eMethods

Cell culture treatments

Primary fibroblasts were plated and after 24 hours treated with different protease inhibitors (5 μM E64, 10 μM Bestatin, 5 μM Pepstatin (Sigma Aldrich)) and the treatments were repeated after 24hours. 48 hours after, cells were harvested and lysed for biochemical assays.

Aggregate detection

1) Soluble and insoluble fractions in SDS-PAGE

Ultrasonication: Primary fibroblasts from controls and patients were lysed by using Dounce homogenizer in 100 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8 and protease inhibitor cocktail (Sigma Aldrich), then passed through a syringe and centrifuged at 1000g x 10 minutes. Post-nuclear supernatant (PNS) (soluble fraction) was collected, and pellet (insoluble fraction) was resuspended in 500 μL lysis buffer and sonicated (20 seconds pulse-10 seconds stop; repeated twice). PNS and pellet were quantified, and an equal amount (60 μg) of each fraction was loaded on SDS-PAGE.

Laemmli buffer resuspension: An equal number of primary fibroblasts from controls and patients were lysed by using Dounce homogenizer in 100 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8 and protease inhibitor cocktail (Sigma Aldrich), then passed through a syringe and centrifuged at 1000g x 10 minutes. PNS (soluble fraction) and pellet (insoluble fraction) were directly resuspended in Laemmli sample buffer 1X (diluting the 6X in PBS: TrisHCl pH 6,8 0.35 M, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% BBP) and loaded on SDS-PAGE.

2) *Total homogenates in agarose-acrylamide gel*

Whole cellular pellets were lysed in Laemmli sample buffer 2X and sonicated to break the DNA as previously described in the ultrasonication method. Total homogenates were loaded in acrylamide-agarose mixed-gels, which have much larger pores than pure acrylamide gels, allowing the passage and an acceptable resolution of large complexes.[1] Stacking gel was prepared at 2% acrylamide, while Resolving gel at 2,7% adding a 0,5% agarose solution. 0,1 mg of total proteins per lane were loaded.

3) *Urea solubilized-cell lysates*

24 hours after MG-132 treatment, total cellular pellets were lysed in 8 M Urea, 100 mM Tris HCl and ultrasonicated as previously described in the ultrasonication method. Homogenates were loaded in two different acrylamide-concentrated gels (6% or 12%) to detect possible

truncated sacsin proteins of any molecular weight. AbN was used to hybridize the membranes compatibly with putative small and large truncated products.

Peripheral blood mononuclear cells

Blood samples were collected from healthy people (one male and one female, respectively 56 and 55 years old). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Lymphoprep (STEMCELL Technologies). RNA was extracted with ZR-Duet DNA/RNA MiniPrep plus kit (Zymo Research), quantified with Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and checked for quality with Agilent 2100 Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies). For WB analysis, PBMCs pellet was lysed in 1% Triton X-100. qPCR was performed following the procedure described in Methods.

Immunofluorescence experiments

Primary mouse embryonic fibroblasts (MEFs) were plated and after 24 hours were fixed in 4% paraformaldehyde/PBS, then permeabilized and blocked in 10% normal goat serum (NGS), 0.5% Triton X-100 in PBS. Cells were incubated with primary antibodies and species-specific Alexa Fluor Secondary Antibodies (ThermoFisher Scientific) and mounted with FluorSave Reagent (Calbiochem International) on microscope slides for immunofluorescence acquisition performed at optical fluorescence microscopy (40X).

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

A. Quantification graph of sacsin levels normalized on spectrin by using AbC in WB. B. Quantification graph of sacsin levels normalized on spectrin by using AbN in WB.

In (A,B), data are presented as mean ± SEM. *p≤0.05; **p<0.01; ****p<0.0001 (unpaired-two tailed Student's t-test).

eFigure 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 3 hours. Ubiquitinated proteins are used as readout of the treatment. B. Quantification graph relative to A. C. Representative WB of sacsin levels in patient and control fibroblasts upon proteasome plus autophagy blockade, by using 0.5 µM MG-132 and 10 µM CQ for 18 hours. P62 is used as readout of the CQ treatment and ubiquitinated proteins of the MG-132 treatment. D. Representative WB of sacsin levels in control and patient fibroblasts upon protease inhibition by using different protease inhibitors for 48 hours (repeating the treatment at 24 hours).

