

# Toxic response caused by a misfolding variant of the mitochondrial protein short-chain acyl-CoA dehydrogenase

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## Abstract

**Background** Variations in the gene *ACADS*, encoding the mitochondrial protein short-chain acyl CoA-dehydrogenase (SCAD), have been observed in individuals with clinical symptoms. The phenotype of SCAD deficiency (SCADD) is very heterogeneous, ranging from asymptomatic to severe, without a clear genotype–phenotype correlation, which

suggests a multifactorial disorder. The pathophysiological relevance of the genetic variations in the SCAD gene is therefore disputed, and has not yet been elucidated, which is an important step in the investigation of SCADD etiology.

**Aim** To determine whether the disease-associated misfolding variant of SCAD protein, p.Arg107Cys, disturbs mitochondrial function.

**Methods** We have developed a cell model system, stably expressing either the SCAD wild-type protein or the misfolding SCAD variant protein, p.Arg107Cys (c.319 C>T). The model system was used for investigation of SCAD with respect to expression, degree of misfolding, and enzymatic SCAD activity. Furthermore, cell proliferation and expression of selected stress response genes were investigated as well as proteomic analysis of mitochondria-enriched extracts in order to study the consequences of p.Arg107Cys protein expression using a global approach.

**Conclusions** We found that expression of the p.Arg107Cys variant SCAD protein gives rise to inactive misfolded protein species, eliciting a mild toxic response manifested though a decreased proliferation rate and oxidative stress, as shown by an increased demand for the mitochondrial antioxidant SOD2. In addition, we found markers of apoptotic activity in the p.Arg107Cys expressing cells, which points to a possible pathophysiological role of this variant protein.

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Competing interest: None declared.

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## Abbreviations

SCAD	Short-chain acyl-CoA dehydrogenase
Wt	Wild-type
Ut	Untransduced cells
CLSM	Confocal laser scanning microscopy
MFN	Mitofusin
BAX	BCL2-associated X protein

VDAC	Voltage dependent anion channel
SOD2	Superoxide dismutase 2
HO-1	Heme oxygenase 1
Hsp	Heat shock protein
mtHsp	Mitochondrial heat shock protein
SCADD	SCAD deficiency
CNS	Central nervous system
EMA	Ethyl malonic acid
PRDX6	Peroxiredoxin 6
iTRAQ	Isobaric tag for relative and absolute quantitation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

## Introduction

Short-chain acyl-CoA dehydrogenase (SCAD) is the initiating enzyme in the mitochondrial  $\beta$ -oxidation of short-chain fatty acids (Ikeda et al 1985). Like the majority of mitochondrial proteins, SCAD is translated in the cytosol and subsequently transferred to the mitochondria, where it is folded to its native structure with the aid of the mitochondrial chaperonin Hsp60/Hsp10 (Pedersen et al 2003; Corydon et al 2005). SCAD is a flavoenzyme and functions in its active conformation as a homotetramer in complex with the cofactor FAD (flavine adenine dinucleotide). Variations in the SCAD gene, *ACADS* (MIM \*606885), have been found to be associated with elevated urinary excretions of ethylmalonic acid (EMA) derived from detoxification of the accumulated substrate of SCAD, butyryl-CoA (Hegre et al. 1959), as well as clinical symptoms, known as SCAD deficiency (SCADD) (Pedersen et al 2008; Waisbren et al 2008). However, the pathophysiological relevance of *ACADS* variations must be further elucidated, based on the heterogeneity of clinical symptoms associated with *ACADS* variations, and the lack of a clear genotype–phenotype correlation with outcomes ranging from very severe to asymptomatic (van Maldegem et al 2006; Jethva and Ficicioglu 2008; Pedersen et al 2008; Waisbren et al 2008).

Disease-associated variations of the SCAD protein have been shown to be unstable and the process of folding impaired (Corydon et al 1998; Pedersen et al 2003, 2008). Protein misfolding is involved in a variety of diseases, and the research in this field is large and still expanding due to the fact that a number of major neurodegenerative disorders, e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease, are members of the group of protein conformational diseases (Kopito and Ron 2000; Stefani and Dobson 2003). Accumulated misfolded proteins have been

shown to exert a toxic cellular effect leading to oxidative stress (Behl et al 1994; Hsu et al 2000; Gregersen et al 2006; Lin and Beal 2006; Gregersen and Bross 2010) and cell death (Nakamura and Lipton 2009), but the main pathogenic factors of misfolded proteins have not yet been elucidated.

In order to investigate putative factors involved in the pathology of disease associated with a misfolding variation in the *ACADS* gene, we have studied the variant SCAD protein p.Arg107Cys (c.319 C>T). This variation has previously been shown to compromise protein folding in isolated mouse mitochondria (Kragh et al 2007; Pedersen et al 2008) and lack of activity in patient fibroblasts (Tein et al 1999). It is primarily observed in the Ashkenazi Jewish population, with heterogeneous clinical symptoms, though predominantly defined by neuromuscular symptoms (Tein et al 2008; Waisbren et al 2008).

