

# Assessment of Sacsin Turnover in Patients With ARSACS

## Implications for Molecular Diagnosis and Pathogenesis

Fabiana Longo, PhD, Daniele De Ritis, MSc, Annarita Miluzio, PhD, Davide Fraticelli, MSc, Jonathan Baets, MD, Marina Scarlato, MD, Filippo M. Santorelli, MD, Stefano Biffo, PhD, and Francesca Maltecca, PhD

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### Correspondence

Dr. Maltecca  
maltecca.francesca@hsr.it

## Abstract

### Background and Objectives

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is caused by variations in SACS gene encoding saccin, a huge multimodular protein of unknown function. More than 200 SACS variations have been described worldwide to date. Because ARSACS presents phenotypic variability, previous empirical studies attempted to correlate the nature and position of SACS variations with the age at onset or with disease severity, although not considering the effect of the various variations 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 patients with ARSACS, including both new and already published cases, carrying variations of different types affecting diverse domains of the protein.

### Results

We found that saccin is almost absent in patients with ARSACS, regardless of the nature of the variation. As expected, we did not detect saccin in patients with truncating variations. We found it strikingly reduced or absent also in compound heterozygotes carrying diverse missense variations. 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 saccin protein undergoes cotranslational ubiquitination and degradation.

### Discussion

Our results provide a 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 saccin level. Last, we identified preemptive degradation of a mutant protein as a novel cause of a human disease.

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From the Mitochondrial Dysfunctions in Neurodegeneration Unit (F.L., D.D.R., D.F., F.M.) and Department of Neurology (M.S.), Ospedale San Raffaele, Milan, Italy; Istituto Nazionale di Genetica Molecolare (A.M., S.B.), INGM, "Romeo ed Enrica Invernizzi," Milan, Italy; Laboratory of Neuromuscular Pathology (J.B.), Institute Born-Bunge, University of Antwerp; Neuromuscular Reference Centre (J.B.), Department of Neurology, Antwerp University Hospital, Belgium; Molecular Medicine (F.M.S.), IRCCS Fondazione Stella Maris, Pisa, Italy; Department of Biosciences (S.B.), University of Milan; and Università Vita-Salute San Raffaele (F.M., D.D.R.), Milan, Italy.

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## Glossary

**AbC** = C-terminal anti-sacsin antibody; **AbN** = N-terminal anti-sacsin antibody; **ARSACS** = autosomal recessive spastic ataxia of Charlevoix-Saguenay; **QC** = quality control; **qRT-PCR** = Quantitative real-time PCR; **UbL** = ubiquitin-like; **WB** = Western blot.

ARSACS (MIM No. 270550)<sup>1</sup> was first described in the Charlevoix-Saguenay population in Québec, where it shows high prevalence as result of a founder effect. The disease is now present worldwide, and it represents the second most frequent recessive ataxia after Friedreich's ataxia.<sup>2</sup> 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 patients with disease onset in early adult years or with a clinical presentation including only 1 or 2 of the 3 typical symptoms. In addition, a minority of patients present intellectual disability, epileptic seizures, urinary dysfunction, and hearing loss.<sup>3-6</sup>

ARSACS is caused by variations in *SACS* gene (MIM No. \*604490),<sup>1</sup> which is one of the largest of our genome.<sup>7</sup> *SACS* gene encodes the 520-kDa multimodular protein sacsin, highly expressed in the CNS.<sup>8</sup>

From the N-terminus to the C-terminus, the amino acid sequence of sacsin contains the following: an ubiquitin-like (UbL) domain,<sup>9</sup> three sacsin-repeating regions with homology with Hsp90,<sup>10</sup> a xeroderma pigmentosus group C protein binding domain,<sup>11</sup> a DnaJ domain,<sup>9</sup> and a higher eukaryotes and prokaryotes nucleotide-binding domain.<sup>12</sup> Despite these motifs indicate that sacsin may operate in protein quality control (QC), its function remains largely unknown. Previous works suggested a role of sacsin in cytoskeleton and mitochondrial dynamics.<sup>13,14</sup>

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

In the perspective of a precision medicine approach, some studies have tried to correlate the pathogenicity or the position of the *SACS* variations with the age at onset or the clinical severity. A previous work suggested that individuals carrying missense variations in homozygosity or heterozygosity with a null allele exhibit significantly milder phenotypes than those carrying a truncating variation on each allele.<sup>19</sup> In a more recent work, considering all the published ARSACS variations up to 2019, a correlation was identified among the pathogenicity score of the variations, the phenotype severity, and the age at onset.<sup>6</sup> However, these *in silico* studies have major limitations because they did not consider the consequences of the various *SACS* variations on sacsin protein levels.

Compared to null variations, the outcomes of missense variations 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.<sup>20</sup>

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

## Methods

### Participant Consent

Eleven patients with the clinical and genetic diagnosis of ARSACS (Table) 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 University of Antwerp Center for Molecular Neurology. The selection of patients was based on genotype, that is the different nature and position of *SACS* variations, to have a comprehensive picture of ARSACS genetics, as detailed in the Results. All participants 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.<sup>21</sup> In brief, the skin biopsy just picked up was gently transferred from a Falcon test tube in a T25 flask filled with 2 mL Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100U/mL penicillin-streptomycin and monitored daily. 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 until 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 (WBs), for the detection of sacsin (anti-sacsin AbC ab181190, Abcam, Cambridge, UK), spectrin (anti-spectrin MAB1622, Merck Millipore, Burlington, MA), ubiquitinated proteins

