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Assessment of Sacsin Turnover in Patients With ARSACS: Implications for Molecular Diagnosis and Pathogenesis

Author(s):

Fabiana Longo, PhD[1]; Daniele De Ritis, MSc[1]; Annarita Miluzio, PhD[2]; Davide Fraticelli, MSc[1]; Jonathan Baets, MD[3;4]; Marina Scarlato, MD[5]; Filippo M. Santorelli, MD[6]; Stefano Biffo, PhD[2;7]; Francesca Maltecca, PhD[1;8]

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Equal Author Contributions:
n/a

Corresponding Author:
Francesca Maltecca
maltecca.francesca@hsr.it

Affiliation Information for All Authors: 1. Mitochondrial Dysfunctions in Neurodegeneration Unit, Ospedale San Raffaele, Milan, Italy; 2. Istituto Nazionale di Genetica Molecolare, INGM, "Romeo ed Enrica Invernizzi", Milan, Italy;3. Laboratory of Neuromuscular Pathology, Institute Born-Bunge, University of Antwerp, Antwerpen, Belgium; 4. Neuromuscular Reference Centre, Department of Neurology, Antwerp University Hospital, Antwerpen, Belgium;5. Department of Neurology, Ospedale San Raffaele, Milan, Italy; 6. Molecular Medicine, IRCCS Fondazione Stella Maris, Pisa, Italy;7. Department of Biosciences, University of Milan, Milan, Italy; 8. Università Vita-Salute San Raffaele, Milan, Italy.

Contributions:
Fabiana Longo: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data
Daniele De Ritis: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Analysis or interpretation of data
Annarita Miluzio: Major role in the acquisition of data
Davide Fraticelli: Major role in the acquisition of data
Jonathan Baets: Drafting/revision of the manuscript for content, including medical writing for content
Marina Scarlato: Drafting/revision of the manuscript for content, including medical writing for content
Filippo M. Santorelli: Drafting/revision of the manuscript for content, including medical writing for content
Stefano Biffo: Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; Analysis or interpretation of data
Francesca Maltecca: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data

Abstract

Background and Objectives: Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is caused by mutations in *SACS* gene encoding sacsin, a huge multimodular protein of unknown function. More than 200 *SACS* mutations have been described worldwide to date. Since ARSACS presents phenotypic variability, previous empirical studies attempted to correlate the nature and position of *SACS* mutations with the age of onset or with disease severity, though not considering the effect of the various mutations on protein stability. In this work, we studied genotype-phenotype correlation in ARSACS at a functional level.

Methods: We analyzed a large set of skin fibroblasts derived from ARSACS patients, including both new and already published cases, carrying mutations of different type affecting diverse domains of the protein.

Results: We found that sacsin is almost absent in ARSACS patients, regardless of the nature of the mutation. As expected, we did not detect sacsin in patients with truncating mutations. Interestingly, we found it strikingly reduced or absent also in compound heterozygotes carrying diverse missense mutations. In this case, we excluded *SACS* mRNA decay, defective translation or faster post-translational degradation as possible causes of protein reduction. Conversely, our results demonstrate that nascent mutant sacsin protein undergoes cotranslational ubiquitination and degradation.

Discussion: Our results provide one mechanistic explanation for the lack of genotype-phenotype correlation in ARSACS. We also propose a new and unambiguous criterion for ARSACS diagnosis, that is based on the evaluation of sacsin level. Finally, we identified preemptive degradation of a mutant protein as a novel cause of a human disease.

Introduction

ARSACS (MIM #270550)[1] was first described in the Charlevoix-Saguenay population in Québec, where it shows high prevalence as the result of a founder effect. The disease is now present worldwide and it represents the second most frequent recessive ataxia after Friedreich's ataxia.[2] Clinically, ARSACS is characterized by progressive cerebellar ataxia, spasticity and sensorimotor peripheral neuropathy. However, the clinical spectrum is highly variable, with an increasing number of diagnosed subjects with disease onset in early adult-years, or with a clinical presentation including only one or two of the three typical symptoms. Also, a minority of patients present intellectual disability, epileptic seizures, urinary dysfunction and hearing loss.[3-6]

ARSACS is caused by mutations in *SACS* gene (MIM *604490),[1] which is one of the largest of our genome.[7] *SACS* gene encodes the 520 kDa multimodular protein sacsin, highly expressed in the central nervous system.[8]

From the N-terminus to the C-terminus, the amino acid sequence of sacsin contains: an ubiquitin-like (UbL) domain,[9] three sacsin-repeating regions (SRR) with homology with Hsp90,[10] a Xeroderma Pigmentosus group C protein Binding domain,[11] a DnaJ domain,[9] and a Higher Eukaryotes and Prokaryotes Nucleotide-binding domain.[12] Despite these motifs indicate that sacsin may operate in protein quality control, its function remains largely unknown. Previous works suggested a role of sacsin in cytoskeleton and mitochondrial dynamics.[13, 14]

More than 200 mutations have been described worldwide, equally divided in homozygous or compound heterozygous.[6, 15] The majority are missense, followed by small deletions, frameshift and nonsense and spread over the whole gene.[6] ARSACS presents inter- or intrafamilial variability, with no clear-cut genotype-phenotype correlation.[3-5, 16-18]

In the perspective of a precision medicine approach, some studies have tried to correlate the pathogenicity and/or the position of the *SACS* mutations with the age of onset or the clinical severity. Romano *et al.* suggested that subjects carrying missense mutations in homozygosity

or heterozygosity with a null allele exhibit significantly milder phenotypes than those carrying a truncating mutation on each allele.[19] In a more recent work, considering all the published ARSACS mutations up to 2019, Xiromerisiou *et al.* identified a correlation between the pathogenicity score of the mutations, the phenotype severity and the age of onset.[6] However, these *in silico* studies have major limitations, since they did not consider the consequences of the various *SACS* mutations on sacsin protein levels.

In comparison to null mutations, the outcomes of missense mutations are very hard to predict. Indeed, they can hit proteins by altering either their stability or, conversely, their functionality. In the first case, mutant proteins are conventionally targeted to the proteasome or to the autophagic pathway for degradation upon complete synthesis.[20]

In this work, we sought to fill a gap in ARSACS pathogenesis by studying the turnover of mutant sacsin in a large set of ARSACS patient fibroblasts carrying mutations of different nature and position along *SACS* gene.

Methods

Subjects' consent

Eleven subjects with the clinical and genetic diagnosis of ARSACS (Table 1) were retrospectively recruited among the cohort of spastic-ataxic patients referring to each center: Ospedale San Raffaele (Milan, Italy), IRCCS Stella Maris (Pisa, Italy) and VIB Uantwerp Center for Molecular Neurology (Uantwerp, Belgium). The selection of patients was based on genotype, i.e. the different nature and position of *SACS* mutations, to have a comprehensive picture of ARSACS genetics, as detailed in the Results. All subjects included in the study gave their written informed consent according to protocols approved by the respective institutional human ethics review boards according to the Declaration of Helsinki.

