Report

Reduction of sacsin levels in peripheral blood mononuclear cells as a diagnostic tool for spastic ataxia of Charlevoix-Saguenay

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Short title: Diagnosis of ARSACS in PBMCs

Abstract

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is a rare neurodegenerative disease caused by biallelic variants in the *SACS* gene encoding for sacsin. More than 200 pathogenic variants have been identified to date, most of which are missense. It is likely that the prevalence of ARSACS is underestimated due to the lack of an efficient diagnostic tool able to validate variants of uncertain significance (VUS). We have previously shown that sacsin is almost absent in ARSACS patients' fibroblasts regardless of the type of *SACS* variant, because sacsin carrying missense variants is cotranslationally degraded. In this work, we aimed to establish the pathogenicity of *SACS* variants by quantifying sacsin protein in blood samples, with relevant implications for ARSACS diagnosis.

We developed a protocol to assess sacsin protein levels by Western Blot using small amounts of peripheral blood mononuclear cells (PBMCs), which can be propagated in culture and cryopreserved. The study involves eight ARSACS patients (including a novel case) carrying variants of different types and positions along the *SACS* gene, and two parents who are carriers of heterozygous missense variants.

We show that ARSACS patients (carrying either missense or truncating variants) almost completely lacked sacsin in PBMCs. Moreover, both carriers of a *SACS* missense variant showed 50% reduction in sacsin protein levels compared to controls. We also describe a patient with uniparental isodisomy carrying a homozygous nonsense variant near the 3′ end of the *SACS* gene. This resulted in a stable sacsin protein lacking the last 202 aminoacids, probably due to escape of nonsense mediated decay of mRNA.

In conclusion, we have optimized a minimally invasive diagnostic tool for ARSACS in blood samples based on sacsin protein levels assessment. Indeed, our results provide definite evidence that sacsin carrying missense pathogenic variants undergoes cotranslational degradation. The quantitative reduction in sacsin levels in the case of missense VUS allows to

define them as pathogenic variants, something which can't be predicted bioinformatically with high certainty.

Keywords

ARSACS, spastic ataxia, genetics, diagnosis, neurodegeneration

Introduction

ARSACS is a rare, childhood to adult-onset neurodegenerative disease characterized by progressive ataxia, spasticity and neuropathy.[1] It is caused by pathogenic variants in the *SACS* gene encoding for sacsin, a 520 kDa multimodular protein whose function is still poorly understood. Sacsin is composed of an ubiquitin-like (UbL) domain that binds to the proteasome [2], three sacsin repeating regions (SRR) having high homology with Hsp90 [3], a Xeroderma pigmentosum C-binding (XPCB) domain [4], a DnaJ domain that binds Hsc70 [2], and a higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain.[5]

ARSACS is highly prevalent in the Québec French-Canadian community, due to a genetic founder-effect. However, it is also present worldwide, with more than 200 pathogenic variants described [6], suggesting that its diagnosis and prevalence are underexplored especially in low- and middle-income countries, due to the lack of diagnostic tools.

We have previously shown that sacsin is almost absent or strikingly reduced in ARSACS patients' fibroblasts, regardless of the nature of the *SACS* genetic variant.[7] This applies not only to ARSACS patients carrying truncating variants, as expected, but also to patients who are compound heterozygotes for either two diverse *SACS* pathogenic missense variants, or for a missense variant and a truncating *SACS* variant. We identified preemptive cotranslational degradation of mutant sacsin carrying pathogenic missense variants as the underlying mechanism.[7] In this pathway, sacsin mRNA remains at a constant level and is continuously

translated, but the nascent protein chain carrying pathogenetic missense variant is never fully synthesized as it gets soon ubiquitinated and targeted to proteasome-mediated degradation.[7] This mechanism seems to be universal, as the *Sacs* $^{R272C/R272C}$ knockin mouse model presents a constant mRNA level but almost-zero protein level in homozygosity and halved protein level in heterozygosity, as compared to the wildtype in the cerebellum and in the brain.[8]

Therefore, we hypothesized that the measurement of sacsin protein levels in ARSACS patients' blood samples could represent a minimally invasive tool for ARSACS diagnosis and for assessing the pathogenicity of VUS. Consistently, the levels of sacsin should be halved in healthy carriers of a pathogenic missense variant.