When transiently overexpressed in human astrocytes, we have previously shown that SCAD p.Arg107Cys protein elicits a toxic response by disturbing normal mitochondrial function, visualized through a disruption of the normal dynamic equilibrium of fission and fusion of the mitochondrial reticulum, accompanied by oxidative stress (Schmidt et al 2010).

In the present study, we have further investigated the SCAD variant protein p.Arg107Cys using a cell model system stably expressing either the wild-type SCAD protein or the p.Arg107Cys variant protein. In order to elucidate whether this disease-associated variant of SCAD could be involved in the pathophysiology of SCADD, we measured the *ACADS* gene expression, SCAD protein folding/misfolding, SCAD enzyme activity, cell proliferation, and expression of selected stress response genes, in addition to a global approach using quantitative nanoLC-MS/MS proteomic analysis. We report the cellular consequences of stable overexpression of the disease-associated p. Arg107Cys variant of SCAD, including a decreased proliferation rate, increased levels of antioxidants, as well as markers of apoptosis. Taken together, these results show that this misfolded protein is capable of disturbing mitochondrial function.

## Materials and methods

### Cell culturing

The virus packaging cell lines GP+E86 (*Mus musculus*, ATCC # CRL-9642), PG13 (*Mus musculus*, ATCC # CRL-10686) and the host cell line A172 (*Homo sapiens*, ATCC # CRL-1620), were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Life Technologies) supplemented with 10 % (v/v) fetal calf serum (Biological

Industries), 0.29 mg/ml glutamine, 100 units/ml penicillin (Leo Pharmaceutical), and 0.1 mg/ml streptomycin (Leo Pharmaceutica). The cell lines were incubated at 37°C, or 40°C for heat shock experiments, at 5% (v/v) CO<sub>2</sub>.

### Transduction

SCAD Wt or the variant c.319 C>T (p.Arg107Cys) cDNA was cloned into the retroviral vector pBabe-puro (Morgenstern and Land 1990). An ecotropic packaging cell line, GP+E86 (ATCC, CRL-9642) was seeded with 2/3 × 10<sup>6</sup> cells in a 25-cm<sup>2</sup> culture flask (TPP). After 24 h, the cells were transfected with the pBabe-puro constructs by calcium phosphate co-precipitation. Briefly, 15 µg plasmid DNA was diluted in buffer A (0.15 M NaCl, 1 mM EDTA, 0.01 M tris-HCl, pH 7.5) and 0.25 M CaCl (Total volume of 280 µl). The mixture was carefully added to 280 µl T× buffer (0.05 M HEPES pH 7.1, 0.25 M NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>/Na<sub>2</sub>PO<sub>4</sub>), and the precipitate was allowed to form for 30 min at room temperature. The GP+E86 cells were administered with 5 ml new DMEM medium and added to the 560 µl transfection mixture. After 4 h of transfection at 37°C, the cells were administered with 5 ml new DMEM medium. Twenty-four hours later, the medium was exchanged with 2 ml fresh medium, which was harvested 24 h later, supplemented with polybrene, 8 µg/ml (Sigma), and sterile-filtered through a 0.45-µm filter (Millipore, MA, USA). The virus-enriched medium was used in the transduction of an amphotropic packaging cell line, PG13, by centrifugation of plates 30 min at 32°C at 1,000g, and incubated at 37°C overnight. The A172 cells were transduced as above, now using virus-enriched PG13 medium, diluted 10<sup>2</sup>, 10<sup>3</sup>, and 10<sup>4</sup> times to ensure a low viral titer, and consequently a low copy number integrated. After a selection period of 14 days, using conditioned medium with 2 µg/ml puromycin, clones were picked from the plates with the least colonies, and transferred to isolated 6-well culture plates for amplification.

### mRNA expression levels

RNA was isolated using the PerfectPure RNA cultured cell kit (5Prime). cDNA was synthesized from 1 µg RNA using “1st-strand” cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) according to manufacturer’s instructions. mRNA transcription levels were measured in triplicates using the TaqMan<sup>®</sup> Gene expression assay on an ABI PRISM<sup>®</sup> 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of the forward primer, reverse primer, and probes used in each assay were: SCAD; 5’CGCCCCTCAC CAAGCT3’, 5’GGCCAGGGCCATGTCT3’, and 5’CAGGT CATCCAGTTCAAG3’. Hsp60 (HSPD1); 5’GGCTTGCAAACTTTTCAGATGGA3’, 5’GGCATCTG TAACTCTGTCTTTCTTTTCA3’, and 5’TCCCACCA

ACCTTCAGCAC3’. HO-1 (HMOX1); 5’CCAGCGGGC CAGCAA3’, 5’GGGAGCGGGTGTGAGT 3’, and 5’CAAAGTGCAAGATTCTG 3’. β-actin (ACTB #4310881E), Hps70 (HSPA1A, #Hs00271244\_s1), and SOD2 (#Hs00167309\_m1) geneexpression assays were manufactured by Applied Biosystems. The probe used in the β-actin assay was conjugated to a VIC<sup>™</sup> fluorescent label at the 5’ end, while probes of the remaining assays were conjugated to a 6-FAM<sup>™</sup> dye. All probes contained a non-fluorescent quencher at the 3’ end.

