

Proteins were extracted from immortalized lymphoblasts prepared using standard methods. Cells were trypsinized, pelleted and homogenized in 20mM HEPES buffer with protease inhibitors. Protein concentrations were determined using Bradford reagent (BioRad). Homogenates were separated on a 3-8% Tris-Acetate gel (LifeTechnologie, www.lifetechnologies.com) and transferred onto a nitrocellulose membrane (BioRad). The membrane was then probed with a custom in-house affinity purified rabbit polyclonal anti-sacsin antibody (raised against human sacsin protein aa. 4445-4579).

RESULTS

Identification of novel SACS point mutations and genomic rearrangements in French-Canadians ARSACS cases

Since 2000, a growing number of clinically presumed FC ARSACS cases with a single mutation or no mutations were identified². This prompted our systematic search for novel SACS point mutations and genomic rearrangements. We sequenced based on the reference sequence [NM_014363.4] the genomic SACS coding sequence on 15 cases with a single known mutation and five with no mutations presenting classical FC ARSACS phenotype. We also sequenced the full SACS copy DNA (cDNA)

on four of these cases. As summarized in the Table, both mutations were uncovered in 18/20 of the cases (90%) (Figure 1). They consist of seven not previously described and five previously published mutations. These mutations include: five missenses, five insertions and deletions (indels), one nonsense and one large genomic deletion. In this cohort no new carriers of the previously reported c.7504C>T mutation was found, while all previously known carriers were excluded from this study considering that all had two known SACS mutations. The two most common other mutations appear to be c.814C>T (p.R272C), previously described in cases from Maritime Canada¹¹, and the novel c.4744G>A (p.D1582N) (Table 1). We also identified two novel mutations c.6290delG (p.C2097LfsX2113) and c.9086delA (p.N3029IfsX3051) in a Quebec resident of French-Italian origin with a classical FC ARSACS phenotype. We screened using a custom Sequenom iPLEX for 87 SACS mutations in a group of 94 pan-Quebec French-Canadian healthy or diseased non-ataxic controls. We uncovered only three carriers of the common c.8844delT mutation in (1:33), and no carriers of the other mutations. This further supports that the higher prevalence of ARSACS in FC is due uniquely to the high carrier rate of the c.8844delT mutation in FC.

Evidence for a deletion of the entire SACS gene was uncovered early in this study by simple sequencing in one patient considered homozygote for c.8844delT (6594delT) and found to be homozygote for many SNP variants in the SACS

Table: Genotypes of 18 ARSACS cases carrying novel (*) or already published SACS FC mutations

Number of cases	Mutation 1	Mutation 2
5	c.8844delT (p.I2949FfsX2952)	c.4744G>A (p.D1582N) *
2	c.8844delT (p.I2949FfsX2952)	c.814C>T (p.R272C)
1	c.8844delT (p.I2949FfsX2952)	c.12992G>A (p.R4331Q)
1	c.8844delT (p.I2949FfsX2952)	c.237insAfsX (p.S80IfsX98)*
1	c.8844delT (p.I2949FfsX2952)	c.1190insAfsX (p.S397KfsX405)*
1	c.8844delT (p.I2949FfsX2952)	large del 1,41 Mb
1	c.8844delT (p.I2949FfsX2952)	c.5836T>C (p.W1946R)
1	c.8844delT (p.I2949FfsX2952)	c.9508C>T (p.R3170X)*
1	c.5836T>C (p.W1946R)	c.237insAfsX (p.S80IfsX98)*
1	c.5836T>C (p.W1946R)	c.4205A>T (p.D1402V)*
1	c.11242 del688bp (p.3748fs3756X)*	c.11242 del688bp (p.3748fs3756X)
1	c.814C>T (p.R272C)	c.814C>T (p.R272C)
1	c.9497-9498delTT (p.F3166X)*	c.9497-9498delTT (p.F3166X)*