**Table** Clinical Features of Patients With ARSACS Reported in This Study

Patients with ARSACS	SACS variations	Age at 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 y M	nv	++	++	+	Wheelchair	nv	35	-/-/-	—	—	4
PN2	p.[Cys72fsTer4]; [(Arg3636Gln; Pro3652Thr)]	>25 y M	nv	+	+	+	Walking with support	nv	58	-/+/-	+	—	4
PN3	p.[Glu723fsTer15]; [Phe3209fsTer46]	5 y M	nv/SARA score: 10/40	+	-/+	—	Wheelchair	nv	5	+/-/-	+	nv	Unpublished
PN4	p.[Leu2374Ser]; [deleted_allele]	26 mo M	nv/SARA score: 26/40	++	++	+++	Walking difficulties	nv	54	-/+/-+	+++ Vermis	nv	23
PN5	p.[(Gly188Glu; Ser2465Leu)]; [Leu3916Trp]	16 y M	9	+	-/+	+	Walking with support	+	36	-/-/-	+ Vermis	+	24
PN6a	p.[Ser1531fsTer9]; [Arg1645Ter]	18 mo M	17	+	++	+	Walking difficulties	++	24	-/+/-+	++ Vermis	+	Unpublished
PN6b	p.[Ser1531fsTer9]; [Arg1645Ter]	18 mo M	12	++	-/+	+++	Walking with support	++	25	-/-/-	++ Vermis	+	Unpublished
PN7	p.[Ile3755fsTer8]; [Asp3926Gly]	14 mo F	16	++	—	++	Walking with support	++	28	-/-/-	+ Vermis	+	25
PN8a	p.[Asn1586fsTer3]; [deleted_allele]	14 mo F	17	++	—	++	Wheelchair	++	27	-/-/-	++ Vermis	+	Unpublished
PN8b	p.[Asn1586fsTer3]; [deleted_allele]	18 mo F	21	+	—	++	Walking difficulties	++	34	-/+/-	+ Vermis	+	Unpublished
PN9	p.[Pro536Leu]; [Arg632Trp]	16 y M	nv/SARA score: 17/40	—	—	+	Wheelchair	nv	29	-/-/-	+	+	Unpublished

Abbreviations: ARSACS = autosomal recessive spastic ataxia of Charlevoix-Saguenay; DSI = disease severity index; nv: not evaluated; OCT = optical coherence tomography; RNFL = retinal nerve fiber layer; TCC = thin corpus callosum; WM = white matter; - = absent, +- = subtle, + = present, ++ = strongly present, +++ = very strongly present.

(anti-ubiquitin ab134953, Abcam), p62 (anti-p62/SQSTM1 P0067, Merck Millipore), LC3I-II (anti-LC3A/B ab58610, Abcam), and HA (anti-HA Epitope Tag 16b12, Biolegend, San Diego, CA). Secondary antibodies included horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (Amersham Bioscience, Buckinghamshire, UK).

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

### Anti N-Terminal Anti-sacsin Antibody Generation

We generated a new rabbit polyclonal anti-sacsin antibody recognizing the N-terminal portion of sacsins (ID Q9NZJ4 [SACS\_HUMAN]). We decided to develop a new anti-sacsin antibody (N-terminal sacsins antibody [AbN]) raised against a polypeptidic region of sacsins including amino acids from 1 to 728 (all the exons until 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 the CHOPCHOP web tool. The gRNA sequences were as follows: gRNA-1 5' TGCTCCTGCGTTATCAGTA 3' and gRNA-2 5' GTAGGCCATGCAATTCTCAT 3'. Oligos encoding the gRNAs were annealed and cloned into pCas-Guide-EF1a-green fluorescent protein CRISPR/Cas9 plasmid (OriGene, Rockville, MD), according to the manufacturer's instructions. To delete SACS gene, HeLa cells and SH-SY5Y cells were transfected with CRISPR/Cas9 plasmid carrying gRNA-1 or gRNA-2. Green fluorescent protein-positive cells were sorted by FACS Aria Fusion (Becton Dickinson, Franklin Lakes, NJ), and plated at single cell density. Monoclonal cell lines were expanded from single colonies, and sacsins knockout was validated by immunoblot and by Sanger sequencing. Primers for gRNA-1 target region were as follows: forward primer 5' AGCAAAGGAGCAACGTCTG 3' and reverse primer 5' GCTCTTTTCCATCTCCAGACG 3'. Primers for gRNA-2 target region were forward primer 5' AGCCAAAACCTCT TACTGG 3' and reverse primer 5' AGTGGCTCTTTTGT CCTGA 3'.

### RNA Extraction and Real Time-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'. Quantitative real-time PCR (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 as follows: forward primer 5' TTTTCAGTTGCGAGGGGTTG 3' and reverse primer 5' TCCTGGCTTGGGAGGTAAAG 3'.

To normalize SACS mRNA levels, we used TATA binding protein (*TBP*) mRNA levels: *TBP* forward primer 5' ACGCCGAATATAATCCCAAG 3' and reverse primer 5' GCACACCATTTTCCCAGAAC 3'.

### Polysomal Profile Analysis

Polysomal fractions were derived from immortalized growing fibroblasts as described,<sup>22</sup> and reverse transcription was performed. To analyze SACS mRNA, specific primers were used: forward primer 5' GGCAATTTTGTCCCCTTCTCC 3' and 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 Sacsins Fragments

Immortalized fibroblasts (transfected for 16 hours with ubiquitin-HA plasmid or not) were treated for 3 hours with 1  $\mu$ M MG-132 and lysed in radioimmunoprecipitation assay buffer. Lysate (700  $\mu$ g) was immunoprecipitated with Dynabeads Protein (No. 10002D Dynabeads Protein A, ThermoFisher Scientific, Waltham, MA) bound to AbN (6  $\mu$ g antibody per 700  $\mu$ g protein) following the manufacturer's instructions. Ubiquitin-HA plasmid was kindly provided by Dr. Simona Polo's laboratory, IFOM, Milan.

### Statistical Analyses

Continuous variables were summarized by their means and SEMs. Differences in protein levels between patients and controls were assessed by densitometric analysis of WB bands from at least 3 independent experiments with 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–25 years of age) and clinical features (Table). Notably, two sets of siblings are reported. Two brothers with an age difference of 3 years between them, despite carrying the same variation, 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 variations of different nature and localized in different domains of sacsins: compound heterozygous for a missense on one allele and a truncating frameshift on the other allele (PN1, PN2, PN7), compound heterozygous for different truncating variations (PN3, PN6a, PN6b), compound heterozygous for a missense variation and a big deletion (PN4), compound heterozygous for truncating variations and a big

deletion (PN8a, PN8b), and compound heterozygous for missense variations only (PN5, PN9) (Figure 1A).

Among the above-mentioned variations, 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 novel ARSACS causing-variations, at least reported in compound heterozygosity in the same patient. The other variations are already published (PN1 and PN2;<sup>4</sup> PN4;<sup>23</sup> PN5;<sup>24</sup> PN7<sup>25</sup>).