Human primary fibroblast derivation from skin biopsies

Patient-derived skin fibroblasts were obtained during diagnostic procedures by punch skin biopsy performed on distal leg, following standard protocols as described previously.[21] In brief, the skin biopsy just picked up was gently transferred from a Falcon test tube in a T25 flask filled with 2mL of DMEM medium supplemented with 20% Fetal Bovine Serum (FBS), 1mM sodium pyruvate, 2mM L-glutamine and 100U/mL penicillin-streptomycin and daily monitored. After three weeks in culture, when the fibroblasts spreading from the biopsy were almost confluent, the first trypsin passage was performed. Then, the cells were cultured till passage 3, a stage at which fibroblasts are suitable for experiments and freezing.

Antibodies, drugs and reagents

Commercially available antibodies were used in Western blots (WB), for the detection of sacsin (Anti-sacsin AbC ab181190. Abcam, Cambridge, UK), spectrin (Anti-spectrin MAB1622 Merck Millipore, Burlington, MA, USA), ubiquitinated proteins (Anti-ubiquitin ab134953, Abcam), p62 (Anti-p62/SQSTM1 P0067 Merck Millipore), LC3I-II (Anti-LC3A/B ab58610. Abcam), HA (anti-HA Epitope Tag 16b12, Biolegend, San Diego, CA, USA). Secondary antibodies included HRP-conjugated anti-mouse and anti-rabbit IgG (Amersham Bioscience, Buckinghamshire, UK).

In vitro treatments were carried out with different compounds: 1 µM MG-132 24 hours or 3 hours (Merck Millipore), 20 µM Chloroquine (CQ) 24 hours (Merck Millipore) or together (0.25 µM MG-132 + 10 µM CQ, 18 hours). After the treatments, cells were harvested and lysed for biochemical assays.

Anti N-terminal sacsin antibody (AbN) generation

We generated a new rabbit polyclonal anti-sacsin antibody recognizing the N-terminal portion of sacsin (ID Q9NZJ4 (SACS_HUMAN)). We decided to develop a new anti-sacsin antibody (AbN) raised against a polypeptidic region of sacsin including aminoacids from 1 to

728 (all the exons till the giant exon 10). The antibody was produced by the antibody production service of Biomatik (Cambridge, Ontario, Canada).

SACS deletion by CRISPR/Cas9

Traditional 20 bp-NGG spCas9 gRNAs targeting *SACS* coding region were designed with CHOPCHOP web tool. The gRNA sequences were: gRNA-1 5' TGCTCCTGCGGTTATCAGTA 3'; gRNA-2 5' GTAGGCCATGCAATTCTCAT 3'. Oligos encoding the gRNAs were annealed and cloned into pCas-Guide-EF1a-GFP CRISPR/Cas9 plasmid (OriGene), according to the manufacturer instructions. To delete *SACS* gene, HeLa cells and SH-SY5Y cells were transfected with CRISPR/Cas9 plasmid carrying gRNA-1 or gRNA-2. GFP positive cells were sorted by FACS Aria Fusion (Becton Dickinson), and plated at single cell density. Monoclonal cell lines were expanded from single colonies and sacsin knockout (KO) was validated by immunoblot and by Sanger sequencing. Primers for gRNA-1 target region: forward primer 5' AGCAAAAGGAGCAACGTCTG 3'; reverse primer 5' GCTCTTTTCCATCTCCAGACG 3'. Primers for gRNA-2 target region: forward primer 5' AGCCAAAACCCTCTTACTGG 3'; reverse primer 5' AGTGGCTCTCTTTGTCCTGA 3'.

RNA extraction and Real Time-PCR (qRT- PCR)

Total mRNA from primary fibroblasts was extracted and retro-transcribed using standard procedures. *GAPDH* primers: forward primer 5' CCACCCAGAAGACTGTGGAT 3'; reverse primer 5' GTTGAAGTCAGAGGAGACCACC 3'. qRT- PCR was performed based on SYBR Green chemistry (Light Cycler 480. SYBR Green I master, Roche). *SACS* primers for the analysis of total *SACS* mRNA levels in patients and controls were: forward primer 5' TTTTCAGTTGCGAGGGGTTG 3'; reverse primer 5' TCCTGGCTTGGGAGGTAAAG 3'.

To normalize *SACS* mRNA levels, we used TATA Binding Protein (*TBP*) mRNA levels. *TBP* forward primer 5' ACGCCGAATATAATCCCAAG 3'; reverse primer 5' GCACACCATTTTCCCAGAAC 3'.

Polysomal profile analysis

Polysomal fractions were derived from immortalized growing fibroblasts as described,[22] and reverse transcription was performed. To analyze *SACS* mRNA, specific primers were used: forward primer 5' GGCAATTTTGTCCCCTTCTCC 3' reverse primer 5' GGTCTTCCTCGGGTTTGGG 3'. qRT-PCR for *TBP* was used as control of translation. For the analysis of targets from monosome and polysome fractions, the data were quantified as the percentage of expression in each fraction.

Immunoprecipitation of N-terminal sacsin fragments

Immortalized fibroblasts (transfected for 16 hours with Ubiquitin-HA plasmid or not) were treated for 3 hours with 1 μM MG-132 and lysed in RIPA buffer. 700 μg of lysate was immunoprecipitated using Dynabeads Protein (#10002D Dynabeads Protein A, ThermoFisher Scientific) bound to AbN (6 μg of antibody per 700 μg of protein) following the manufacturer instructions. Ubiquitin-HA plasmid was kindly provided by S. Polo's lab, IFOM, Milan.

Statistical analyses

Continuous variables were summarized by their mean values and Standard Error of the Mean (SEM). Differences in protein levels between patients and controls were assessed by densitometric analysis of WB bands from at least three independent experiments using Image J, followed by student *t*-test analysis.

Data availability

Data are available on request to the corresponding author.

Results

Clinical features and genetics of the patients

The cohort studied in this work resembles the wide ARSACS phenotypic spectrum in terms of onset (from 14 months to 25 years of age) and clinical features (Table 1). Notably, two sets of siblings are reported. Two brothers with an age difference of 3 years among them, despite carrying the same mutation, have a completely different phenotype: one with clear spastic paraparesis and the other with neuropathic/ataxic gait.

To have a broad picture of ARSACS genetics, we selected patients carrying mutations of different nature and localized in different domains of sacsin: compound heterozygous for a missense on one allele and a truncating frameshift on the other allele (PN1, PN2, PN7), compound heterozygous for different truncating mutations (PN3, PN6a, PN6b), compound heterozygous for a missense mutation and a big deletion (PN4), compound heterozygous for truncating mutations and a big deletion (PN8a, PN8b), compound heterozygous for missense mutations only (PN5, PN9) (Figure 1A).