Abbreviations: +: treated; -: vehicle; BEST: bestatin; PEPS: pepstatin.

eFigure 3. Mutant sacsin does not aggregate

A. Representative WB of sacsin levels in control and patient soluble and insoluble fractions, obtained by pellet sonication. The asterisk represents a leak of material from the adjacent well. B. Representative WB of sacsin levels in control and patient soluble and insoluble fractions directly homogenized and resuspended in Laemmli sample buffer. C. Total homogenates from control and patient fibroblasts obtained by lysis in Laemmli sample buffer 2X and sonicated, then loaded in an agarose-acrylamide gel at 2.7 %. D. Representative WB of sacsin protein (revealed by using the AbN) in control and patient fibroblasts treated or not with 1µM MG-

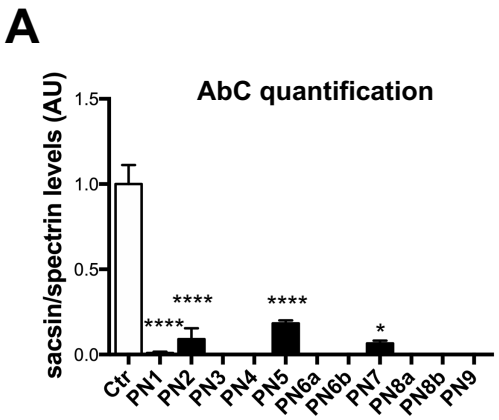
132 and solubilized in 8M Urea and ultrasonicated. The upper panel is a 6% acrylamide gel, the bottom one a 12%, suitable to detect any possible truncated misfolded sacsin proteins. Abbreviations: PNS: post-nuclear surnatant; IF: insoluble fraction.

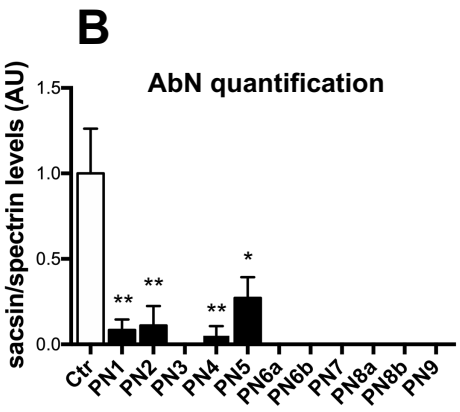
eFigure 4. Halved amount of sacsin in haploinsufficient murine embryonic fibroblasts (MEFs) does not result in intermediate filament remodeling

A. Representative WB of the sacsin levels in sacsin wild-type (Sacs+/+), heterozygous (Sacs +/-) and KO (Sacs-/-) primary MEFs normalized on spectrin levels. B. Representative immunofluorescence images showing the severe vimentin remodeling in Sacs-/- primary MEFs, a typical sign of ARSACS pathology, which is absent in Sacs+/+ and Sacs+/- primary MEFs. Arrows indicate the perinuclear vimentin accumulation.