### Full-Length Sacsin Protein Is Dramatically Reduced in Fibroblasts From Patients With ARSACS Regardless of the Nature of the Variation

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

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

Strikingly, with both antibodies, we found that full-length saccin protein is dramatically reduced or totally absent in all patients with ARSACS, regardless of the nature of SACS variations (Figure 1, B and C and eFigure 1, A and B, links.lww.com/WNL/B619). This finding was unexpected for patients carrying monoallelic missense variations and even more unexpected for those carrying biallelic missense variations.

We then used the AbN to identify putative truncated products in patients carrying frameshift variations on SACS gene. AbN recognizes a large epitope spanning the first saccin methionine residue, until the end of exon 9 (residue 1–728). This epitope contains the saccin UbL domain, a fairly common domain among proteins predicted to interact with the proteasome.<sup>9</sup> 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 variation, the expected molecular weights of the saccin fragments are reported in the Figure 1 legend and highlighted as asterisks in AbN WBs in Figure 1C. Despite that AbN efficiently recognized full-length saccin protein, we were not able to appreciate any truncated saccin fragments in WB by using AbN in any of the patients carrying frameshift variations (Figure 1C).

### SACS mRNA Is Reduced in ARSACS Fibroblasts Carrying Frameshift Variations, While It Is Stable or Even Increased in Those Carrying Missense Variations

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

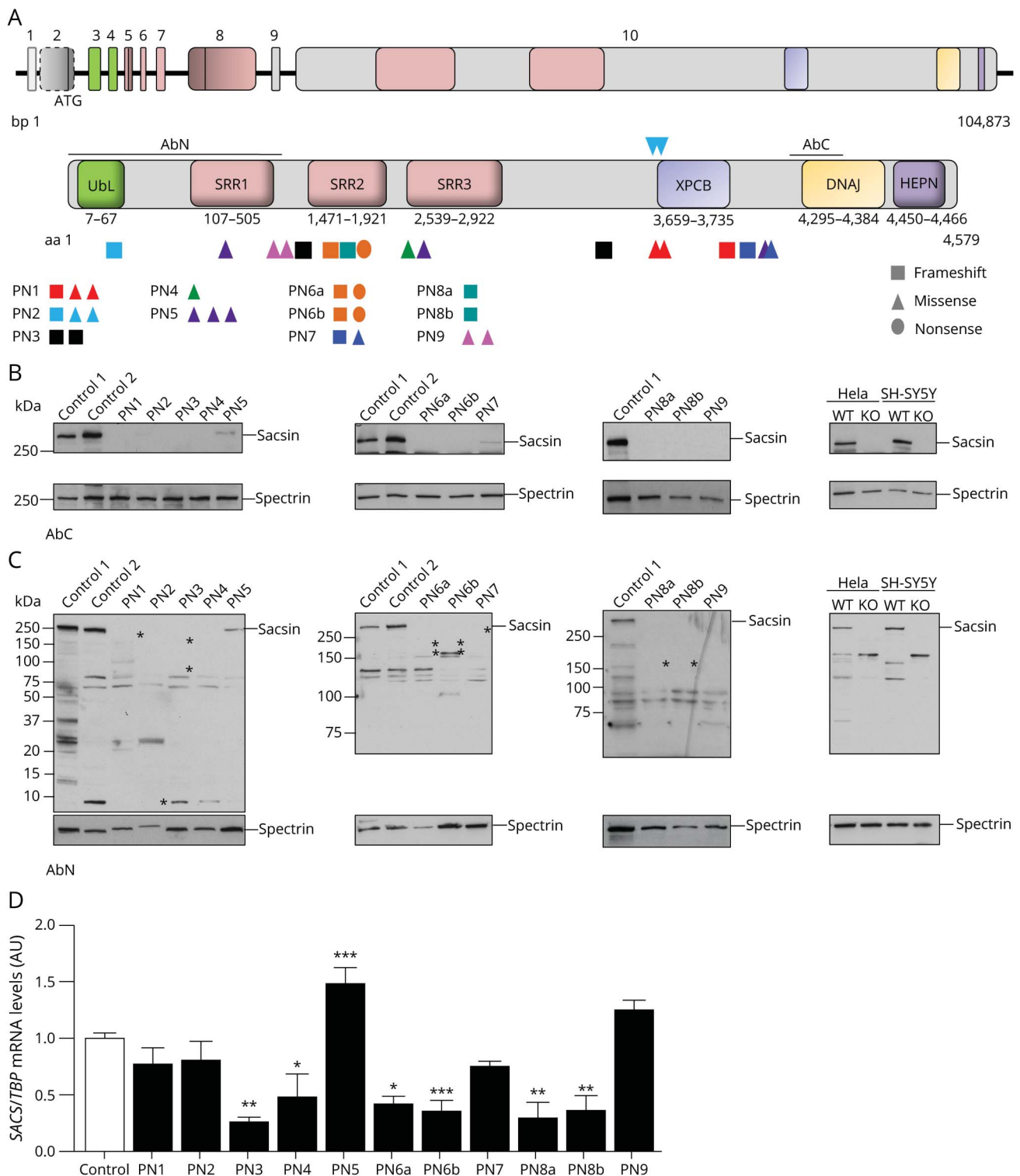
In patients with ARSACS carrying only truncating variations, SACS mRNA was evidently reduced compared to controls (PN3, PN8a, PN8b, PN6a, and PN6b), suggesting a nonsense mediated decay of the mRNA. On the other hand, mRNA was stable (PN4, PN9) or even increased (PN5) in patients carrying missense variations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

### Inhibition of Degradative Systems Does Not Rescue Saccin in Patients With ARSACS Carrying Missense Variations

Because SACS mRNA is stable and saccin protein is dramatically reduced in patients with ARSACS carrying missense variations, we checked whether mutant saccin could undergo a faster posttranslational degradation. Unfolded or aberrant proteins are usually targeted by the proteasome system or the autophagic system, the main molecular pathways involved in protein QC and maintenance of cellular proteostasis.<sup>20</sup> To test this hypothesis, we blocked cellular degradative systems by treating patient cells either with a proteasome inhibitor (MG-132, 1  $\mu$ M, 24 hours) or an autophagy inhibitor (chloroquine, 20  $\mu$ M, 24 hours) (Figure 2, A–D). Both treatments did not rescue mutant saccin in fibroblasts from patients with ARSACS carrying missense variations. By blocking the proteasomal pathway for 24 hours, a statistically significant reduction of wild-type saccin levels was observed in controls (Figure 2B). This suggested a possible inhibition of protein synthesis due to prolonged MG-132 treatment and consequent cellular stress induction.<sup>26</sup> We thus repeated the blockade of the proteasome reducing the time of treatment to 3 hours. In this condition, saccin was stable in controls but again not rescued in patients (eFigure 2, A and B, links.lww.com/WNL/B619). We also blocked proteasome and autophagy together, but also in this case, we did not see any increase in the amount of saccin protein in patients (eFigure 2C, links.lww.com/WNL/B619).