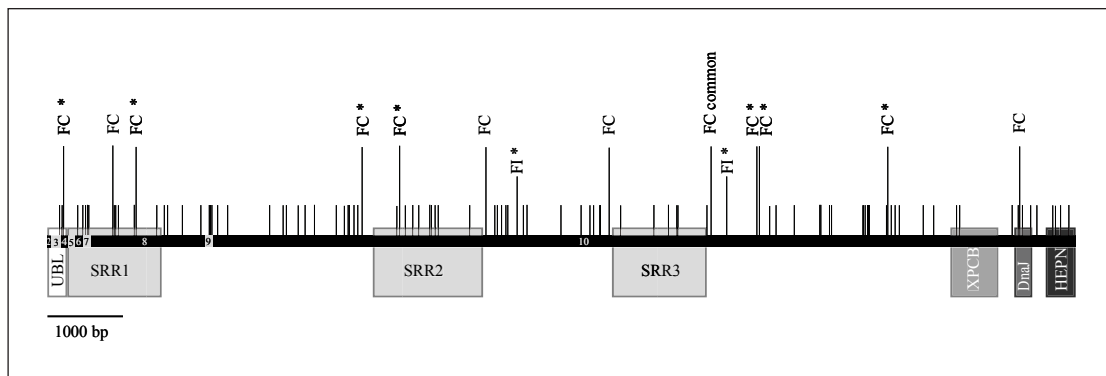


Figure 1: Schematic representation of the *SACS* mRNA showing the localization of 104 *SACS* reported mutations. Positions are based on the longest mRNA transcript containing 9 coding exons numbered from 2 to 10 based on reference sequence NM_014363.4. Novel (*) French-Canadian (FC) and French-Italian (FI) mutations described in this study in Quebec ARSACS cases are identified as are previously described FC mutations. *SACS* sequences coding for predicted or known saccin domains are represented as boxes. The three large deletions reported including the entire *SACS* gene are not illustrated. UBL: ubiquitin-like domain; SRR: saccin repeating region; XPCB: xeroderma pigmentosum complementation groupe C binding domain; DnaJ: heat shock protein DnaJ; HEPN: higher eukaryotes and procaryotes nucleotide binding domain; bp: base pair.

gene (Table). Copy number variation (CNV) analysis using the Illumina Human 1M Duo chip established in this case the existence of a 1.41 Mb deletion (position on chromosome 13: 23511581-24928468), which includes *SACS* and five other genes, which share similar general boundaries as reported in other large regional deletion¹²⁻¹⁴. This may correspond to the same historical deletion observed in a homozygote FC cases described with both ARSACS and LGMD2C due to the combined loss of *SACS* and *SGCG*¹². To establish if such deletion may have led to an erroneous diagnosis of homozygote carrier for the common c.8844delT we screened 25 such ARSACS cases using 24 pre-designed ABI®-based Copy Number Assays mapped to the *SACS* region and six surrounding genes. We were able to confirm the existence of the large deletion in our single deletion proven case (Table). No rearrangements were found in the 25 presumed homozygotes for the c.8844delT mutation, or in 15 cases with only one mutation at the onset of this study (Table). Together, these results suggest that large genomic deletions of *SACS* are rare in the FC population. We did observe small exonic deletions or duplications in many cases, as was described by Baets et al⁹, but their presence in many of 68 controls, 62 spastic ataxia and 43 patients already known for two mutations suggest that they correspond to common CNV in populations of European descent and should not be considered pathological mutations. Despite the different methods used, two clinically affected ARSACS cases have only one uncovered mutation. This may suggest that we have not yet found the second mutation or that in fact they are affected by other ataxias and are simply carriers of the common c.8844delT *SACS* mutation by chance.