Among the above-mentioned mutations, those carried by PN3 (p.[Glu723fsTer15];[Phe3209fsTer46]), PN6a and PN6b (p.[Ser1531fsTer9];[Arg1645Ter]), PN8a and PN8b (p.[Asn1586fsTer3];[deleted_allele]) and PN9 (p.[Pro536Leu];[Arg632Trp]) are reported in this study for the first time, at least in compound heterozygosity in the same patient. The other mutations are already published (PN1 and PN2;[4] PN4;[23] PN5;[24] PN7[25]).

Full-length sacsin protein is dramatically reduced in ARSACS patient fibroblasts regardless of the nature of the mutation

To study the impact of *SACS* mutations on sacsin levels, we derived skin fibroblasts from eleven compound heterozygous ARSACS patients, for a total of nineteen different mutations analyzed (Figure 1A).

To examine sacsin protein levels in the different ARSACS patients, we performed WB using a commercially available C-terminal anti-sacsin antibody (AbC) (Figure 1B) and a newly developed N-terminal anti-sacsin antibody (AbN) (Figure 1C) (epitopes shown on the sacsin protein scheme in Figure 1A). AbN was designed and produced by our group, as no sacsin N-terminal antibodies efficiently recognizing sacsin in WB are commercially available. To validate the specificity of AbN, we used sacsin KO HeLa and SH-SY5Y cells we engineered by CRISPR-Cas9 technology (right panel, Figure 1C).

Strikingly, with both antibodies we found that full-length sacsin protein is dramatically reduced or totally absent in all ARSACS patients, regardless of the nature of *SACS* mutations (Figure 1B-C, eFigure 1A-B). This finding was unexpected for patients carrying monoallelic missense mutations, and even more for those carrying biallelic missense mutations.

We then employed the AbN to identify putative truncated products in patients carrying frameshift mutations on *SACS* gene. AbN recognizes a large epitope spanning the first sacsin methionine residue, till the end of exon 9 (residue 1-728). This epitope contains the sacsin UbL domain, a fairly common domain among proteins predicted to interact with the proteasome.[9] AbN indeed recognizes other proteins carrying UbL domains (bands shared by controls and PNs in the WB in Figure 1C). Considering the position of the premature stop codon for each mutation, the expected molecular weights of the sacsin fragments are reported in Figure 1 legend and highlighted as asterisks in AbN WBs in Figure 1C. Despite the AbN efficiently recognized full-length sacsin protein, we were not able to appreciate any truncated sacsin fragments in WB by using AbN in any of the patients carrying frameshift mutations (Figure 1C).

SACS mRNA is reduced in ARSACS fibroblasts carrying frameshift mutations, while it is stable or even increased in those carrying missense mutations

To understand if the absence of full-length sacsin (and of truncated sacsin products) in ARSACS fibroblasts was due to mRNA instability, we first analyzed *SACS* mRNA levels by real-time PCR (qRT-PCR).

In ARSACS patients carrying only truncating mutations, *SACS* mRNA was evidently reduced compared to controls (PN3, PN8a, PN8b, PN6a and PN6b), suggesting a Non-sense Mediated Decay (NMD) of the mRNA. On the other hand, mRNA resulted stable (PN4, PN9) or even increased (PN5) in patients carrying missense mutations compared to controls. In ARSACS patients who are compound heterozygous for a missense and a frameshift mutation (PN1, PN2, PN7) we found no significant difference in the amount of *SACS* mRNA compared to controls, suggesting that the contribution of allele carrying the missense change determines the conserved amount of *SACS* mRNA (Figure 1D).

Inhibition of degradative systems does not rescue sacsin in ARSACS patients carrying missense mutations

Since *SACS* mRNA is stable and sacsin protein is dramatically reduced in ARSACS patients carrying missense mutations, we checked if mutant sacsin could undergo a faster post-translational degradation. Unfolded or aberrant proteins are usually targeted by the proteasome system or the autophagic system, the main molecular pathways involved in protein quality control and maintenance of cellular proteostasis.[20] To test this hypothesis, we blocked cellular degradative systems by treating patient cells either with a proteasome inhibitor (MG-132, 1 μM, 24 hours) or an autophagy inhibitor (Chloroquine (CQ), 20 μM, 24 hours) (Figure 2A-D). Both treatments did not rescue mutant sacsin in ARSACS patient fibroblasts carrying missense mutations. By blocking the proteasomal pathway for 24 hours, a statistically significant reduction of wild-type sacsin levels was observed in controls (Figure 2B). This suggested us a possible inhibition of protein synthesis due to a prolonged MG-132 treatment and consequent cellular stress induction.[26] We thus repeated the blockade of the proteasome reducing the time of treatment to 3 hours. In this condition, sacsin was stable in

controls, but again not rescued in patients (eFigure 2A-B). We also blocked proteasome and autophagy together, but also in this case we did not see any increase in the amount of sacsin protein in patients (eFigure 2C).

We then investigated if sacsin could be degraded by specific proteases. We inhibited cysteine proteases with E64, amino peptidases with bestatin and aspartyl proteases with pepstatin. The low amount of mutant sacsin in ARSACS patients was not modified by any of these treatments (eFigure 2D). We took in consideration that sacsin carrying missense mutations could be completely translated, but undetectable by standard WB procedures due to its misfolding and/or aggregation. To address this hypothesis, we performed different experiments to solve aggregates in biochemical assays. We checked for putative mutant sacsin aggregates by loading onto SDS-PAGE both the soluble and the insoluble fractions of control and patient fibroblasts (eFigure 3A-B). However, we were unable to detect sacsin aggregates in the insoluble fractions in patient cells. We also performed WB using mixed acrylamide-agarose gels to solve aggregates, or solubilized the cellular pellets with 8M Urea buffer, again without rescuing mutant sacsin levels (eFigure 3C-D, eMethods).

Altogether, these results indicate that the drastic reduction of mutant sacsin in ARSACS patients is not caused either by a faster post-translational degradation of the protein or aggregation.