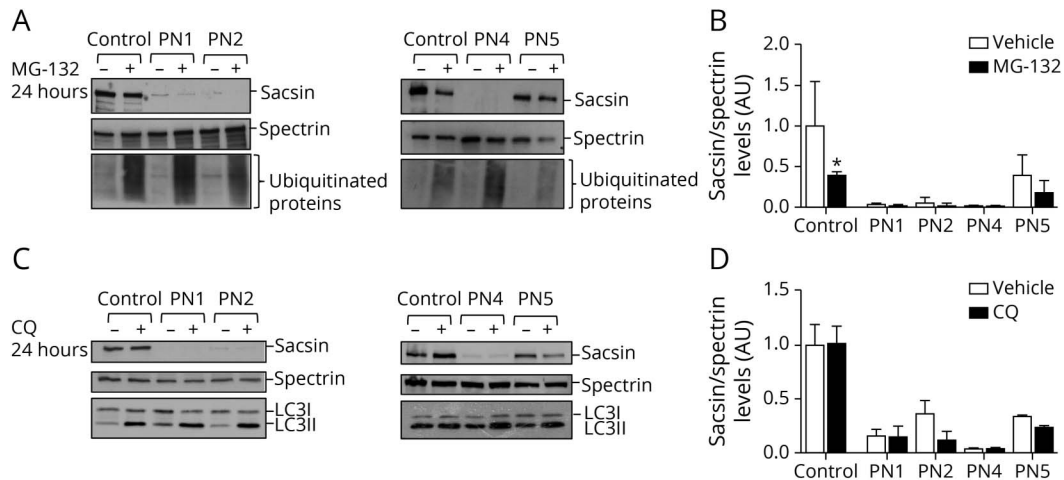
We then investigated whether saccin could be degraded by specific proteases. We inhibited cysteine proteases with E64, amino peptidases with bestatin, and aspartyl proteases with pepstatin. The low amount of mutant saccin in patients with ARSACS was not modified by any of these treatments (eFigure 2D, links.lww.com/WNL/B619). We took into

**Figure 1** Sacsin Protein Is Drastically Reduced in ARSACS Fibroblasts Carrying Different SACS Variations



(A) Scheme of SACS gene and saccsin protein illustrating the identified functional domains and corresponding exons. For each patient (see Table), the type and position of the variations on saccsin protein are indicated with a symbol and a color code. N- and C-terminal anti-saccsin antibody (AbN and AbC) used in this study are shown (bottom). (B) Representative Western blot (WB) showing residual saccsin protein in ARSACS patient-derived primary fibroblasts vs controls by using a AbC. Spectrin is used as loading normalizer. (C) Representative WB showing residual saccsin protein in ARSACS patient-derived primary fibroblasts vs controls by using AbN. Asterisks indicate the expected truncated saccsin protein: PN1 = 412 kDa; PN2 = 8 kDa; PN3 = 81, 358 kDa; PN6a and PN6b = 170, 180 kDa; PN7 = 410 kDa; PN8a and PN8b = 175 kDa. (D). SACS mRNA quantification by qRT-PCR in ARSACS patient-derived primary fibroblasts vs controls, normalized on TBP mRNA levels. In (D), data are presented as mean  $\pm$  SEM. \* $p \leq 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (unpaired-two tailed Student's *t* test). aa = aminoacid; ARSACS = autosomal recessive spastic ataxia of Charlevoix-Saguenay; bp = base pair; HEPN = higher eukaryotes and prokaryotes nucleotide-binding domain; KO= knockout; PN = patient; SRR = saccsin repeating region; UbL = ubiquitin-like domain; XPCB = xeroderma pigmentosus group C protein binding domain; WT = wild-type; KO= knockout.

**Figure 2** Mutant Sacsin Is Not Posttranslationally Degraded



(A) Representative Western blot (WB) of saccin levels in patient and control fibroblasts after proteasome blockade experiments by using 1  $\mu$ M MG-132 for 24 hours. Ubiquitinated proteins are used as readout of the treatment, and spectrin is used as loading normalizer. (B) Quantification graph relative to panel (A). (C) Representative WB of saccin levels in patient and control fibroblasts after autophagy blockade experiments by using 20  $\mu$ M chloroquine (CQ) for 24 hours. LC3I conversion in LC3II is used as readout of the treatment. (D) Quantification graph relative to C. In panels (B and D), data are presented as mean  $\pm$  SEM. n = at least 3. \* $p$   $\leq$  0.05 (unpaired 2-tailed Student  $t$  test). + = treated; - = vehicle.

consideration that saccin carrying missense variations could be completely translated but undetectable by standard WB procedures due to its misfolding or aggregation. To address this hypothesis, we performed different experiments to solve aggregates in biochemical assays. We checked for putative mutant saccin aggregates by loading onto sodium dodecyl sulphate–polyacrylamide gel electrophoresis both the soluble and insoluble fractions of control and patient fibroblasts (eFigure 3, A and B, links.lww.com/WNL/B619). However, we were unable to detect saccin 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 saccin levels (eFigure 3, C and D and eMethods, links.lww.com/WNL/B619).

Together, these results indicate that the drastic reduction of mutant saccin in patients with ARSACS is not caused by either a faster posttranslational degradation of the protein or aggregation.