ARSACS phenotype in French-Canadians

By selecting cases with a “classical FC ARSACS” phenotype with at least one unknown mutation, this study was not designed to explore phenotype/genotype correlation in FC cases with two confirmed mutations. The more overlapping phenotypes in this

cohort of 18 confirmed cases probably reflects that 89% (16/18) carry one copy of the common FC c.8844delT mutation (n:13) or at least one nonsense mutation (n:3). We confirmed the absence of saccin on western blot for the homozygotes for the nonsense c.8844delT mutation while there is still some degree of expression in cases with at least one missense mutation (Figure 2). This raises the possibility that some carriers of at least one missense mutation with preservation of expression of mutated saccin may have a partial loss of function and a milder phenotype. Indeed all cases with later onset of ARSACS reported by Baets et al have at least one missense mutation⁹.

To explore if other FC spastic ataxia patients with non-ARSACS phenotypes carried other *SACS* mutations we combined two methods to cheaply screen a large number of

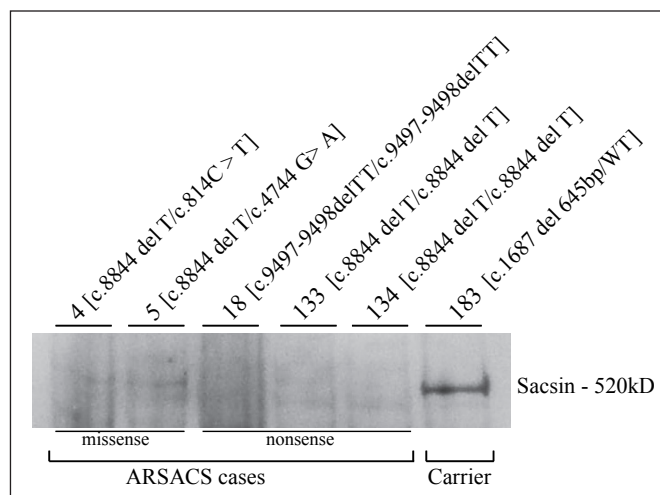


Figure 2: Western blot showing the partial preservation of saccin in lymphoblasts from cases 4 and 5 carrying one missense mutation.

cases for 87 *SACS* mutations, including the seven potential novel mutations found in our cohort, and exclude a large genomic deletion of the *SACS* region. First, we designed a custom Sequenom iPLEX Gold technology (Sequenom, San Diego, CA, USA) based assay that screens plates of 92 samples for 87 *SACS* mutations. The genotype of 45 ARSACS samples was successfully confirmed using this assay. Using this technology we also screened for *SACS* mutations 104 samples from a heterogeneous group of FC spastic ataxic patients without a molecular diagnosis. One carried the common c.8844delT, while two were heterozygotes for the c.4744G>A. Furthermore, on 62 samples, none were found to carry a genomic regional *SACS* rearrangement using a custom ABI®-based Copy Number Assays for human CNV assay, further supporting that this deletion is rare in FC.

DISCUSSION

This study establishes that even within a founder population like the French-Canadians there is a great diversity of *SACS* mutations that cause a “classical FC ARSACS” phenotype. Though clearly the higher prevalence of ARSACS in this population is due to the high carrier rate of the common c.8844delT (6594delT) mutation, five other mutations are also recurrent: c.7504C>T, c.4744G>A, c.814C>T, c.237insAfsX and c.5836T>C. In the case of the c.814C>T mutation, this may well be due to an Acadian introduction considering that the same mutation was found in Maritime Canada¹¹. As observed in other populations, many mutations appear to be private familial mutations. This mutation diversity and positional scattering along the gene suggests that the large size of *SACS* by itself is responsible for the large number of mutations (Figure 1). Large genomic deletions that include *SACS* and other neighbouring genes have now been reported by many groups¹²⁻¹⁴. It is still unknown if these are all independent deletion events or a few rare historical deletions shared by all these cases. More detailed comparisons of the break points will be needed to settle this issue. Our study demonstrates that these large deletions are not common in the French-Canadian population, by this supporting that PCR based detection of the common *SACS* mutations are unlikely to have missed heterozygotes for a point mutations and a large deletion. Significantly, the uncovering that an homozygote carrier of two large deletions suffers from a combined ARSACS and LGMD2C established that a complete knock out of saccin is not lethal¹². The growing number of missense *SACS* mutations suggests that predicted partial loss of function may lead to different ARSACS phenotypes.