We have previously demonstrated that *SACS* gene is expressed in human blood samples from healthy controls, and in particular sacsin mRNA and protein are mostly abundant in the PBMCs fraction.[7] Although sacsin is expressed at lower levels in PBMCs than in human fibroblasts, we hypothesized that the level is still quantifiable and allows reliable measurements, even of the 50% reduction expected in healthy carriers.

Here, we present the setup and validation of a novel protocol for the diagnosis of ARSACS in blood samples from patients which is based on the biochemical assessment of sacsin protein reduction.

Materials and Methods

Participant Consent

Eight patients (four males and four females) with the clinical and genetic diagnosis of ARSACS (**Table 1**), along with two parents, each related to a different one of these patients, were retrospectively recruited among the cohort of spastic-ataxic patients referring to each center: IRCCS Ospedale San Raffaele (Milan, Italy), Department of Neurology, Antwerp University Hospital, Antwerpen, Belgium and CHU de Quebec- Université Laval (Canada). All

participants gave their written informed consent according to protocols in force by respective institutional human ethics review boards and Declaration of Helsinki. All reported pathogenic variants (both of patients and parents) were re-confirmed by Sanger-sequencing.

NGS sequencing

The proband's DNA (Patient1) was screened using a targeted Next Generation Sequencing (NGS) approach with a gene panel including 231 genes: all known causative genes for hereditary spastic paraplegia (HSP), the known genes for recessive ataxia and for spinocerebellar ataxias (excluding those forms related to repeat expansion), the most frequently mutated genes in neuropathies and the known genes for familial ALS. Targeted NGS panel description is reported in **Supplementary Material.**

The variants identified were verified by Sanger sequencing. Numbering of the pathogenic variants in *SACS* gene is based on *SACS* cDNA and protein Acc. N. NM_014363.4 and NP_055178.3, respectively.

PBMCs isolation and in vitro culturing

Fresh venous blood was collected in Vacutainer-EDTA tubes (BD) and PBMCs were isolated after density gradient centrifugation (Lymphoprep, STEMCELL Technologies). PBMCs were then either cryopreserved in medium with 10% DMSO or harvested at $2*10^6$ cells/mL density, up to 21 days in vitro (DIV), with X-VIVO™ medium (Lonza) supplemented with 5% human AB male serum (ECS0219D, Euroclone), 1 mM sodium pyruvate, 2 mM l-glutamine, 100 U/mL penicillin-streptomycin, 50 UI/mL human recombinant Interleukin-2 (IL-2, Sigma), phyto-hemo-agglutinin (PHA, Merck) at 1 µg/mL. From DIV3, cells in suspension (which are mainly lymphocytes) are washed and incubated with medium without PHA and with 100 UI/mL IL-2. Immunophenotyping at DIV6 by flow-cytometry confirms high frequency of live cells comparable to fresh PBMCs (DIV0) and conserved CD3+ T cells percentage of lymphocytes (around 60%). Also, in CD3+ T cells the CD4+/CD8+ ratio was normal (around

1.1:1 at DIV6 compared to 2.3:1 at DIV0) (*data not shown*). Control PBMCs were collected as above from six different healthy volunteers (age ranging 30-40 years old, both males and females).

Western Blot

Sacsin protein level was measured by Western Blot. Briefly, 10 to 30 µg PBMCs lysed with 1% TritonX-100 were loaded on 6% or 5% SDS-PAGE. Antibodies: anti-sacsin (Abcam 181190), anti-calnexin (Sigma C4731), Horseradish Peroxidase (HRP)-conjugated anti-rabbit IgG (GE Healthcare #GEHNA9341ML).

Densitometric analyses of Western Blot chemiluminescence signal were performed with ImageJ, normalizing sacsin to either calnexin or ponceau S staining signal levels.

Immunofluorescence on ARSACS fibroblasts

Primary fibroblasts from controls, PN6 and PN7 biopsies were cultured as we previously described.[7] After fixation and permeabilization, cells were incubated with anti-vimentin antibodies (Abcam Ab92547) and then Alexa Fluor-488 Secondary Antibodies (Thermo Fisher Scientific) and counterstained with DAPI. Images were acquired with Zeiss Axio-Observer (20X 0.5) and Volocity 6.3 software.

Statistical analyses

ONE-way ANOVA with Tukey's correction for multiple comparison was always used to compare normalized sacsin levels among controls, carriers and patients' groups. Unpaired t-test was used to measure alteration of sacsin levels in PN7 compared to controls. Analyses were performed with GraphPad Prism 8.