### Translation of Mutant Saccin Carrying Missense Variations Is Not Blocked in Patients With ARSACS

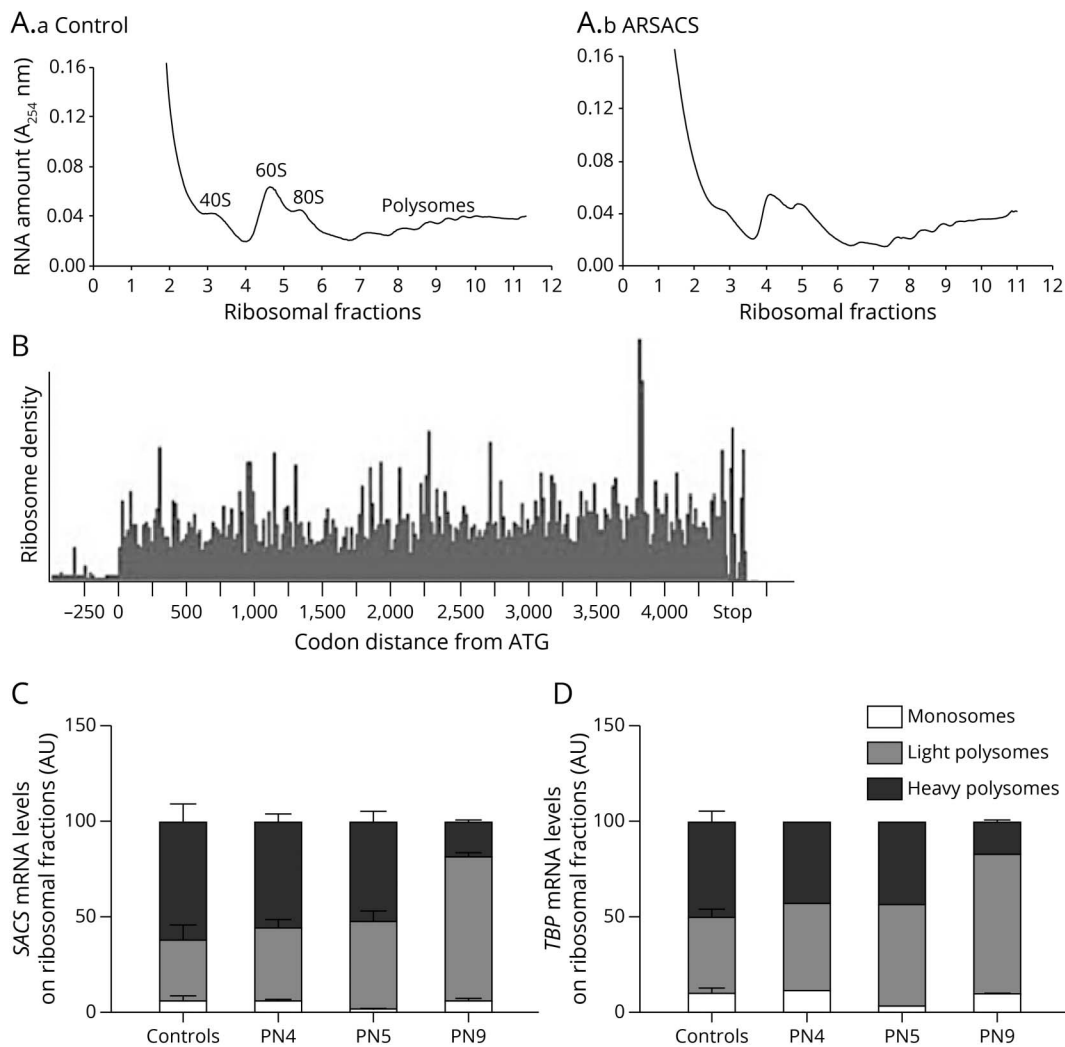
At this point, we considered that the absence of saccin-carrying missense variations could be due to its inefficient translation. We first excluded a general problem in translation because polysomal profiles showed a similar pattern in patients with ARSACS and controls (Figure 3A). To know whether wild-type 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.<sup>27,28</sup> This analysis showed

little accumulation of ribosomes in the 5' untranslated region of SACS mRNA and a homogenous density of ribosomes across the 13,737-nucleotide-long mRNA sequence, suggesting the absence of strong regulatory sequences in the 5' untranslated region (Figure 3B). To specifically assess the translation of saccin, we investigated whether SACS mRNA carrying missense variations was associated to intact ribosomes (polysomes) or split ribosomes (monosomes, due to a blockade of translation). We thus performed qRT-PCR for saccin on RNA extracted from the different ribosomal fractions (monosomes, light polysomes, heavy polysomes) for patients with ARSACS and controls. We found that SACS mRNA was associated with the polysomal fractions (the actively translating fractions) in patients with ARSACS (PN4, PN5, PN9) and 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 Saccin Products Are Cotranslationally Ubiquitinated and Degraded

At this point, having experimentally excluded all the possible molecular mechanisms accounting for saccin reduction in patients with ARSACS, we considered that mutant saccin could be degraded during translation. Two different QC mechanisms exist during translation, both leading to the ubiquitination and degradation of nascent proteins. The first one, the ribosomal QC, is associated with the mRNA degradation and ribosome stalling/splitting (the nonsense-mediated decay, the no-go decay, etc).<sup>29</sup> The other one, the cotranslational QC, senses the correct folding of the nascent chain, promoting the degradation of the protein while it is

**Figure 3** SACS mRNA Carrying Missense Variations Is Associated With the Polysomal Fractions



(A) Representative ribosomal profile graphs in control (A.a) and autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) patient fibroblasts (A.b). (B) Ribosome density on the human saccin mRNA, retrieved and analyzed from available riboseq datasets. (C) Quantitative real-time PCR data of the SACS mRNA distribution in different ribosomal fractions: monosomes and light and heavy polysomes in fibroblasts from patients with ARSACS (PN4, PN5, PN9) compared to 3 different controls. (D) *TBP* mRNA distribution is shown as housekeeping gene analyzed in the same cells.