Translation of mutant sacsin carrying missense mutations is not blocked in ARSACS patients

At this point we considered that the absence of sacsin carrying missense mutations could be due to its inefficient translation. We first excluded a general problem in translation, as polysomal profiles showed a similar pattern in ARSACS patients and controls (Figure 3A). To know if wild-type *SACS* mRNA could be subjected to a translational regulation *per se*, we performed meta-analysis of ribosome profiling data downloaded and analyzed exploiting the GWIPS-viz platform.[27, 28] This analysis showed little accumulation of ribosomes in the

5'UTR of *SACS* mRNA, and a homogenous density of ribosomes across the 13737 nucleotide long mRNA sequence, suggesting the absence of strong regulatory sequences in the 5'UTR (Figure 3B). To specifically assess the translation of sacsin, we investigated if *SACS* mRNA carrying missense mutations was associated to intact ribosomes (polysomes) or split ribosomes (monosomes, due to a blockade of translation). We thus performed qRT-PCR for sacsin on RNA extracted from the different ribosomal fractions (monosomes, light polysomes, heavy polysomes) for ARSACS patients and controls. We found that *SACS* mRNA was associated to the polysomal fractions (the actively translating fractions) in ARSACS patients (PN4, PN5, PN9) as well as in controls (Figure 3C), demonstrating that there is no blockade of its translation. The same mRNA distribution on the different ribosomal fractions was observed for a housekeeping gene (*TBP*) tested as control (Figure 3D).

Nascent mutant sacsin products are cotranslationally ubiquitinated and degraded

At this point, having experimentally excluded all the possible molecular mechanisms accounting for sacsin reduction in ARSACS patients, we considered that mutant sacsin could be degraded during translation. Two different quality control (QC) mechanisms exist during translation, both leading to the ubiquitination and degradation of nascent proteins. The first one, the ribosomal QC, is associated to the mRNA degradation and ribosome stalling/splitting (i.e the NMD, the No-Go Decay etc.).[29] The other one, the cotranslational QC, senses the correct folding of the nascent chain, promoting the degradation of the protein while it is being translated, and occurs in the presence of a stable mRNA associated to intact ribosomes.[30] This second scenario is the most plausible in the case of mutant sacsin carrying missense mutations, since we did not detect any reduction of the mRNA. Although poorly characterized, cotranslational QC is predicted to occur for big and multimodular proteins, whose folding takes place cotranslationally and proceeds in a domain-wise manner.[31] The

players involved in nascent protein degradation associated to cotranslational QC are completely unknown so far.

As formal proof of the cotranslational degradation of sacsin carrying missense mutations, we looked for the presence of ubiquitinated degradation products of nascent mutant sacsin in ARSACS patient cells carrying missense mutations only. Thus, we immunoprecipitated sacsin with AbN (in the view of recognizing N-terminal sacsin fragments of unpredictable molecular weights) in ARSACS patients (PN5, PN9) versus controls. Since putative mutant sacsin intermediates should be ubiquitinated and degraded by the proteasome, before the immunoprecipitation (IP) we treated cells with the proteasome inhibitor MG-132 (1μM, 3 hours) to improve their detection. We efficiently immunoprecipitated full-length sacsin with AbN in control and (to a lower extent as expected) in patient cells (Figure 4A). In ARSACS fibroblasts, sacsin degradation products are detectable especially in the higher part of the gel, in particular with the anti-ubiquitin immunodecoration (Figure 4A, right). To better resolve the higher part of the gel we decided to reload an independent sacsin IP in a 6% gel (Figure 4B). Sacsin degradation products were clearly visible in the IP lanes of ARSACS patients, especially when immunodecorated with the AbN (Figure 4B, left). To enhance sacsin ubiquitination, we transfected control and patient cells with Ubiquitin-HA construct and immunoprecipitated sacsin in the same conditions as before. We reconfirmed the detection of sacsin degradation products in ARSACS patients (Figure 4C) and, interestingly, we noticed that the differential bands present in the IP samples of patients were more evident by immunodecoration with anti-HA (revealing the ubiquitinated sacsin products) (Figure 4C, right).

According to our results, we can arrange a final model of the first event of ARSACS pathogenesis: in the presence of frameshift or nonsense mutations, *SACS* mRNA is degraded; in the presence of missense mutations, sacsin fails to fold and undergoes cotranslational degradation (Figure 5). In both cases the result is the absence/striking reduction of sacsin.

Discussion

In this work, we studied in detail for the first time the effects of different ARSACS-causing mutations on sacsin stability, at both mRNA and protein level. The cohort of ARSACS patients that we analyzed encompasses a wide range of diverse types of *SACS* mutations localized in different regions of the gene. Our results demonstrate that sacsin is barely detectable in ARSACS patients regardless of the nature or position of the mutation along the gene. While this result was expected for patients carrying biallelic truncating mutations (whose mRNA is unstable and degraded), it was not anticipated for patients carrying missense mutations, especially on both alleles. We indeed discovered that sacsin carrying missense mutations in any site of its sequence is cotranslationally ubiquitinated and degraded, rarely reaching its mature size. We surmise that this mechanism prevents the even more dangerous production of a huge misfolded protein, potentially highly prone to aggregation in the crowded cytosolic environment.

Since ARSACS presents variability in its clinical presentation, efforts have been dedicated to find a genotype-phenotype correlation, to better tailor a precision medicine approach, in terms of disease prognosis, family counselling and for future clinical trials. Our data however do not confirm previous reports only based on *in silico* analyses correlating the nature and/or position of the *SACS* mutations with the severity of the disease.[6, 19] Here, we demonstrated that loss of function in ARSACS is caused by loss/striking reduction of sacsin, independently of the mutation.

Excluding the Québec cohort, where the original c8844delT *SACS* mutation is highly prevalent, the 2020 worldwide ARSACS scenario shows that most mutations are missense, followed by small deletions, frameshift, nonsense and small insertions.[6] In our cohort of compound heterozygous patients, two of them carry biallelic missense mutations and five of them monoallelic missense, for a total of nine different missense changes. In all cases, the full-length protein is almost absent in patients with missense mutations (hitting different regions of the protein, from the N-terminus to the C-terminus) as well as in patients with

truncating mutations. Only in PN5 a residue of sacsin was detected with both AbC and AbN antibodies, which is anyway about 20% of controls, whereas in PN9 (who carries biallelic missense as well) sacsin is not detectable.

Our findings are supported by the recently published of $Sacs^{R272C/R272C}$ knockin mouse model, carrying a human pathogenetic mutation in the first SRR domain.[8] This model presents a scarcely detectable sacsin protein despite a stable mRNA and, accordingly, a phenotype overlapping with the one of the *Sacs* knockout mouse,[8, 13] confirming the conservation of the cotranslational degradation of mutant sacsin also in cerebellum. In addition, a striking reduction of sacsin was shown in other two reports of ARSACS mutations in patient fibroblasts or lymphoblasts,[14, 32] with no further mechanistic insights.

In this work, we could not directly test RNA and protein levels in heterozygous parents of patients carrying missense mutations. However, sacsin protein is halved in the heterozygous $Sacs^{R272C/+}$ mouse,[8] supporting that sacsin carrying the R272C missense mutation likely undergoes co-translational degradation. Halved amount of sacsin seems to be anyway sufficient to supply to cellular needs, as demonstrated by the fact that both $Sacs^{R272C/+}$ mice, as well as $Sacs^{+/-}$ mice, do not show any obvious phenotype,[8, 13] like healthy human carriers. Vimentin is indeed strikingly affected in $Sacs^{-/-}$ MEFs, forming abnormal perinuclear bundles as expected, while no difference is observed between the $Sacs^{+/-}$ and wild-type MEFs (eFigure 4A-B).