The measurements were performed on cell cultivations performed in triplicates at 3 separate days (i.e. nine cultivations and mRNA expression studies performed for each clone). Statistical analysis was performed with an analysis of variance test (ANOVA), with a stochastic level for each observation.

### Western blotting

SDS-PAGE: Cell pellets were incubated 30 min in lysisbuffer on ice [50 mM Tris-HCl, pH 7.8, 5 mM EDTA, pH 8.0, 1 mM DTT, 10 µg/ml Aprotinin (Sigma-Aldrich), 1 mg/ml trypsin inhibitor (Bie & Berntsen), tablet of protease inhibitors (Roche) in 10 ml, and 0.5% Triton X-100], followed by three times freeze/thaw and 40 sec of sonication (Branson, Ultrasonic cleaner). The lysate was separated into Triton X-100 soluble and insoluble fractions by 15 min of centrifugation at 10,000g, 4°C. Ten µg of the soluble protein fraction, as well as equal volume of the insoluble fraction, was subjected to SDS-PAGE gel electrophoresis on 12.5% Tris-HCl Criterion pre-cast gels (Bio-Rad). Proteins were blotted onto a PVDF membrane (Immobilon-P<sup>™</sup>, Millipore, 0.45 µm) by semi-dry electro-blotting (2117 Multiphor II, Pharmacia) at 220 mA for 45 min. Native-PAGE: As with SDS-PAGE, except the lysis-buffer contains 250 mM sucrose. Then, 15 µg total protein lysate was loaded on a 4–15% Tris-HCl Criterion pre-cast gel (Bio-Rad). In the blotting procedure, 0.1% v/v SDS was added to the transfer buffer, and blotting was carried out at 110 mA for 90 min.

The PVDF membranes were incubated 1 h in 5% non-fat skim milk (Difco), and further 24 h with polyclonal rabbit anti-SCAD antibodies (Ikeda et al 1985), following 1 h with secondary goat anti-rabbit-HRP antibodies (Dako). For detection of protein, ECL plus Western Blotting Detection System (Amersham Biosciences) was used, according to manufacturer’s recommendations, and visualized by Phosphor Imaging (STORM 840, Molecular Dynamics). Statistical analysis was performed using a *t* test.

### Immunolocalization

Cells were incubated 30 min at 37°C with 100 nM MitoTracker<sup>®</sup> Red CMXRos probe (Molecular Probes) at

30% confluence in 10-cm<sup>2</sup> slideflasks (Nunc, Roskilde, Denmark), followed by fixation in 1.5 ml 4% (w/v) paraformaldehyde (Merck). Slides were incubated with primary polyclonal anti-SCAD antibodies (Ikeda et al 1985) for 1 h, and subsequently 1 h with secondary Alexa 488-labeled goat anti-rabbit IgG antibodies (Molecular Probes). Cells were treated for 20 min with 1 mg/ml RNase A (Roche), and nuclear labeling was carried out using 1 μM TO-PRO-3 iodide (Molecular Probes). A drop of SlowFade<sup>®</sup> Golden antifade reagent (Molecular Probes) was added before imaging by confocal laser scanning microscopy, using a Leica TCS SL microscope. Imaging was done by using a 488-nm line of a multiline argon laser (detection of Alexa-488), the 543-nm line of a green helium-neon laser (detection of MitoTracker Red CMXRos), and the 633-nm line of a red helium–neon laser (detection of TO-PRO-3).

#### Activity measurements

Cell pellets were homogenized and sonicated in PBS plus 50 μmol/l flavin adenine dinucleotide (FAD). The protein concentration was adjusted to 2 mg/ml, and 10 μl was incubated at 37°C in medium, containing 200 mmol/l Tris-HCl (pH 8.0), 50 μmol/l FAD, 400 μmol/l ferricinium hexafluorophosphate, 25 μmol/l butyryl-CoA, and 200 μmol/l isovaleryl-CoA (to eliminate unspecific conversion of butyryl-CoA by the isovaleryl-CoA dehydrogenase enzyme). The reaction was terminated by adding HCl to a final concentration at 200 mmol/l. After centrifugation, the supernatant was neutralized with 2 mol/l KOH and 600 mmol/l morpholinoethane sulfonic acid (MES), and acyl-CoA esters were resolved by HPLC followed by calculation of reaction rates from the amount of crotonyl-CoA and 3-hydroxybutyryl-CoA formed (Bok et al 2003). By performing parallel reactions, supplemented with anti-SCAD antibodies, the calculations were corrected for unspecific 3-hydroxybutyryl-CoA production. All measurements were performed in triplicates, and expressed as nmol per minute per milligram total protein. Statistical analysis was performed using a *t*-test.