Results

Our aim was to validate the reduction of sacsin protein levels in blood samples as a diagnostic tool for *SACS* pathogenic variants. To this end, we firstly optimized a protocol for the detection

of sacsin in PBMCs and secondly we provided evidence that sacsin carrying missense pathogenic variants is cotranslationally degraded in the very same blood cells, both in ARSACS patients and healthy-carriers. All the patients analyzed in this study were re-evaluated by neurologists of each center in 2023-2024 and the clinical scores are reported in **Table 1**. The *SACS* pathogenic variants analyzed are reported in **Fig. 1A**.

We started analyzing a novel compound heterozygote ARSACS patient (PN1) that we identified by NGS (**Table 1**) carrying a missense pathogenic variant on one allele and a frameshift on the other allele (p.Pro3095Leu; p.Leu1171Glyfs*8). Sacsin protein level was measured by Western Blot in PBMCs isolated from fresh venous blood and maintained in culture for diverse days in vitro (DIVs): DIV8, DIV11, DIV16 (**Fig. 1B**). There was around a 50% reduction in the abundance of sacsin protein in Parent1 (carrier of the missense p.Pro3095Leu), as the sacsin level mean in Parent1 in respect to controls was: 59%±10%SD when normalized to calnexin ($P<0.01$) (**Fig. 1C**) and 52%±11%SD when normalized to total ponceau S staining ($P<0.05$) (**Fig. 1D**). These data: i) confirm that sacsin encoded by the allele carrying the missense pathogenic variant undergoes cotranslational degradation also in the PBMCs of the healthy carrier; ii) corroborate the high reproducibility and reliability of our quantitative protocol. Consistently, sacsin levels in PBMCs of PN1 were reduced by 91%±4%SD when normalized to calnexin ($P<0.0001$) and 91%±5%SD when normalized to Ponceau S ($P<0.001$) (**Fig. 1C-D**). Since sacsin levels did not change between the different DIVs, we conducted subsequent experiments between DIV6-DIV8.

To further enforce this evidence, we then analyzed PBMCs from another compound heterozygous Patient (PN2) carrying a missense pathogenic variant on one allele and a frameshift on the other allele (p.Asp3926Gly; p.Ile3755fs*8)[9] (**Table 1**). Western Blot on PBMCs again showed that sacsin protein level normalized to calnexin was reduced (**Fig. 2A**) by 57%±22%SD in Parent2 harboring the monoallelic missense pathogenic variant

(p.Asp3926Gly) compared to controls (P<0.01), and by 94%±3%SD in PN2 compared to controls (P<0.0001) (**Fig. 2B**). Also, normalizing to total ponceau S reconfirmed 56%±8%SD reduction in Parent2 (P<0.01) and 95%±4%SD reduction in PN2 (P<0.0001) (**Fig. 2C**).

We then analyzed PBMCs from other ARSACS patients that included: two compound heterozygote siblings with two truncating variants (PN3a, PN3b) (p.Ser1531fs*9; Arg1645X)[7] who are expected to present the complete absence of sacsin protein; one compound heterozygote with two missense *in cis* and a truncating variant (PN4) (p.Arg3636Gln, p.Pro3652Thr; p.Leu3745fs*1)[10], one compound heterozygote with two different missense (PN5) (p.Asp1402Val; p.Trp1946Arg)[11] and one homozygote with a truncating variant (p.Ser1531fs*9) (PN6).[12] All patients displayed a complete or striking reduction of sacsin levels in PBMCs, regardless of the type of pathogenic variant (**Fig. 2D-E**). Sacsin level was not quantifiable at all in PN3a-b and in PN6 as expected, while it was reduced by 96%±4%SD in PN4 (P<0.0001) and by 82%±7%SD in PN5 (P<0.0001), normalizing both to calnexin (**Fig. 2F**) and Ponceau S (**Fig. 2G**). We also treated controls and PN4 PBMCs with the proteasome inhibitor MG-132 at 1 μM for 3 hours. As we previously showed in the fibroblasts of the same patient [7], the levels of full length sacsin were identical with or without MG-132 (**Supplementary Figure 1A**), indicating that post-translational degradation is not the mechanism accounting for mutant sacsin reduction in PBMCs of ARSACS patients.