We found that *SACS* mRNA is stable or even increased also in the ARSACS compound heterozygous patients carrying biallelic or monoallelic missense mutations, excluding mRNA degradation as the possible explanation for the absence of sacsin. The failure in rescuing full-length sacsin upon the inhibition of all cellular degradative systems and several proteases excluded faster post-translational degradation. Also, mRNA of sacsin turned out to be associated with polysomes in ARSACS patients as well as in controls, rejecting the hypothesis of defective translation. We instead discovered that sacsin protein carrying missense mutations is cotranslationally ubiquitinated and degraded.

To preserve proteostasis, eukaryotic cells must not only promote accurate folding, but also prevent the accumulation of misfolded species that may arise from inefficient folding, errors in translation, and aberrant mRNAs. A growing body of evidence indicates that large and multimodular proteins start folding cotranslationally. For such proteins, domain-wise cotranslational folding helps reaching their native states and may reduce the probability for off-pathways and aggregation-prone conformations.[31, 33, 34] Also, many studies indicate that a sizable portion of nascent chains are cotranslationally ubiquitinated and degraded by the proteasome in physiological conditions, both in yeast,[35, 36] and in mammalian cells.[37] This likely applies to wild-type sacsin, a 520 kDa protein with a complex multimodular architecture containing repeated motifs, whose folding may take minutes to hours to complete. We hypothesize that cotranslational folding and degradation occur physiologically for sacsin as mechanisms of QC. In the case of sacsin carrying a missense mutation, after the unsuccessful attempts of chaperones to fold the nascent chain, the latter is constitutively ubiquitinated and degraded, preventing the synthesis of a misfolded full-length protein. This compartmentalized surveillance mechanism results in a loss-of-function, avoiding a potentially more dangerous toxic gain-of-function of mutant sacsin in the cytosol. Differently from pathogenetic missense mutations, missense variants present in healthy population probably do not impact the cotranslational folding of sacsin, but this hypothesis requires experimental validation.

We provided the formal proof of cotranslational degradation of mutant sacsin with the IP with AbN in condition of proteasome inhibition, which revealed the presence of several specific ubiquitinated sacsin products at lower molecular weight compared to the full-length in ARSACS patients carrying missense mutations.

The low residual amount of mutant sacsin that is present in some patients (PN5 and PN7), even if barely detectable, may be due to a quote of mutant protein escaping from cotranslational degradation, because C-terminally localized mutations are presumably more permissive.

Few studies observed cotranslational ubiquitination of mammalian proteins, although only in non-physiological conditions, i.e. *in vitro* translation, overexpression conditions or in the presence of artificial (non-pathogenic) mutations.[38,39] In cystic fibrosis (CF), this mechanism was observed for the CF transmembrane conductance regulator (CFTR), but only with the ΔF508 mutation (and not with other mutations).[40] Here we report *in vivo* the cotranslational degradation of a cytosolic protein (carrying any type of missense mutation) as the cause of a human disease.

Preemptive mutant sacsin degradation in ARSACS may be a paradigmatic example of a mechanism which can potentially underlie many other genetic diseases with loss of function. Our data identifies lack of sacsin protein as unifying mechanism shared by different *SACS* mutations, with multiple important implications for ARSACS disease managing.

For the diagnosis, we propose that the evaluation of sacsin levels should be included in the clinical genetics practice to establish a definite ARSACS diagnosis, or even as pre-screening in high probable cases to avoid expensive next-generation sequencing panel analysis, and certainly to validate variants of uncertain clinical significance (VUS). In a previous report, it was suggested that mitochondrial network abnormalities in patient fibroblasts could be evaluated when *SACS* genetics and molecular results are difficult to interpret.[41] However, this approach is experimentally complex and time consuming, compared to the evaluation of sacsin protein level that we propose here. We indeed found that sacsin is well detected in Peripheral Blood Mononuclear Cells (PBMCs) at both RNA and protein levels, making feasible the development of a diagnostic approach for ARSACS aimed at testing sacsin amount in peripheral blood (see eFigure 5A-C). For designing therapeutic strategies, cotranslational degradation of mutant sacsin makes unproductive any post translational approach. While our study excludes the nature/position of the *SACS* mutation as a cause of the diverse ARSACS phenotype, further studies are needed to explain the pronounced clinical variability. The genetic background is certainly playing a role in this regard. In the genetically relatively homogeneous Québec population, ARSACS typically starts with ataxia

and spasticity in the first decade of life, followed by neuropathy in the second decade, despite many different mutations have been identified.[32] However, in other countries 20% of ARSACS patients show atypical presentations with disease onset after the age of 20, a Charcot–Marie–Tooth (CMT)-like disorder, or absence of one of the three defining clinical features,[4, 5] epilepsy or hearing loss.[3, 6] Both inter- and intra-familial heterogeneity of this complex clinical condition might be explained with the combined effect of modifier genes, risk alleles or multilocus variations (including mutations/copy number variants (CNVs) of other genes) that can modify the phenotype in terms of penetrance, expressivity and age of onset. The unexpected huge variation in the human genome on a population level indeed challenges the canonical Mendelian disease analysis, pointing to a more comprehensive model in which variants with different effect size and environmental factors contribute to determine the phenotype of the genetic disease. A genome wide screen in ARSACS patients, aggregated based on phenotypic similarities, may provide a more complete genetic view of the disease. This approach is already in place for other rare Mendelian diseases, such as CMTs and others.[42-45]

Figure Titles and Legends

**Figure 1. Sacsin protein is drastically reduced in ARSACS fibroblasts carrying different *SACS* mutations**

A. Scheme of *SACS* gene and sacsin protein illustrating the identified functional domains and corresponding exons. For each patient (see Table 1), the type and position of the mutations on sacsin protein are indicated with a symbol and a color code. N and C-terminal anti-sacsin antibody (AbN and AbC) antibodies used in this study are shown (bottom). B. Representative WB showing residual sacsin protein in ARSACS patient-derived primary fibroblasts versus controls by using a AbC. Spectrin is used as loading normalizer. C. Representative WB showing residual sacsin protein in ARSACS patient-derived primary fibroblasts versus controls by using AbN. Asterisks indicate the expected truncated sacsin protein: PN1=412 kDa; PN2=8 kDa; PN3=81kDa,358 kDa; PN6a and PN6b=170 kDa,180 kDa; PN7=410 kDa;

PN8a and PN8b=175 kDa. D. *SACS* mRNA quantification by qRT-PCR in ARSACS patient-derived primary fibroblasts versus controls, normalized on *TBP* mRNA levels.