#### Mitochondrial enrichments

Each cell line was cultivated in triplicates, and pelleted into three pools of the clones Wt 1–3, three pools of the p.Arg107Cys expressing clones 1–3, as well as three pools of untransfected cells. Cell pellets (each corresponding to cells from three 150-cm<sup>2</sup> culture flask (TPP)) were resuspended in 11 ml hypotonic RSB buffer (10 mM NaCl, 1.5 mM MgCl<sub>2</sub>) and cells were allowed to swell for 15 min before disruption of the cellular membrane with 20 strokes in an ice-cold 15-ml Dounce homogenizer, using pestle B.

Immediately after, 8 ml 2.5 × MS-buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 7.5)) were added to attain an isotonic solution. After addition of 10 ml 1 × MS-buffer, mitochondrial enrichments were performed using differential centrifugation (10 min, 750g, repeated twice, supernatant centrifuged 15 min, 10,000g). All steps performed at 4°C or on ice.

#### Proteomic analyses

For large-scale relative quantification of proteins in samples enriched for mitochondria, iTRAQ (isobaric tag for relative and absolute quantitation) was applied. The analyses and data treatment were performed as previously described (Palmfeldt et al 2009) applying nano-liquid chromatography (Easy nLC; Proxeon, Odense, Denmark) separation coupled to tandem mass spectrometry (MS/MS) detection (LTQ Orbitrap; Thermo Fisher Scientific, Waltham, USA). The analyses were performed in triplicate starting with material from three separate cultivations. In each iTRAQ analysis, the relative amounts between the three samples; Wt, p.Arg107Cys, and untransduced, were determined for each protein. The samples were analyzed in duplicate on nanoLC-MS/MS and data from the two analyses were merged, giving increased robustness to the quantification. Proteins were reported to have statistically altered levels when they passed two criteria (1) two-tailed student *t* test ( $p < 0.05$ ,  $n = 3$ ) and (2) a threshold test, based on  $2 \times$  global standard error, requiring at least 11.6% altered level.

#### Proliferation study

Samples of 250,000 cells of each clone were seeded into 25-cm<sup>2</sup> culture flask (TPP). Cell numbers were evaluated using the Nucleocounter, Chemometec, according to manufacturers' recommendations. Measurements performed on 37°C cell cultivations were performed once for each clone, and statistical analyses were performed using a *t* test. Measurements performed on 40°C cell cultivations were performed in triplicates (i.e. three cultivations for each clone), and statistical analyses were performed with an analysis of variance test (ANOVA), with a stochastic level for each observation.

## Results

Considering the neuromuscular symptoms of patients with variations in the *ACADS* gene, we have chosen to study the effects of p.Arg107Cys SCAD protein in human astrocytes. The astrocyte cell type is the only cell type in the central nervous system (CNS) which harbors the enzymes of the

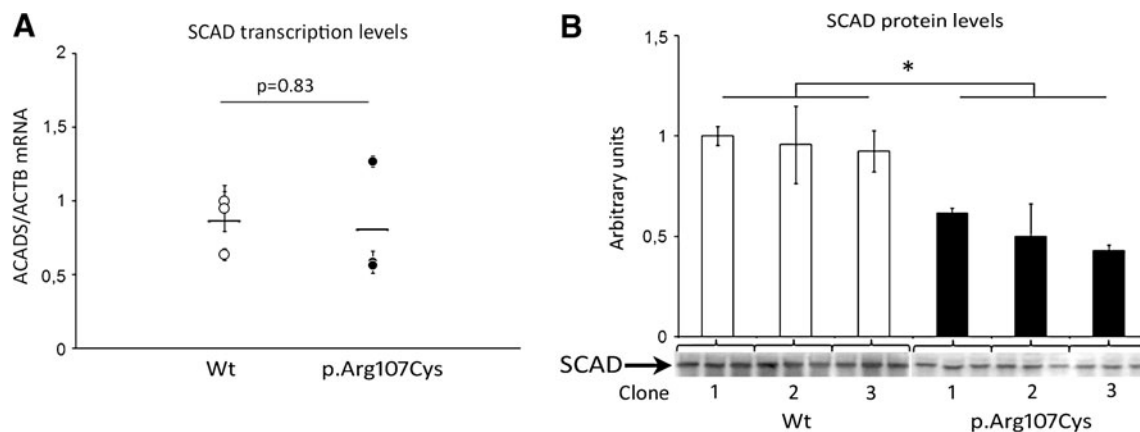
mitochondrial  $\beta$ -oxidation, and is thus the expected CNS cell type to be affected by the proposed accumulations of misfolded SCAD proteins (Auestad et al 1991; Ebert et al 2003). A human cell line stably expressing the SCAD wild-type (Wt) or the variant p.Arg107Cys protein was developed by retroviral transduction of the astrocytic cell line A172 (American Type Culture Collection # CRL-1620). A172 cells have the advantage of a downregulated *ACADS* expression and therefore do not express SCAD protein endogenously. In order to minimize SCAD overexpression of the transduced A172 cells, a low viral titer was utilized in the transduction experiments to diminish the number of cells receiving more than one copy of the *ACADS* cDNA. To circumvent differences between Wt and p.Arg107Cys expressing cells to be caused by different integration sites, several transduced clones of each cDNA type were established.