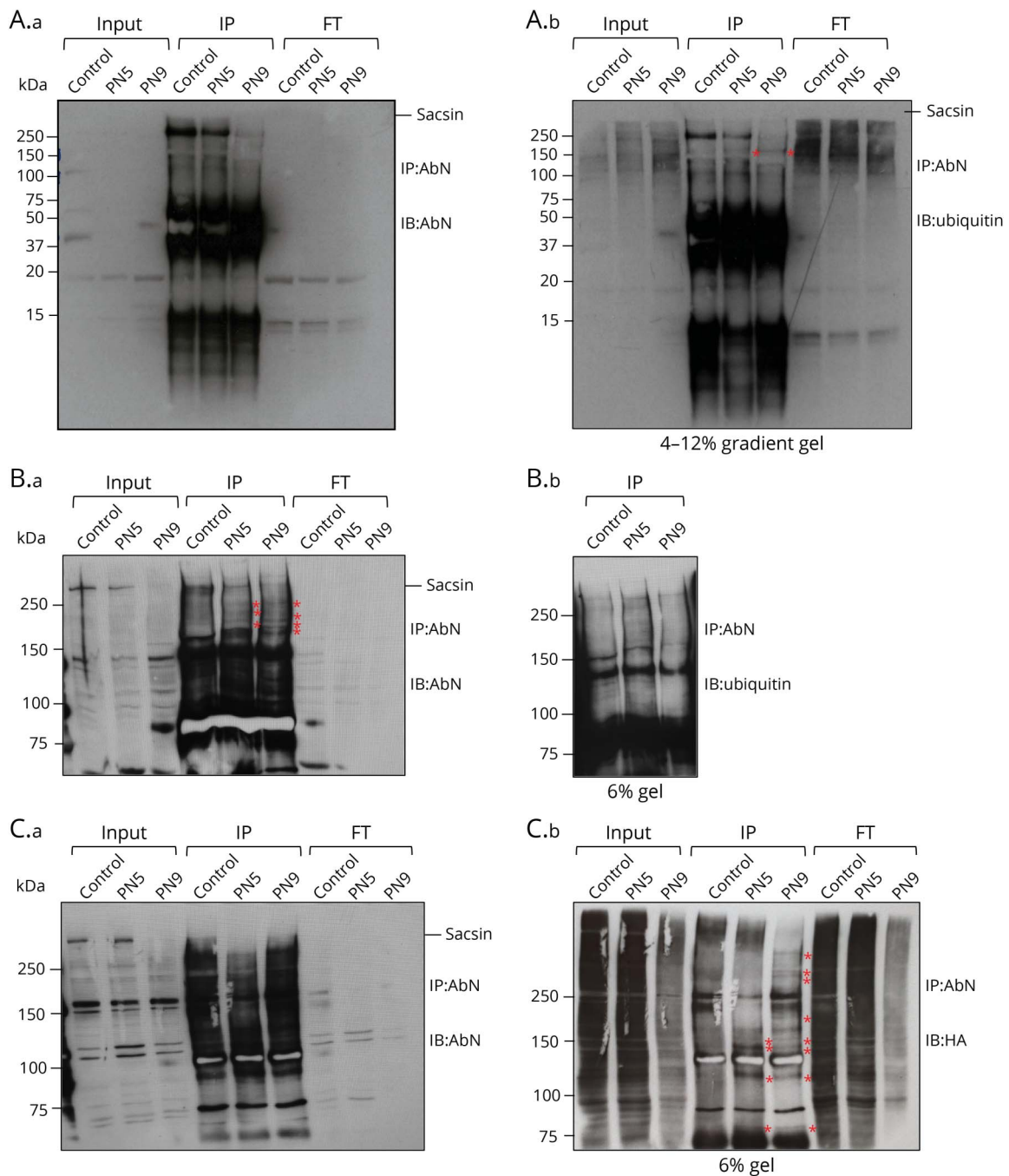
being translated, and occurs in the presence of a stable mRNA associated with intact ribosomes.<sup>30</sup> This second scenario is the most plausible in the case of mutant saccin carrying missense variations because we did not detect any reduction of the mRNA. Although poorly characterized, cotranslational QC is predicted to occur for big and multimodular proteins, the folding of which takes place cotranslationally and proceeds in a domain-wise manner.<sup>31</sup> The players involved in nascent protein degradation associated with cotranslational QC are completely unknown so far.

As formal proof of the cotranslational degradation of saccin carrying missense variations, we looked for the presence of ubiquitinated degradation products of nascent mutant saccin in cells from patients with ARSACS carrying missense variations only. Thus, we immunoprecipitated saccin with AbN (in the view of recognizing N-terminal saccin fragments of

unpredictable molecular weights) in patients with ARSACS (PN5, PN9) vs controls. Because putative mutant saccin intermediates should be ubiquitinated and degraded by the proteasome, before the immunoprecipitation (IP), we treated cells with the proteasome inhibitor MG-132 (1  $\mu$ M, 3 hours) to improve their detection. We efficiently immunoprecipitated full-length saccin with AbN in control and (to a lower extent as expected) patient cells (Figure 4A). In ARSACS fibroblasts, saccin degradation products are detectable especially in the higher part of the gel, in particular with the anti-ubiquitin immunodecoration (Figure 4A.b). To better resolve the higher part of the gel, we decided to reload an independent saccin IP in a 6% gel (Figure 4B). Saccin degradation products were clearly visible in the IP lanes of patients with ARSACS, especially when immunodecorated with the AbN (Figure 4B.a). To enhance saccin ubiquitination, we transfected control and patient cells with



**Figure 4** Sacsin Protein Carrying Missense Variations Is Cotranslationally Ubiquitinated and Degraded



(A) Sacsin immunoprecipitation (IP) by using AbN in fibroblasts from patients with ARSACS carrying missense variations (PN5, PN9) and a control treated with 1  $\mu$ M MG-132 for 3 hours. Four percent to 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 controls. 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 fibroblasts from patients with ARSACS carrying missense variations (PN5, PN9) and a control on ubiquitin-HA overexpression, treated with 1  $\mu$ M MG-132 for 3 hours. Six percent 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. FT = flow-through fractions; IB = immunoblotting.

ubiquitin-HA construct and IP Sacsin in the same conditions as before. We reconfirmed the detection of Sacsin degradation products in patients with ARSACS (Figure 4C), and 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.b).

According to our results, we can arrange a final model of the first event of ARSACS pathogenesis: in the presence of

frameshift or nonsense variations, *SACS* mRNA is degraded; in the presence of missense variations, saccsin fails to fold and undergoes cotranslational degradation (Figure 5). In both cases the result is the absence/striking reduction of saccsin.