In (D), data are presented as mean ± SEM. *p≤0.05; **p<0.01; ***p<0.001 (unpaired-two tailed Student's t-test).

Abbreviations: PN: patient; Ctr: control; AbN/C: N/C-terminal anti-sacsin antibody; UbL: Ubiquitin-Like domain; SRR: Sacsin Repeating Region; XPCB: Xeroderma Pigmentosus group C protein Binding domain; HEPN: Higher Eukaryotes and Prokaryotes Nucleotide-binding domain; bp: base pair; aa: aminoacid.

A

AbN

AbC

B

C

D
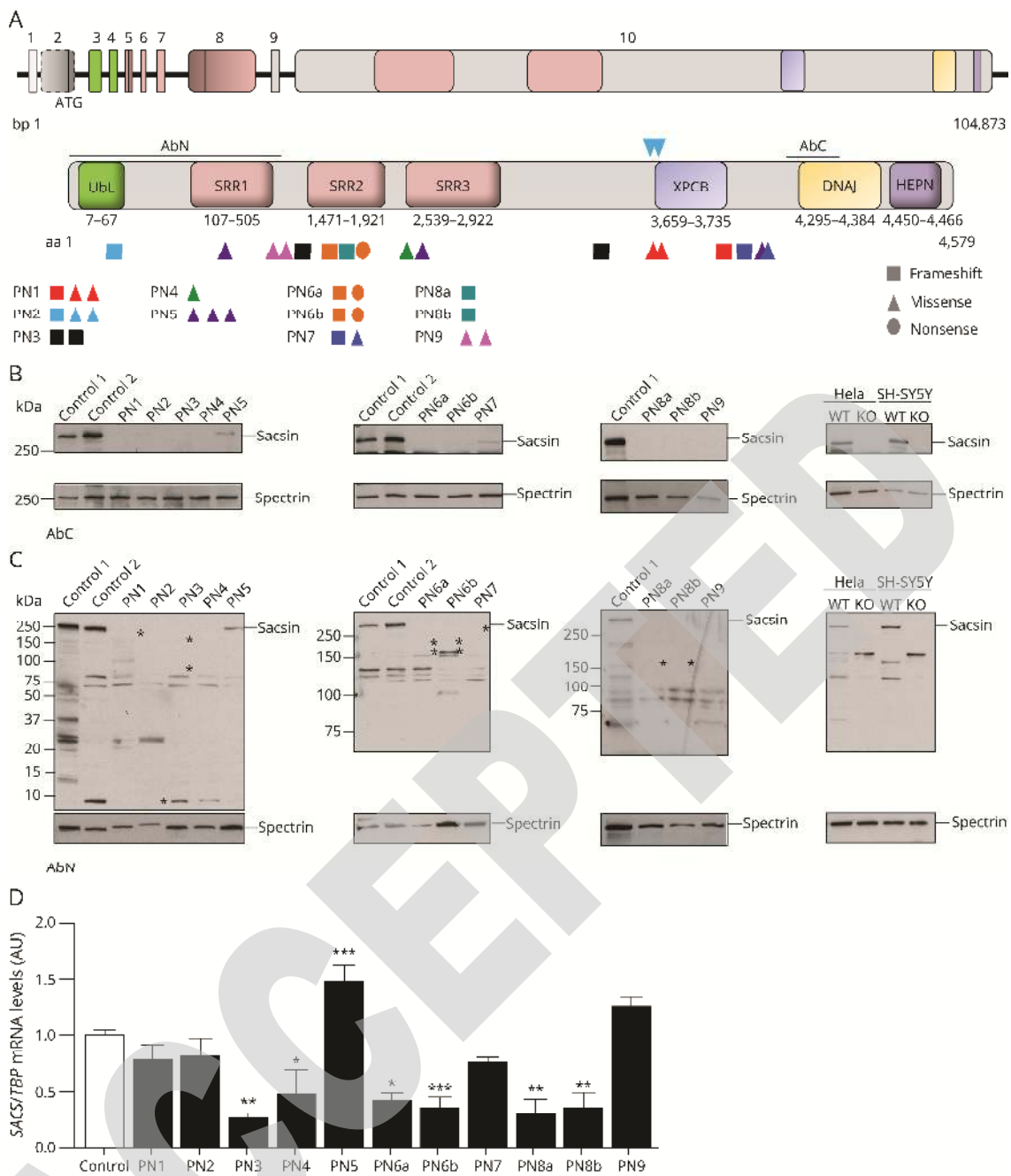
**Figure 2. Mutant sacsin is not post-translationally degraded**

A. Representative WB of sacsin levels in patient and control fibroblasts after proteasome blockade experiments, by using 1 µM MG-132 for 24 hours. Ubiquitinated proteins are used as readout of the treatment and spectrin as loading normalizer. B. Quantification graph relative to A. C. Representative WB of sacsin levels in patient and control fibroblasts after autophagy blockade experiments, by using 20 µM Chloroquine (CQ) for 24 hours. LC3I conversion in LC3II is used as readout of the treatment. D. Quantification graph relative to C. In (B,D) data are presented as mean ± SEM. n=at least 3, *p≤0.05 (unpaired-two tailed Student's t-test).

Abbreviations: +: treated; -: vehicle.
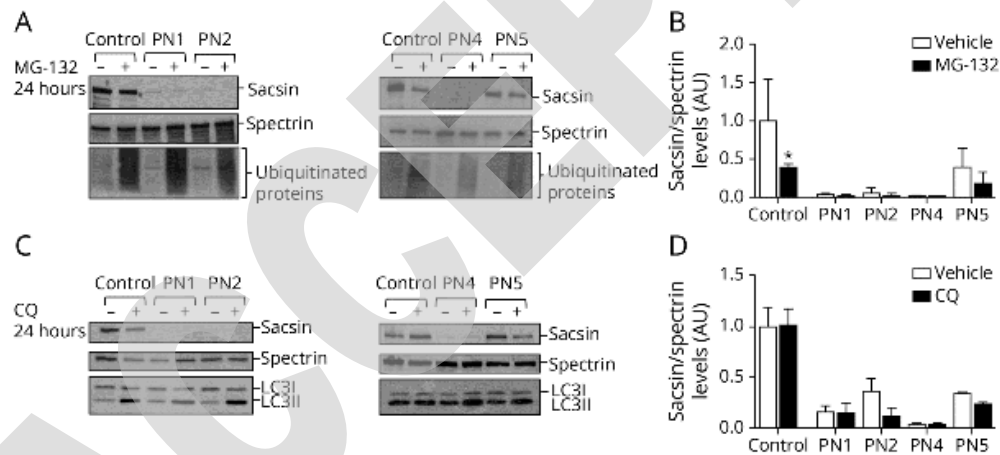
Figure 3. *SACS* **mRNA carrying missense mutations is associated to the polysomal fractions**