SCAD mRNA expression levels were quantified in all clones, relative to  $\beta$ -actin. To be able to compare the phenotypic outcome, three Wt clones (Wt 1–3) and three p.Arg107Cys clones (p.Arg107Cys 1–3) from each transduction experiment were selected based on comparable SCAD mRNA transcription levels (Fig. 1a; *t* test performed on mean values,  $p=0.83$ ). In addition, we evaluated the SCAD protein products from the six selected clones by SDS-PAGE followed by western blotting of cellular lysates, using anti-SCAD antibody (Fig. 1b). Quantitative SCAD-protein analysis of monomeric SCAD protein amounts in three independent cell cultures of each clone revealed that the three p.Arg107Cys expressing clones (black bars) contain approximately half the amount of steady state level

SCAD protein, compared with the Wt (white bars, *t* test performed on mean values,  $p<0.01$ ). Subsequently, mitochondrial concentrations of SCAD protein were visualized by confocal laser scanning microscopy (CLSM), using the mitochondrial-targeted probe MitoTracker Red CMXRos and anti-SCAD antibody, showing a decreased amount of SCAD protein in the p.Arg107Cys expressing cells compared with Wt expressing cells. Correct localization of the introduced SCAD protein in the mitochondria was confirmed by merging anti-SCAD image (green color) with MitoTracker (red), rendering a yellow color when colocalized. Fig. 2 shows an example of one Wt and one p.Arg107Cys expressing clone.

Native-PAGE analysis was subsequently used to investigate whether the SCAD proteins were properly folded to the native tetrameric form (Fig. 3a). The blot shows an intense band corresponding to Wt tetrameric SCAD (lanes 1–3), whereas the corresponding band of the variant protein is weak (lanes 4–6), close to the level of the untransduced cells (Ut, lane 7). We next analyzed the Wt and the p.Arg107Cys expressing clones, as well as the untransduced cell line for SCAD enzyme activity (Fig. 3b). The enzyme activity of the SCAD Wt transduced cells was increased as expected, but the enzyme activity of the p.Arg107Cys transduced cells was close to the background levels of the untransduced cells (Ut). This is in accordance with the lowered SCAD-protein tetramer levels detected by the immunoblot analysis (Fig. 3a).

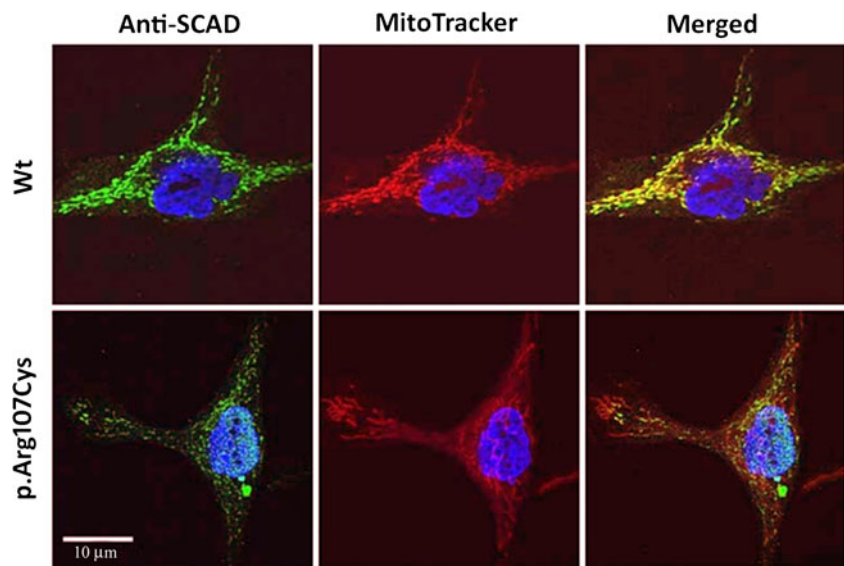
To examine whether these misfolded inactive protein variants elicit an effect on the cells, a proliferation study



**Fig. 1** SCAD mRNA and protein expression levels. **a** Quantitative PCR performed on cDNA from three selected Wt clones (white circles) and three p.Arg107Cys clones (black circles). The SCAD transcription is represented relative to  $\beta$ -actin and normalized to the Wt-1 clone. Error bars reflect the standard deviations of three measurements on the same cDNA pool of each clone. A *t* test on the mean values of each clone revealed no difference in the SCAD mRNA expression-levels between the Wt and p.Arg107Cys expressing cells ( $p=0.83$ ). The vertical bar represents the mean value of the three clones. **b** SDS-PAGE and western blot of the selected clones.