## Discussion

In this work, we studied in detail the effects of different ARSACS-causing variations on saccsin stability at both the mRNA and protein level. The cohort of patients with ARSACS that we analyzed encompasses a wide range of diverse types of *SACS* variations localized in different regions of the gene. Our results demonstrate that saccsin is barely detectable in patients with ARSACS regardless of the nature or position of the variation along the gene. While this result was expected for patients carrying biallelic truncating variations (whose mRNA is unstable and degraded), it was not anticipated for patients carrying missense variations, especially on both alleles. We indeed discovered that saccsin carrying missense variations 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.

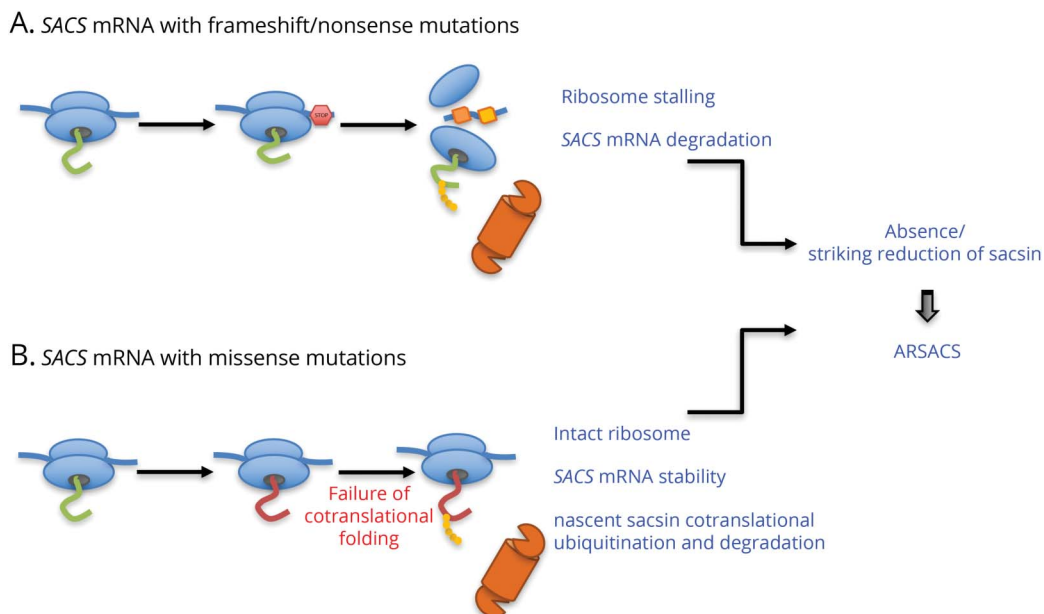
Because ARSACS presents variability in its clinical presentation, efforts have been dedicated to find a genotype-phenotype correlation to better tailor a precision medicine approach in terms of disease prognosis and family counseling

and for future clinical trials. Our data, however, do not confirm previous reports based only on *in silico* analyses correlating the nature or position of the *SACS* variations with the severity of the disease.<sup>6,19</sup> Here, we demonstrated that loss of function in ARSACS is caused by loss/striking reduction of saccsin, independently of the variation.

Excluding the Québec cohort, in whom the original c8844delT *SACS* variation is highly prevalent, the 2020 worldwide ARSACS scenario shows that most variations are missense, followed by small deletions, frameshift, nonsense, and small insertions.<sup>6</sup> In our cohort of compound heterozygous patients, 2 of them carry biallelic missense variations and 5 of them carry monoallelic missense, for a total of 9 different missense changes. In all cases, the full-length protein is almost absent in patients with missense variations (hitting different regions of the protein, from the N-terminus to the C-terminus) and in patients with truncating variations. Only in PN5 there was a residue of saccsin detected with both AbC and AbN antibodies, which is  $\approx 20\%$  of controls, whereas in PN9 (who carries biallelic missense as well), saccsin is not detectable.

Our findings are supported by the recently published *Sacs*<sup>R272C/R272C</sup> knock-in mouse model, carrying a human pathogenetic variation in the first saccsin repeating region domain.<sup>8</sup> This model presents a scarcely detectable saccsin protein despite a stable mRNA and, accordingly, a phenotype overlapping with that of the *Sacs* knockout mouse,<sup>8,13</sup>

**Figure 5** Full-Length Saccsin Protein Is Almost Absent in Patients With ARSACS, Independently of the Nature of the Variations



Final model explaining why mutant saccsin is almost absent in patient cells. In (1), saccsin is prevented from being translated because the *SACS* mRNA carrying frameshift variations is degraded by nucleases, while in (2), the *SACS* mRNA carrying missense variations is stable and saccsin is cotranslationally degraded. The proteasome is the degradative system implied in these pathways (drawn in orange). Green nascent chain indicates before sensing the variation; red nascent chain, after sensing domain misfolding. ARSACS = autosomal recessive spastic ataxia of Charlevoix-Saguenay.

confirming the conservation of the cotranslational degradation of mutant saccin also in cerebellum. In addition, a striking reduction of saccin was shown in two other reports of ARSACS variations in patient fibroblasts or lymphoblasts,<sup>14,32</sup> with no further mechanistic insights.

In this work, we could not directly test RNA and protein levels in heterozygous parents of patients carrying missense variations. However, saccin protein is halved in the heterozygous *Sacs*<sup>R272C/+</sup> mouse,<sup>8</sup> supporting that saccin carrying the R272C missense variation likely undergoes cotranslational degradation. A halved amount of saccin seems to be sufficient to supply to cellular needs, as demonstrated by the fact that both *Sacs*<sup>R272C/+</sup> mice and *Sacs*<sup>+/-</sup> mice do not show any obvious phenotype,<sup>8,13</sup> like healthy human carriers. Vimentin is indeed strikingly affected in *Sacs*<sup>-/-</sup> mouse embryonic fibroblasts, forming abnormal perinuclear bundles as expected, while no difference is observed between the *Sacs*<sup>+/-</sup> and wild-type mouse embryonic fibroblasts (eFigure 4, A and B, [links.lww.com/WNL/B619](https://www.lww.com/WNL/B619)).