Finally, we also analyzed sacsin protein levels from an unusual case that was previously described, in which the Patient (PN7) harbors a nonsense variant in homozygosity due to paternal uniparental isodisomy (c.13132C>T, p.Arg4378*).[13] This truncating variant is noteworthy as it occurs near the 3' end of *SACS* gene, leaving out the last part of the DnaJ domain and the HEPN domain in sacsin protein (see **Fig. 1A** and **Supplementary Figure 1B**). Sacsin was detected in PBMCs from PN7 at levels comparable to those of the controls (**Fig. 3A**), without significant changes (**Fig. 3B**), suggesting that very terminal truncating variants

can escape mRNA instability and degradation, and can produce a non-functional protein. The expected molecular weight shift of 22 kDa due to the truncation was not observable by performing SDS-PAGE (even with a 5% gel), suggesting that higher-resolution methodologies are necessary to observe the shift between 520 and 500 kDa. The p.Arg4378X variant has been already reported *in trans* with another pathogenic variant in the *SACS* gene (c.1178_1181delAT; p.Leu393Cysfs*17) in a patient with ARSACS.[14] We further validated the pathogenicity of this variant by assaying vimentin remodeling by immunofluorescence in primary fibroblasts from PN7 and comparing to PN6 and healthy controls. Intermediate filament remodeling is a hallmark of ARSACS pathogenesis, with abnormal bundles of vimentin in ARSACS-patient fibroblasts [15] and of neurofilament (NFs) in brain autopsies of ARSACS patients and in the *Sacs*$^{-/-}$ mouse.[8,16] While in the controls vimentin appeared evenly distributed in the cells, in PN6 and PN7 we observed abnormal reorganization of vimentin filaments, which appear to form bundles or to be more densely packed at the periphery and were indistinguishable between the two patients (**Fig. 3C-D**). Considering that PN6 has no residual sacsin protein, this experiment suggests that the homozygous p.Arg4378X variant in PN7 impairs sacsin function.

Discussion

Classifying missense variants into pathogenic or benign remains a major challenge in the context of personalized medicine. Although improvements have been made in prediction of pathogenicity via computational algorithms [17], only appropriate functional assessments can give definite proof. Here, we show that depletion of sacsin due to cotranslational degradation in the presence of pathogenetic missense variants can be exploited for ARSACS diagnosis. We previously formally demonstrated this mechanism in ARSACS fibroblasts [7], and here we precisely quantified sacsin protein reduction in PBMCs from ARSACS patients and healthy

carriers that had one or two pathogenic missense variants. Indeed, sacsin protein was reduced by more than 80% in these ARSACS patients compared to controls, as well as in ARSACS patients with truncating variants that eliminate sacsin production. These data support our hypothesis that cotranslational degradation is likely a universal mechanism for pathogenetic *SACS* missense variants.

We also previously demonstrated that sacsin depletion was due to mRNA degradation and instability in the case of truncating variants.[7] Here, we report an unusual case (PN7)[13] in which the truncating p.Arg4378X variant escapes this mechanism. This is likely due to the fact that this variant localizes at the very 3' end of the *SACS* gene, while in our previous report the truncating variants were located further upstream.[7] Since the p.Arg4378X is expected to result in the loss of part of the DnaJ domain and of the HEPN domain, this finding raises the intriguing hypothesis that both these domains are central in ARSACS pathogenesis. At the best of our knowledge, this is the first time that such a hypothesis is corroborated by functional data in ARSACS patients. Because the HEPN domain binds nucleotides, it has been proposed that the it is essential for the function of other sacsin domains (e.g the Hsp40-like DnaJ domain that binds Hcs70 in vitro, and the Hsp90-like SRR domains) by increasing local nucleotide concentration.[5] Interestingly, in eukaryotes, the HEPN domain is almost exclusively present in sacsin-like proteins always C-terminal to the DnaJ domain [3], suggesting that the combination of both domains is essential for sacsin function.