A. Representative ribosomal profile graphs in control (A.a) and ARSACS (A.b) patient fibroblasts. B. Ribosomal density on the human sacsin mRNA, retrieved and analyzed from available riboseq datasets. C. qRT-PCR data of the *SACS* mRNA distribution in different ribosomal fractions: monosomes, light and heavy polysomes in ARSACS patient fibroblasts (PN4, PN5, PN9) compared to 3 different controls. D. *TBP* mRNA distribution is shown as housekeeping gene analyzed in the same cell.
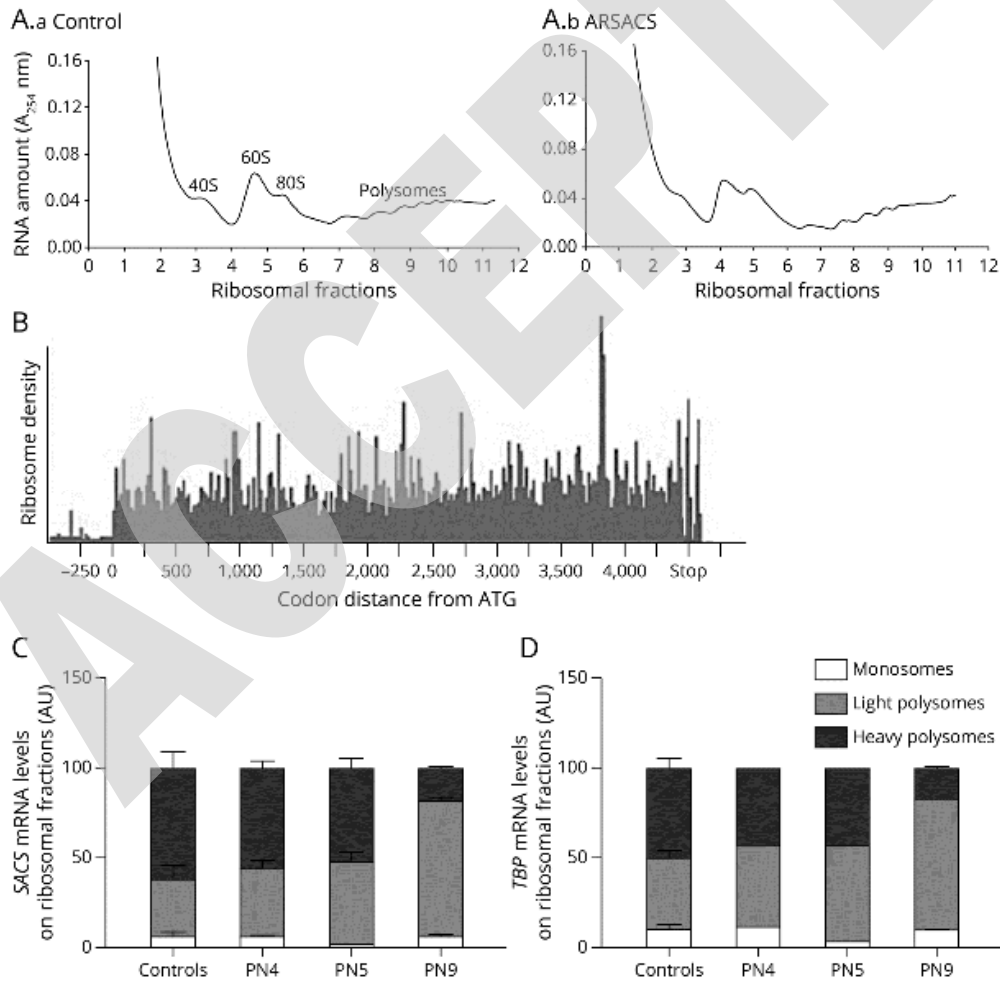
Figure 4. Sacsin protein carrying missense mutations is co-translationally ubiquitinated and degraded