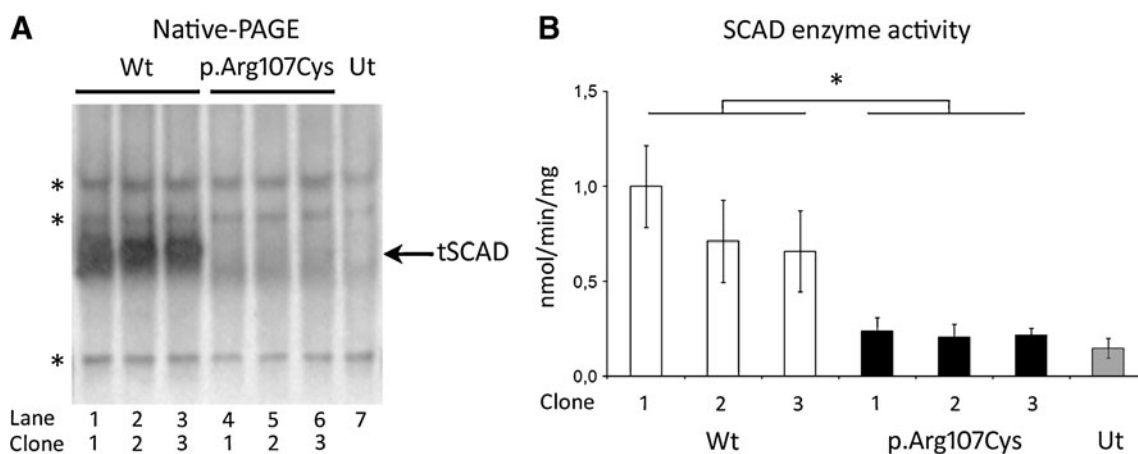
The schematic diagram is depicting the mean and standard deviation of three independent cell-culture flasks, by quantitation of the blot, and normalized to the Wt-1 clone. Error bars reflect standard deviations of triplicate cell cultivations of each clone (to be seen on the western blot). A *t* test performed on the mean values of each clone showed a statistically significant difference in the SCAD protein-levels between Wt and p.Arg107Cys expressing cells (\* $p=0.0016$ ). No SCAD bands were detected by western blotting of untransduced cells (data not shown)

**Fig. 2** SCAD colocalizes with the mitochondria. Confocal laser scanning microscopy of a Wt and a p.Arg107Cys expressing clone after immunolabeling with anti-SCAD antibody (green color) and MitoTracker Red CMXRos (red color). The image is a representative example of one of each clone, confirming colocalization of the exogenic SCAD protein with the mitochondria



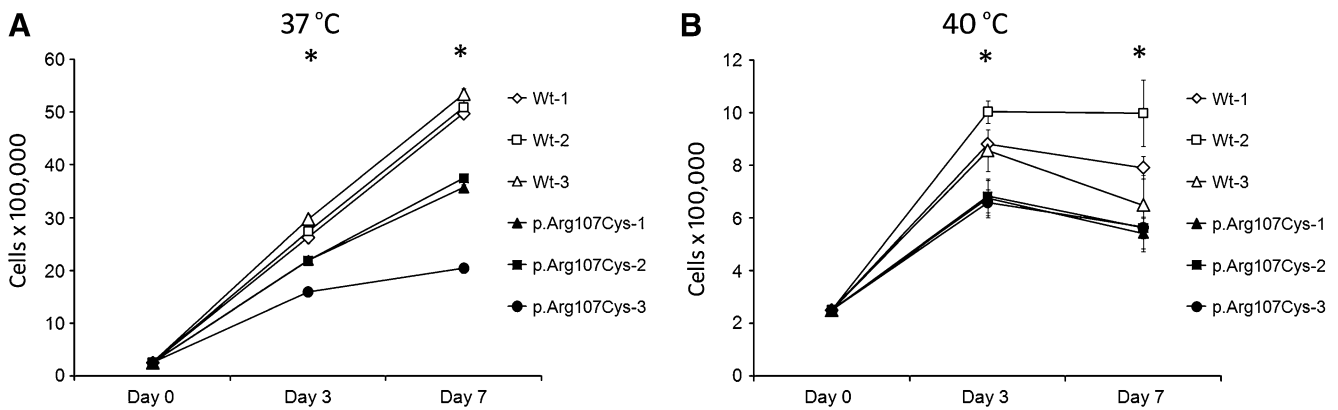
was performed (Fig. 4). Preliminarily, 250,000 cells of each clone were seeded in 25-cm<sup>2</sup> culture flasks, resulting in approximately 25% confluence, and the number of cells was evaluated after 3 and 7 days, respectively. At 37°C, the mean cell number of the three Wt clones (white marks) was found to be consistently higher than the p.Arg107Cys expressing clones (black marks), which points to an inhibited growth of the p.Arg107Cys expressing cell lines, compared with the Wt-expressing cells (Fig. 4a). Protein unfolding and misfolding is known to be exacerbated at higher temperatures. In order to test possible differences in how the cells respond to stress, the proliferation study was repeated in triplicate in cells grown at 40°C (Fig. 4b). At 40°C, proliferation was inhibited in both cell lines, compared with 37°C. Importantly, all six clones revealed