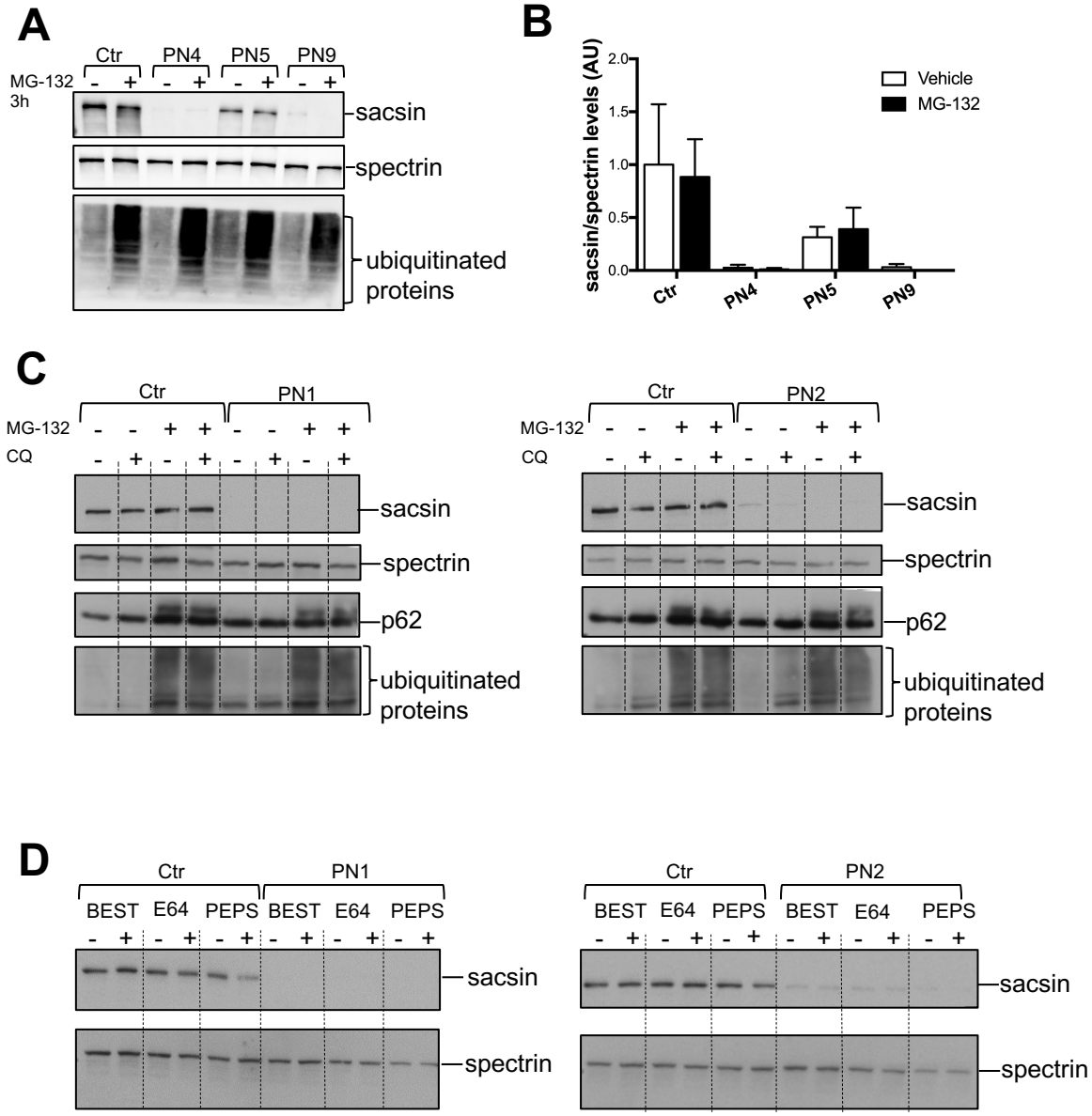
eFigure 5. Sacsin is expressed in human peripheral blood mononuclear cells

A. Detection of *SACS* mRNA by RT-PCR in PBMCs from two different human subjects. Total RNA from the same extraction which was not retrotranscribed (RT-) underwent the same amplification as negative control, to check for genomic contamination. B. Quantification by qRT-PCR of *SACS* mRNA level in PBMCs compared to control fibroblasts used in this work, normalized on *TBP* mRNA. C. Representative WB showing sacsin expression in human PBMCs, compared to fibroblasts, by using AbC antibody; spectrin was used as normalizer. Abbreviations: RT: retro-transcriptase; PBMCs: Peripheral Blood Mononuclear Cells.