We found that *SACS* mRNA is stable or even increased in the ARSACS compound heterozygous patients carrying biallelic or monoallelic missense variations, excluding mRNA degradation as the possible explanation for the absence of saccin. The failure in rescuing full-length saccin on inhibition of all cellular degradative systems and several proteases excluded faster posttranslational degradation. In addition, mRNA of saccin turned out to be associated with polysomes in patients with ARSACS and in controls, rejecting the hypothesis of defective translation. We instead discovered that saccin protein carrying missense variations 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 them reach their native states and may reduce the probability for off-pathways and aggregation-prone conformations.<sup>31,33,34</sup> In addition, many studies indicate that a sizable portion of nascent chains are cotranslationally ubiquitinated and degraded by the proteasome in physiological conditions, both in yeast<sup>35,36</sup> and in mammalian cells.<sup>37</sup> This likely applies to wild-type saccin, a 520-kDa protein with a complex multimodular architecture containing repeated motifs, the folding of which may take minutes to hours to complete. We hypothesize that cotranslational folding and degradation occur physiologically for saccin as mechanisms of QC. In the case of saccin carrying a missense variation, 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

saccin in the cytosol. Unlike pathogenetic missense variations, missense variants present in healthy population probably do not affect the cotranslational folding of saccin, but this hypothesis requires experimental validation.

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

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

Few studies observed cotranslational ubiquitination of mammalian proteins, although only in nonphysiological conditions, that is, in vitro translation, overexpression conditions, or the presence of artificial (nonpathogenic) variations.<sup>38,39</sup> In cystic fibrosis, this mechanism was observed for the cystic fibrosis transmembrane conductance regulator, but only with the  $\Delta$ F508 variation (not with other variations).<sup>40</sup> Here, we report in vivo the cotranslational degradation of a cytosolic protein (carrying any type of missense variation) as the cause of a human disease.

Preemptive mutant saccin degradation in ARSACS may be a paradigmatic example of a mechanism that can potentially underlie many other genetic diseases with loss of function.

Our data identify lack of saccin protein as unifying mechanism shared by different *SACS* variations, with multiple important implications for ARSACS disease managing.

For the diagnosis, we propose that the evaluation of saccin levels should be included in clinical genetics practice to establish a definite ARSACS diagnosis or even as prescreening in high probable cases to avoid expensive next-generation sequencing panel analysis and certainly to validate variants of uncertain clinical significance. A previous report was suggested that mitochondrial network abnormalities in patient fibroblasts could be evaluated when *SACS* genetics and molecular results are difficult to interpret.<sup>41</sup> However, this approach is experimentally complex and time-consuming compared to the evaluation of saccin protein level that we propose here. We indeed found that saccin is well detected in peripheral blood mononuclear cells at both the RNA and protein level, making feasible the development of a diagnostic approach for ARSACS aimed at testing the amount of saccin in peripheral blood (eFigure 5, A–C, [links.lww.com/WNL/B619](https://www.lww.com/WNL/B619)). For designing therapeutic strategies, cotranslational degradation of mutant saccin makes unproductive any posttranslational approach. While our study excludes the nature/position of the *SACS* variation 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; however, many different variations have been identified.<sup>32</sup> In other countries, 20% of patients with ARSACS show atypical presentations with disease onset after 20 years of age, a Charcot-Marie-Tooth-like disorder, absence of 1 of the 3 defining clinical features,<sup>4,5</sup> epilepsy, or hearing loss.<sup>3,6</sup> Both interfamilial and intrafamilial heterogeneity of this complex clinical condition might be explained by the combined effect of modifier genes, risk alleles, or multilocus variations (including variations/copy number variants of other genes) that can modify the phenotype in terms of penetrance, expressivity, and age at 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 sizes and environmental factors contribute to determine the phenotype of the genetic disease. A genome-wide screen in patients with ARSACS, aggregated according to 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 Charcot-Marie-Tooth disease and others.<sup>42-45</sup>

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## Disclosure

F. Longo, D. De Ritis, A. Miluzio, D. Fraticelli, J. Baets, M. Scarlato, F.M. Santorelli, S. Biffo, and F. Maltecca report no disclosures relevant to the manuscript. Go to [Neurology.org/N](http://Neurology.org/N) for full disclosures.

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## Appendix Authors

Name	Location	Contribution
<b>Fabiana Longo, PhD</b>	Mitochondrial Dysfunctions in Neurodegeneration Unit, Ospedale San Raffaele, Milan, Italy	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
<b>Daniele De Ritis, MSc</b>	Mitochondrial Dysfunctions in Neurodegeneration Unit, Ospedale San Raffaele, Milan, Italy; Università Vita-Salute San Raffaele, Milan, Italy	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data
<b>Annarita Miluzio, PhD</b>	Istituto Nazionale di Genetica Molecolare, INGM, "Romeo ed Enrica Invernizzi," Milan, Italy	Major role in the acquisition of data
<b>Davide Fraticelli, MSc</b>	Mitochondrial Dysfunctions in Neurodegeneration Unit, Ospedale San Raffaele, Milan, Italy	Major role in the acquisition of data
<b>Jonathan Baets, MD</b>	Laboratory of Neuromuscular Pathology, Institute Born-Bunge, University of Antwerp, Belgium; Neuromuscular Reference Centre, Department of Neurology, Antwerp University Hospital, Belgium	Drafting/revision of the manuscript for content, including medical writing for content
<b>Marina Scarlato, MD</b>	Department of Neurology, Ospedale San Raffaele, Milan, Italy	Drafting/revision of the manuscript for content, including medical writing for content
<b>Filippo M. Santorelli, MD</b>	Molecular Medicine, IRCCS Fondazione Stella Maris, Pisa, Italy	Drafting/revision of the manuscript for content, including medical writing for content
<b>Stefano Biffo, PhD</b>	Istituto Nazionale di Genetica Molecolare, INGM, "Romeo ed Enrica Invernizzi," Milan, Italy; Department of Biosciences, University of Milan, Italy	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data
<b>Francesca Maltecca, PhD</b>	Mitochondrial Dysfunctions in Neurodegeneration Unit, Ospedale San Raffaele, Milan, Italy; Università Vita-Salute San Raffaele, Milan, Italy	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

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