a complete stop in cell division after 3 days of heat stress, following a reduction in cell number up to day 7, indicating cell death. As with the proliferation curves at 37°C, the proliferation curves of cells grown at 40°C show a significantly slower proliferation rate in the p.Arg107Cys expressing clones compared with the SCAD Wt expressing clones. The difference between the Wt and the p.Arg107Cys expressing clones was nevertheless not enhanced by heat stress (differences at day 3,  $p=0.025$  and  $p=0.013$ , and at day 7  $p=0.021$  and  $p=0.031$  at 37 and 40°C, respectively). To further study the effect of heat stress, one Wt-expressing clone as well as one p.Arg107Cys expressing clone were subjected to growth at 40°C, and harvested at eight time points over a period of 45 h. This preliminary study was carried out to find



**Fig. 3** Tetramerization and activity measurements. **a** Native-PAGE of Wt and p.Arg107Cys expressing clones, as well as of untransduced cells (*Ut*). The blot shows an impaired tetramerization ability of the p.Arg107Cys proteins. *tSCAD* tetrameric SCAD, \*unspecific protein signals witnessing of equal protein loading. **b** SCAD enzyme activity

measurements, expressed in nmol/min/mg total protein. Error bars reflect the standard deviation of three activity measurements on the same protein lysate. A *t* test on the mean values show a significant difference in SCAD activity between Wt and p.Arg107Cys expressing cells ( $*p=0.006$ )



**Fig. 4** Inhibited cell proliferation of the p.Arg107Cys expressing cells. 250,000 cells were seeded at day 0, and the cell number was evaluated at day 3 and day 7. At both 37 and 40°C, the p.Arg107Cys expressing clones were proliferating significantly slower than the Wt expressing clones (37°C, \*day 3:  $p=0.025$ ; day 7  $p=0.021$ , evaluated by  $t$  test),

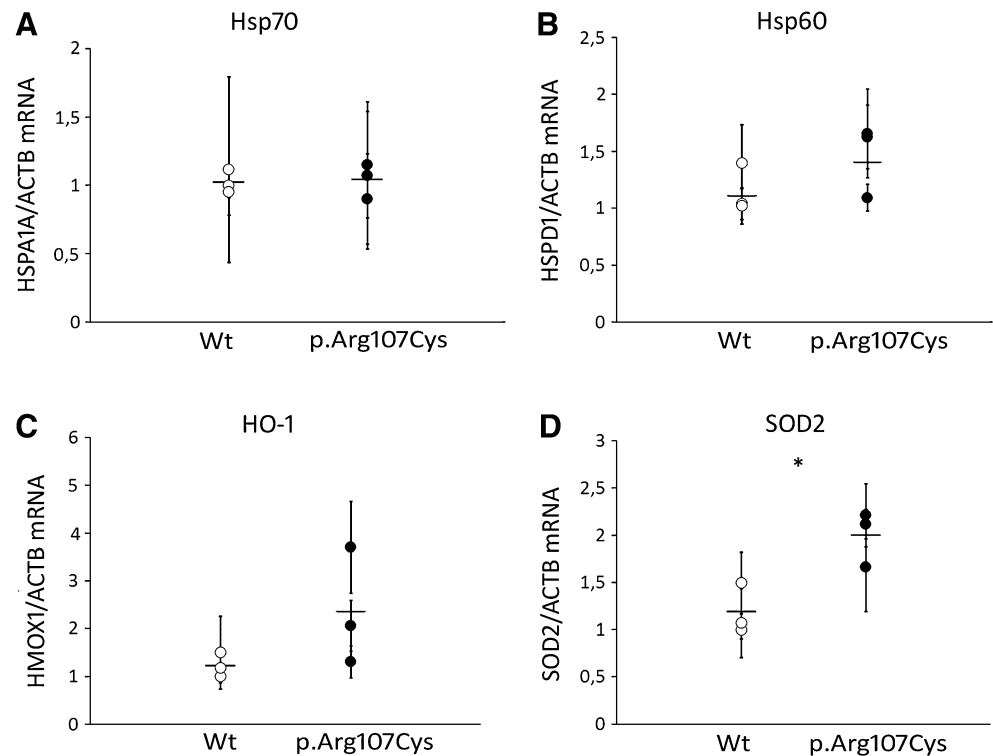
(40°C, \*day 3:  $p=0.013$ ; day 7:  $p=0.031$ , evaluated by an ANOVA test with a stochastic level for each observation). Error bars on the 40°C proliferation study reflect standard deviations of triplicate cell cultivations of each clone