Overall, these data show that assessment of sacsin protein levels could be implemented in diagnostics for ARSACS. The quantitative reduction of sacsin levels in case of missense VUS allows defining them as pathogenic variants. Compared to truncating variants, the real effect of pathogenic missense variants on sacsin protein can be much harder or impossible to predict bioinformatically. This is even more relevant in ARSACS given that missense changes are the great majority of ARSACS-causing variants.[6]

The use of blood samples, and particularly PBMCs, for biomarker and diagnostic research has raised significant attention in recent years, as blood tissue is easily accessible and allows minimally invasive and cost-effective analyses that could be set up in diagnostics laboratories. The protocol we developed allows for the culturing, repeated expansion and freezing of PBMCs, and is also set up for very low amount of material, down to 10 µg of lysate. The robustness and reliability of our protocol allow for the quantitative measure of 50% sacsin level reduction in healthy carriers, even with this low amount of protein extract. Noteworthy, this approach enables the repeated use and storage of samples for later functional analysis, without necessitating to recall patients. Although we detailed the protocol in the Methods allowing reproducibility by other laboratories, we are aware that the overall approach could not be immediately implementable for diagnostic laboratories without specialized knowledge anyway, and this could represent a limitation. In conclusion, this study demonstrates the effectiveness of measuring sacsin protein levels as a direct and efficient ARSACS diagnostic outcome, exploiting the underlying cotranslational degradation mechanism.

Data availability statement

Anonymized data not published within this article can be made available upon reasonable request from any qualified investigator.

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Competing interests

Nothing to report.

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Figure legends

Figure 1. **Sacsin levels are drastically reduced in ARSACS patients' and carriers' PBMCs carrying pathogenic missense variant**. (A) Schematics of sacsin protein (light green) structure; the relative positions of sacsin domains (Ubl, SRR1-3, DnaJ and HEPN) and pathogenic variants reported in this study are shown; color code represents the different Patients; shape represent the different types of pathogenic variants (triangle = frameshift, circle = missense, square=noncoding). Corresponding positions of coding exons (abbreviated as Ex) is also shown above the protein. (B) Western Blot showing sacsin levels in PBMCs from controls, Parent1 and PN1; three biological replicates were collected at DIV8, DIV11 and DIV16. Calnexin and Ponceau S were used as loading controls. (C-D) Quantification, relative to (B), of sacsin levels normalized to calnexin (C), and ponceau S staining (D). Data are presented as mean±SD; * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 (one-way ANOVA with Tukey's correction for multiple comparison).

Figure 2. **Sacsin levels are drastically reduced in ARSACS patients' PBMCs regardless of the type of genetic pathogenic variant.** (A) Representative Western Blot showing sacsin levels in PBMCs from controls, Parent2 and PN2; calnexin was used as loading marker. The upper part of Ponceau S staining is also shown as loading control (see also Supplementary Material for uncropped images). (B-C) Quantification of sacsin levels normalized to calnexin (B) and total Ponceau S staining (C). Data are presented as mean±SD; N=3 independent Western Blots; * P<0.05; ** P<0.01; **** P<0.0001 (one-way ANOVA with Tukey's correction for multiple comparison). (D-E) Representative Western Blots showing sacsin levels in PBMCs from a panel of ARSACS Patients and controls. Calnexin was used as loading marker. The upper part of Ponceau S staining is also shown as loading control (see also Supplementary Material for uncropped images). (F-G) Quantification of sacsin levels normalized to calnexin (F) and total Ponceau S (G) in controls, PN3a and b, PN4, PN5 and

PN6. N=3 independent Western Blots. Data are presented as mean±SD; **** P<0.0001 (one-way ANOVA with Tukey's correction for multiple comparison).

Figure 3. Sacsin levels are conserved in an unusual ARSACS patient carrying a homozygous truncating pathogenic variant near the 3′ end of the *SACS* gene. (A) Representative Western Blot showing sacsin levels in controls and PN7. SDS-PAGE was performed with polyacrylamide gel at 5%; however, this was not resolutive enough to observe the shift at this high molecular weight (from 520kDa to 500kDa). Ponceau S staining is shown as loading control. (B) Quantification of sacsin levels normalized to Ponceau S staining signal in PN7 PBMCs compared to controls; data are presented as mean±SD; N=3 independent Western Blots; sacsin level in PN7 is not significantly altered in respect to controls (unpaired t-test). (C) Representative immunofluorescence images at 20X of primary fibroblasts from controls, PN6 and PN7, stained with anti-vimentin antibody (green) and DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (magenta). White arrows indicate examples of cells in which vimentin is bundled or displaced to the periphery. Scale bar = 40μm (D) Quantification of the percentage of cells presenting vimentin remodeling as in (C), in PN6 and PN7 compared to controls; N=3 independent experiments, at least 100 cells were acquired for each experiment. ** P<0.01 (one-way ANOVA with Tukey's correction for multiple comparisons).

Table 1 Clinical features of ARSACS patients reported in this study.