A. Sacsin IP by using AbN in ARSACS patient fibroblasts carrying missense mutations (PN5, PN9) and a control treated with 1 μM MG-132 for 3 hours. 4-12 % gradient gel showing proteins from high molecular weights to 15 kDa, immunodecorated with AbN (A.a) or ubiquitin (A.b). B. Independent experiment conducted in the same conditions loaded onto a 6% gel to resolve putative differential N-terminal sacsin bands in patients compared to control. Immunodecoration with AbN (B.a WB) or ubiquitin (B.b WB, red asterisks indicate N-terminal sacsin products visible only in the patient IP samples). C. Sacsin IP by using AbN in ARSACS patient fibroblasts carrying missense mutations (PN5, PN9) and a control upon Ubiquitin-HA overexpression, treated with 1 μM MG-132 for 3 hours. 6 % gel showing immunodecoration with AbN (C.a) and with anti-HA revealing ubiquitinated proteins (C.b). Red asterisks indicate differential N-terminal sacsin products visible only in the patient IP samples. Abbreviations: IP: immunoprecipitation; FT: Flow through fractions; IB: immunoblotting.
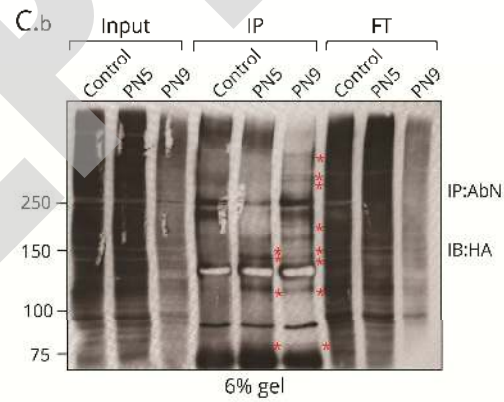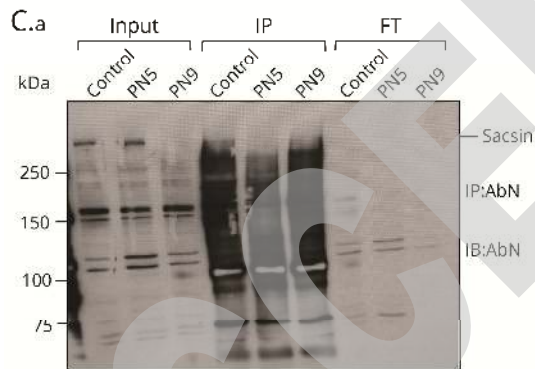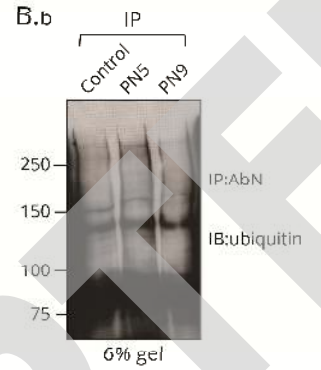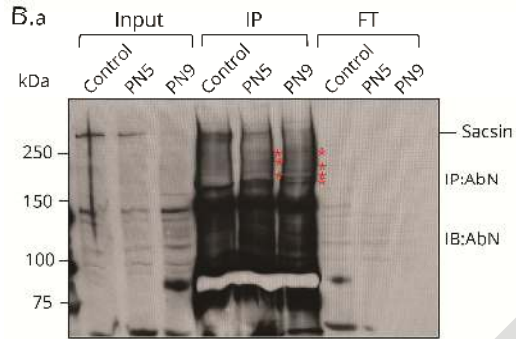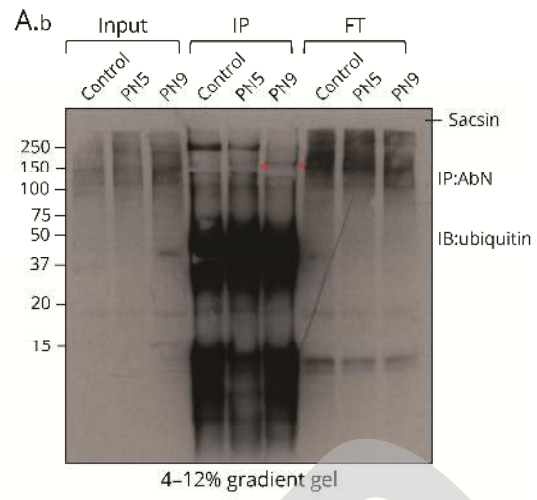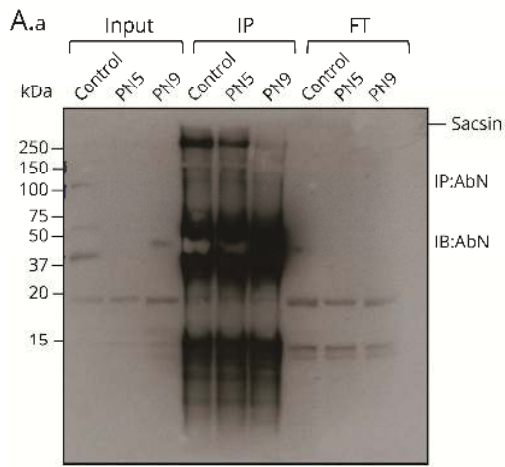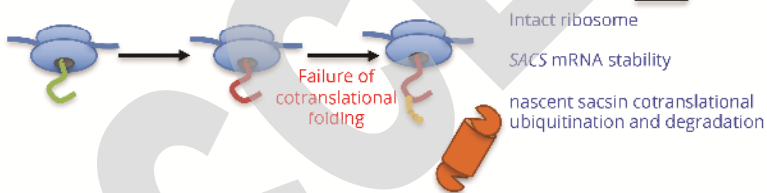
Figure 5. Full length sacsin protein is almost absent in ARSACS patients, independently of the nature of the mutations

Final model explaining why mutant sacsin is almost absent in patient cells. In (1) sacsin is prevented to be translated because the *SACS* mRNA carrying frameshift mutations is degraded by nucleases, while in (2) the *SACS* mRNA carrying missense mutations is stable and sacsin is cotranslationally degraded. The proteasome is the degradative system implied in these pathways (drawn in orange). Green nascent chain: before sensing the mutation; red nascent chain: after sensing domain misfolding.

A. *SACS* mRNA with frameshift/nonsense mutations

Ribosome stalling

SACS mRNA degradation

Absence/striking reduction of sacsin

ARSACS

B. *SACS* mRNA with missense mutations

Failure of cotranslational folding

Intact ribosome

SACS mRNA stability

nascent sacsin cotranslational ubiquitination and degradation

ARSACS patients	*SACS* mutations	Age of onset and sex	DSI-ARSACS	ataxia	dysarthria	sensory loss	walking difficulties/ support/ wheelchair	RNFL thickness by OCT	age at MRI	TCC/cerebral atrophy/WM changes	cerebellar atrophy (hem/vermis)	spinal cord atrophy	Reference
PN1	p.[(Arg3636Gln;Pro3652Thr)]; [Leu3745fsTer1]	15 yrs M	nv	++	++	+	wheelchair	nv	35	-/-/-	-	-	4
PN2	p.[Cys72fsTer4]; [(Arg3636Gln;Pro3652Thr)]	>25 yrs M	nv	+	+	+	walking with support	nv	58	-/+/-	+	-	4
PN3	p.[Glu723fsTer15];[Phe3209fsTer46]	5 yrs M	nv / SARA score: 10/40	+	-/+	-	wheelchair	nv	5	+/-/-	+	nv	Unpublished
PN4	p.[Leu2374Ser];[deleted_allele]	26 months M	nv / SARA score: 26/40	++	++	+++	walking difficulties	nv	54	-/ +-/+-	+++ vermis	nv	23
PN5	p.[(Gly188Glu;Ser2465Leu)]; [Leu3916Trp]	16 yrs M	9	+	-/+	+	walking with support	+	36	-/-/-	+ vermis	+	24
PN6a	p.[Ser1531fsTer9];[Arg1645Ter]	18 months M	17	+	++	+	walking difficulties	++	24	-/+/+-	++ vermis	+	Unpublished
PN6b	p.[Ser1531fsTer9];[Arg1645Ter]	18 months M	12	++	-/+	+++	walking with support	++	25	-/-/-	++ vermis	+	Unpublished
PN7	p.[Ile3755fsTer8];[Asp3926Gly]	14 months F	16	++	-	++	walking with support	++	28	-/-/-	+ vermis	+	25
PN8a	p.[Asn1586fsTer3];[deleted_allele]	14 months F	17	++	-	++	wheelchair	++	27	-/-/-	++ vermis	+	Unpublished
PN8b	p.[Asn1586fsTer3];[deleted_allele]	18 months F	21	+	-	++	walking difficulties	++	34	-/ +-/-	+ vermis	+	Unpublished
PN9	p.[Pro536Leu];[Arg632Trp]	16 yrs M	nv / SARA score: 17/40	-	-	+	wheelchair	nv	29	-/-/-	+	+	Unpublished

Table 1. Clinical features of ARSACS patients reported in this study. – absent, +- subtle, + present, ++ strongly present, +++ very strongly present, nv not evaluated. Abbreviations: DSI=disease severity index; MRI=magnetic resonance imaging; TCC=thin corpus callosum; WM= white matter; RNFL=retinal nerve fiber layer; OCT=optical coherence tomography

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Assessment of Sacsin Turnover in Patients With ARSACS: Implications for Molecular Diagnosis and Pathogenesis

Fabiana Longo, Daniele De Ritis, Annarita Miluzio, et al.

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