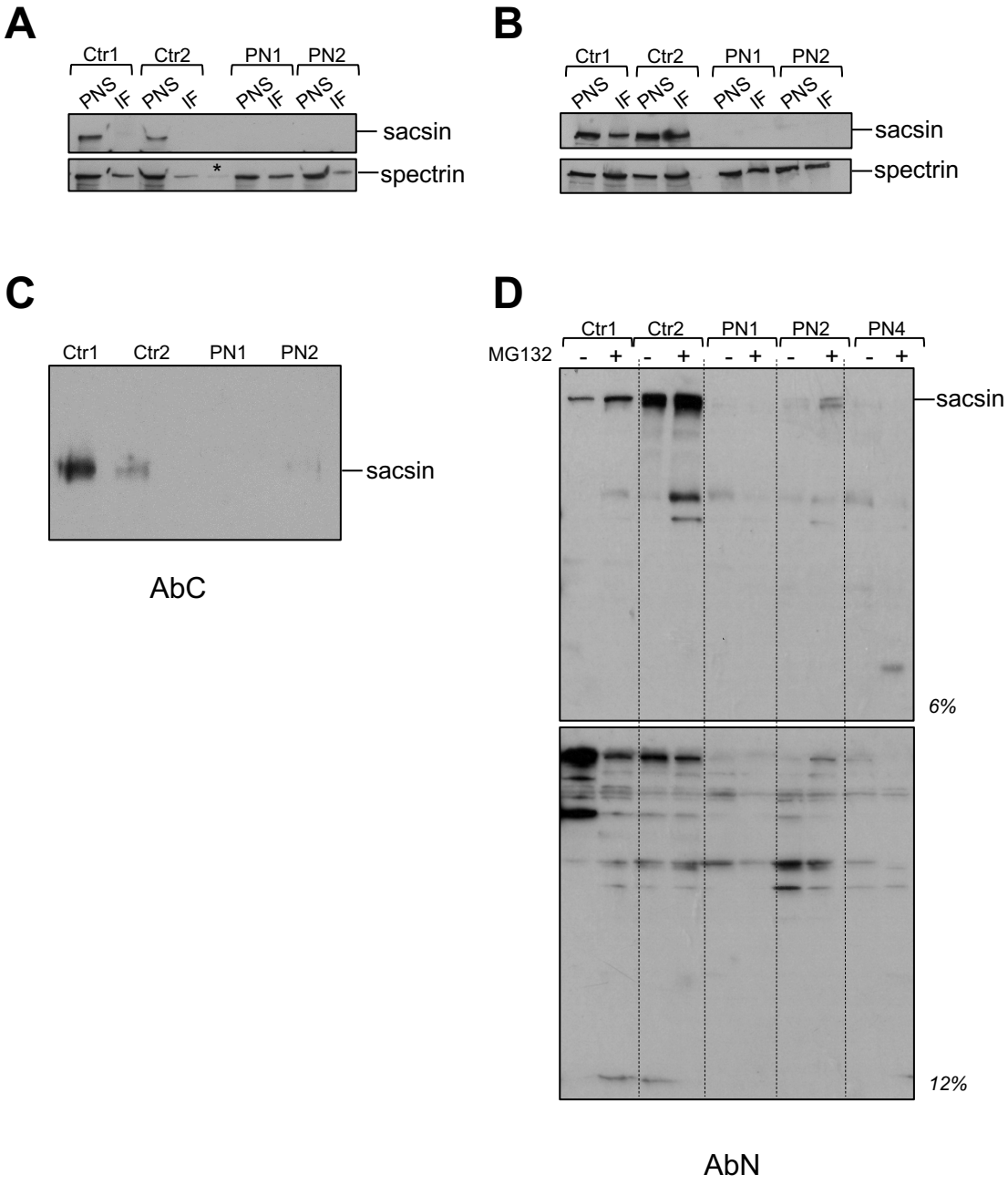

A
AbC quantification
sacsin/spectrin levels (AU)
1.5
1.0
0.5
0.0
****
****
****
*
Ctr
PN1
PN2
PN3
PN4
PN5
PN6a
PN6b
PN7
PN8a
PN8b
PN9


B
AbN quantification
sacsin/spectrin levels (AU)
1.5
1.0
0.5
0.0
**
**
**
*
Ctr
PN1
PN2
PN3
PN4
PN5
PN6a
PN6b
PN7
PN8a
PN8b
PN9