ARSACS patients	*SACS* pathogenic variants	Spasticity UL/LL	Ataxia	Dysarthria	Sensory loss	Walking difficulties/ support/ wheelchair	Reference (pathogenic variant description)
PN1	c.9284C>T, p.Pro3095Leu; c.3511_3512delTT, p.Leu1171Glyfs*8	-/++	+	-	-	walking with support	Unpublished
PN2	c.11777A>G, p.Asp3926Gly; c.11265delAT, p.Ile3755fs*8	-/++	++	-	++	walking with support	Masciullo, 2012
PN3a	c.4593dupA, p.Ser1531fs*9; c.4933C>T , p.Arg1645fs*	-/+++	++	++	+++	walking difficulties	Longo, 2021
PN3b	c.4593dupA, p.Ser1531fs*9; c.4933C>T, p.Arg1645fs*	-/++	++	-/+	+++	walking with support	Longo, 2021
PN4	[c.10907G>A, p.Arg3636Gln + c.10954C>A, p.Pro3652Thr]; c.11234_11235delTT, Leu3745fs*1	-/++	++	++	+	wheelchair	Baets, 2010
PN5	c.4205A>T, p.Asp1402Val; c.5836T>C, p.Trp1946Arg	-/++	++	+	-	walking with support (walker)	Thiffault, 2013
PN6	c.4593dupA, p.Ser1531fs*9 homozygous	+-/+	+++	++	+++	wheelchair	Prodi, 2013
PN7	c.13132C>T, p.Arg4378X homozygous	-/+++	+++	+	+	walking with support/ wheelchair for longer distances	Anesi, 2010

– absent, +- subtle, + present, ++ strongly present, +++ very strongly present. Abbreviations: UL/LL= upper limbs/lower limbs.

All patients were evaluated in 2023 or 2024.

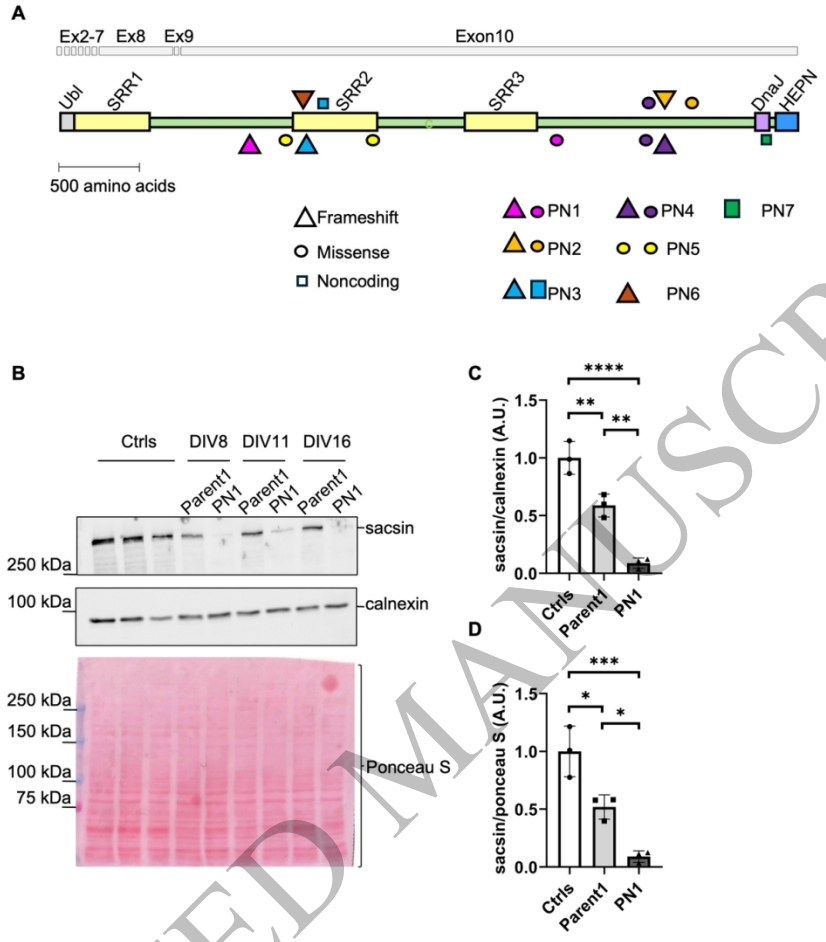


Figure 1

101x146mm (300 x 300 DPI)