Now that clinicians are aware that ARSACS may be a common form of spastic ataxia worldwide, the challenge is to ensure that its molecular diagnosis can be readily performed at low cost. The multimodal approach used in this study provides a way to more rapidly screen larger numbers of samples for a wider range of mutations than the previously proposed Confirmation Sensitive Capillary Electrophoresis-Based Mutation Scanning System (CSCE)¹⁵. The screening for common mutations in certain populations by inexpensive PCR-based methods should still be the first step in establishing a diagnosis. However, with the declining cost of new generation sequencing and the increasing number of *SACS* mutations being described, we would favour the full sequencing of the gene

combined to CNV analysis if one parent is not found to carry the child’s presumed homozygote mutation as the way to establish if patients are affected by ARSACS.

The growing number of reported ataxic patients who carry two *SACS* mutations that do not have a “classical FC ARSACS” phenotype in other countries has expanded the saccinopathy phenotypes⁹. However, our screening of 104 FC spastic ataxia cases of unknown cause did not uncover cases with two *SACS* mutations. Therefore, in this population ARSACS presents a relatively homogenous phenotype likely because of the predominant common c.8844delT (6594delT) mutation. The high prevalence of ARSACS and Friedreich ataxia in Quebec justifies that mutations in both *SACS* and *FXN* genes should be done first in all FC childhood-onset cases of spastic ataxia with a polyneuropathy. Other genes that can be screened in FC childhood-onset ataxia cases considering their relatively higher frequencies in this population are: the *SETX* gene in cases with a polyneuropathy without significant spasticity responsible for Ataxia Oculomotor Apraxia 2 (AOA2)¹⁶, and the *MARS2* gene responsible for the very variable Autosomal recessive Spastic Ataxia with frequent Leukoencephalopathy (ARSAL) in which a polyneuropathy is not a feature in the early stages of the disease^{17,18}. This study confirms that in ARSACS, as for all other ataxias with higher regional prevalence in Quebec described to date such as AOA2¹⁶, ARSAL^{17,18} and Autosomal Recessive Cerebellar Ataxia (ARCA1)^{19,20}, there is a wide spectrum of mutations. However, as for the other ataxias with Quebec founder effects, despite the large number of living cases, these cohorts have a limited power for genotype/phenotype correlation because of the high prevalence of a common founder mutation. Our observation that some saccin is present in carriers of at least one missense mutation raise the possibility that partial preserved function can influence severity. In fact, all cases with later onset of ARSACS reported by Baets et al have at least one missense mutation⁹. Clinical and mutation data sets from combined international series are required to complete a definitive genotype/phenotype correlation. Though uncovering how *SACS* mutations lead to ARSACS was hampered by the large size of the saccin protein (520 KDa), recent studies have started to unravel saccin’s potential dimeric structure²¹ and its chaperone function²²⁻²⁴. Furthermore, the Fondation de l’Ataxie de Charlevoix-Saguenay-CIHR ARSACS Emerging Team recently established that saccin localizes to the surface of mitochondria and that knock down or knock out of saccin in cellular and mice models lead to a disruption of mitochondrial transport into dendrites with ensuing abnormal dendritic morphology in neurons, in particular in Purkinje cells of the cerebellum, that precedes their death²⁵. Therefore, mitochondrial dysfunction/mislocalization are the likely cellular bases for ARSACS, supporting that, as in Friedreich ataxia and a growing of other ataxias and neurodegenerative disorders, mitochondrial dysfunction plays a central role in their pathophysiology²⁶.

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