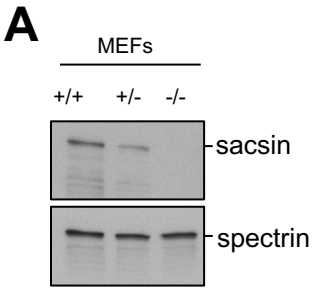
eFigure 1

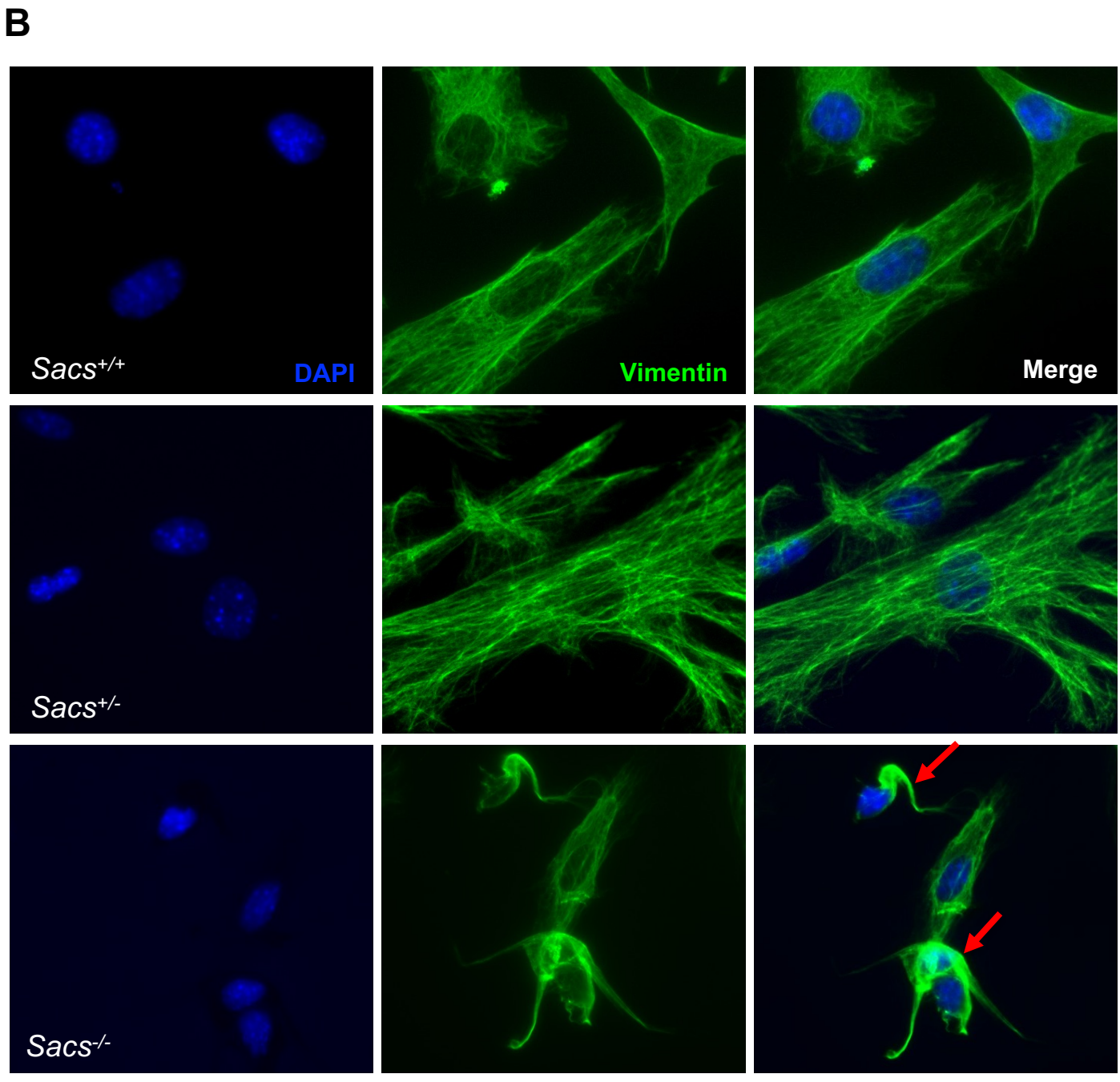

A
Ctr
PN4
PN5
PN9
MG-132
3h
sacsin
spectrin
ubiquitinated proteins
B
sacsin/spectrin levels (AU)
2.0
1.5
1.0
0.5
0.0
Vehicle
MG-132
Ctr
PN4
PN5
PN9
C
Ctr
PN1
MG-132
CQ
sacsin
spectrin
p62
ubiquitinated proteins
Ctr
PN2
MG-132
CQ
sacsin
spectrin
p62
ubiquitinated proteins
D
Ctr
PN1
BEST
E64
PEPS
sacsin
spectrin
Ctr
PN2
BEST
E64
PEPS
sacsin
spectrin

eFigure 2

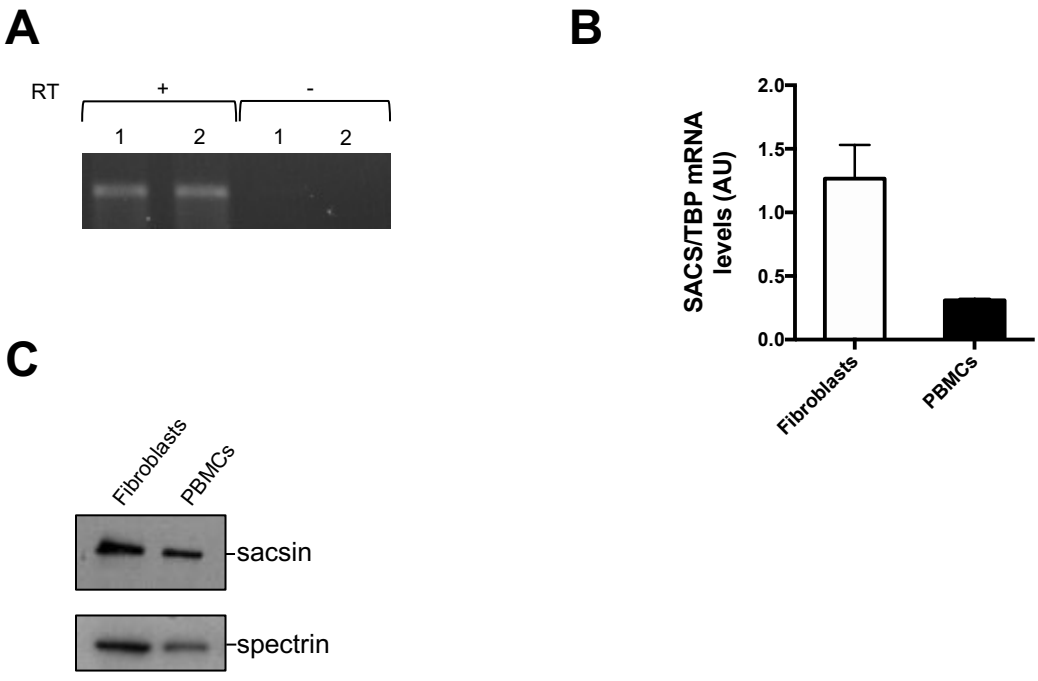

A
Ctr1
Ctr2
PN1
PN2
PNS
IF
sacsin
spectrin
*
B
C
AbC
D
MG132
PN4
6%
12%
AbN

eFigure 3





eFigure 4


A
RT
+
-
1
2
1
2
B
SACS/TBP mRNA levels (AU)
2.0
1.5
1.0
0.5
0.0
Fibroblasts
PBMCs
C
Fibroblasts
PBMCs
sacsin
spectrin

eFigure 5

eReference

1. Weiss A, Klein C, Woodman B, et al. Sensitive biochemical aggregate detection reveals aggregation onset before symptom development in cellular and murine models of Huntington's disease. *J. Neurochem.* 2008;104:846–858