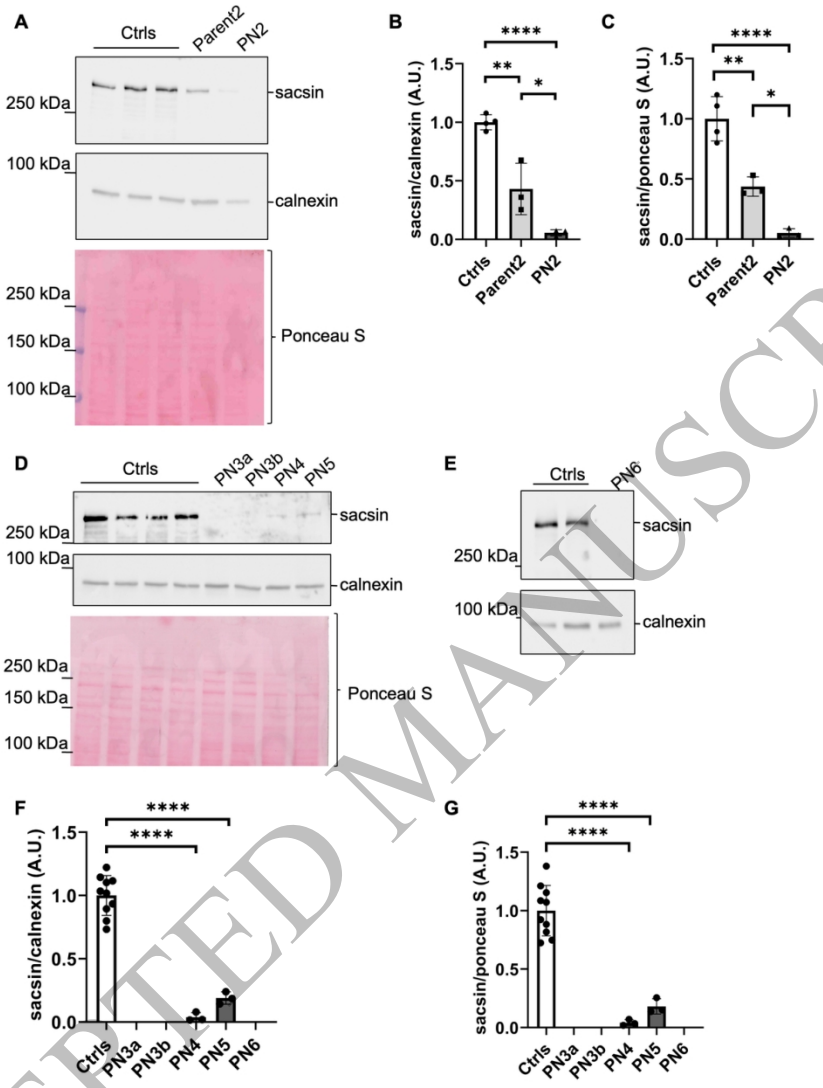


Figure 2

101x146mm (300 x 300 DPI)

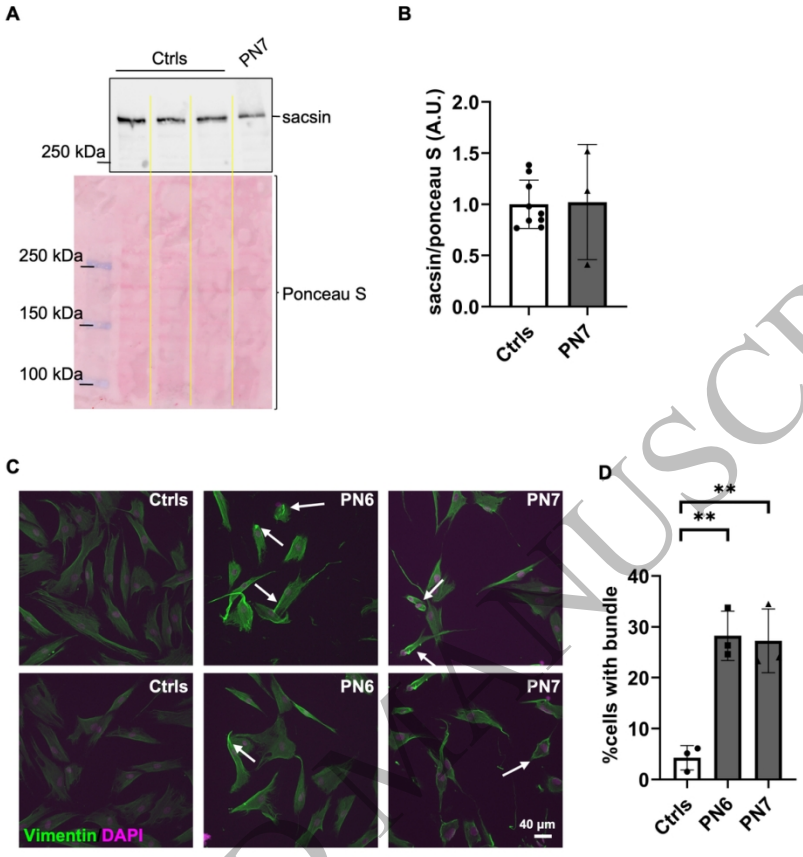
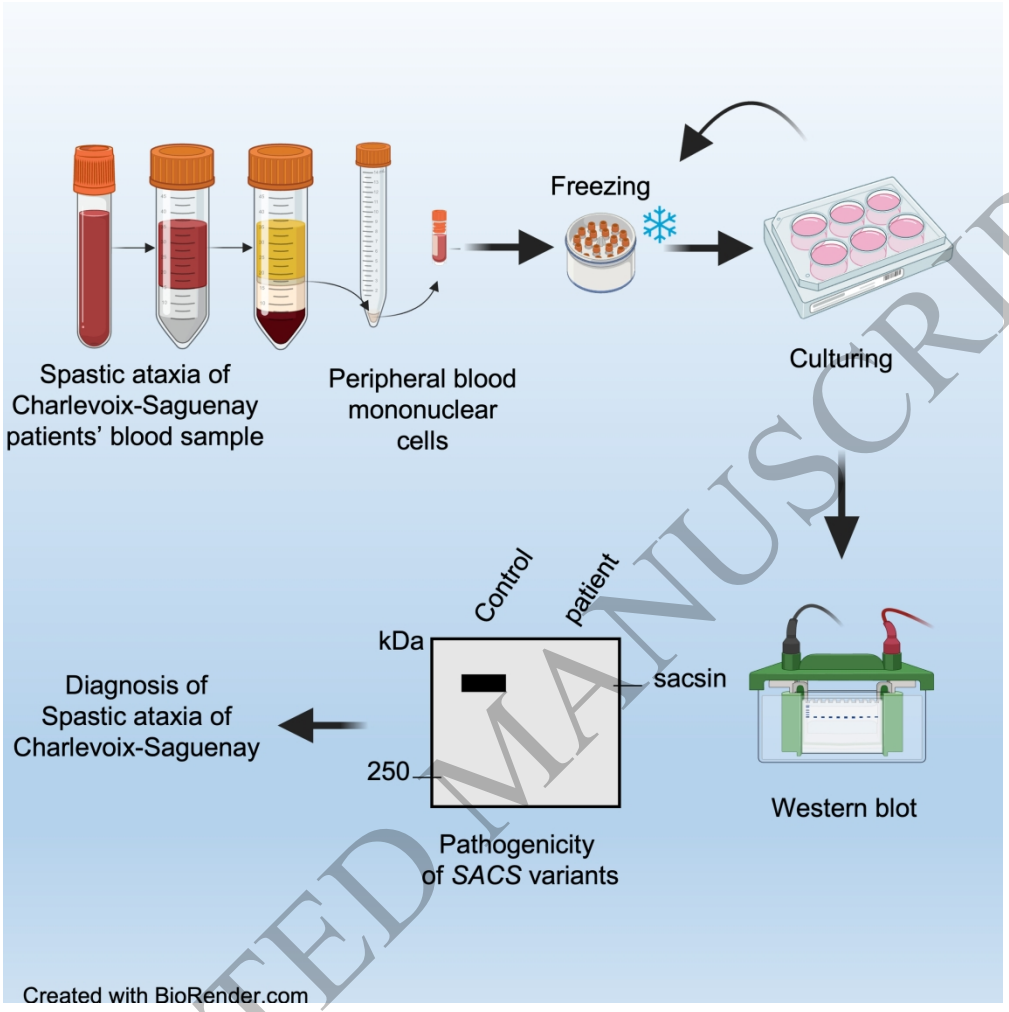


Figure 3

101x146mm (300 x 300 DPI)



Graphical Abstract

119x119mm (600 x 600 DPI)