“appropriate stress-conditions” for further studies, in order to investigate differences in stress responses between the Wt and the p.Arg107Cys expressing cells. The “appropriate stress-conditions” were selected as the 40°C stress duration, in which cells were not inhibited in normal cellular transcription/translation events, but a condition affecting the cellular homeostasis enough to reveal presumed differences in the two types of cells. To measure misfolding stress as well as oxidative stress responses, the cells were analyzed for mRNA transcription levels of the inducible chaperone Hsp70 (*HSPA1A*, cytosolic), the chaperone Hsp60 (*HSPD1*, mitochondrial), as well as for the antioxidants heme oxygenase 1, HO-1 (*HMOX1*, cytosolic), and superoxide dismutase 2, SOD2/MnSOD (*SOD2*, mitochondrial). We found an indication of increased antioxidant levels in the p.Arg107Cys expressing clone after 24 h at 37°C, a finding which was enhanced at 40°C (data not shown). This response was not further increased at prolonged growth at 40°C. We therefore chose the stress-conditions of 24 h at 40°C in the following study. To confirm the result, we subjected the three selected Wt clones and the three p.Arg107Cys clones to 24 h of heat stress at 40°C, and analyzed the mRNA levels of Hsp70, Hsp60, HO-1, and SOD2. The analyses were performed in triplicate, and repeated on 3 separate days ( $n=9$ ) (Fig. 5). The mRNA transcription levels of the two chaperones Hsp70 (Fig. 5a) and Hsp60 (Fig. 5b) were not different in the Wt- and the p.Arg107Cys-expressing cell lines ( $p=0.30$  and  $p=0.60$ , respectively). When analyzing the transcription level of the antioxidant HO-1 (Fig. 5c), expression levels of two clones were higher in the p.Arg107Cys clones compared with the Wt clones, though no significant difference between Wt and p.Arg107Cys clones was detected ( $p=0.21$ ). The expression of the mitochondrial antioxidant SOD2 (Fig. 5d), showed a statis-

tically significant increase in the p.Arg107Cys expressing clones ( $p=0.04$ ), compared with Wt cells after 24 h at 40°C.

To study the consequences of expressing the mitochondrial misfolded protein by a more global approach, we performed a proteomic study on mitochondria-enriched fractions from the transduced cells, cultivated at 37°C, using a nanoLC-MS/MS analysis on iTRAQ-labeled peptides (Palmfeldt et al 2009). This method enables relative quantitative data of differentially expressed proteins in the cell lines. The analysis was performed in triplicate using mitochondria-enriched extracts from a pool of the three Wt expressing clones, a pool of the three p.Arg107Cys expressing clones, and a pool of three cell cultures of untransduced A172 cells (Ut). Identified proteins were compared between the Wt-expressing clones and the Ut cells, between the p.Arg107Cys-expressing clones and the Ut cells, as well as between the p.Arg107Cys-expressing and the Wt-expressing clones. For proteins significantly different up- or down-regulated for the three comparisons, see Supplementary Tables 1–3. The expression ratio of SCAD as well as a selection of proteins associated with mitochondrial misfolding stress is schematically depicted in Fig. 6. The SCAD protein was found to be expressed tenfold higher in the Wt cells compared with the Ut cells, and about five times higher than the p.Arg107Cys protein (Fig. 6a). In addition, the proapoptotic protein BAX was found to be present in higher amounts in the p.Arg107Cys-expressing cells than in the two other cell types (Fig. 6b), as well as the mitochondrial outer membrane pores VDAC1 and VDAC2 (Fig. 6c, d). Among other interesting proteins, we found the mitochondrial fusion protein, mitofusin 1 (MFN1), as well as a member of the antioxidant system, peroxiredoxin 6 (PRDX6), to be increased in the p.Arg107Cys-expressing cells when compared with the Ut cells (Fig. 6e, f). In addition, we found two proteins

**Fig. 5** Increased expression of antioxidants in cells expressing the p.Arg107Cys variant protein. Cells cultivated at 40°C for 24 h, and evaluated for mRNA transcription levels on selected stress response genes **a** HSPA1A (*Hsp70*), **b** HSPD1 (*Hsp60*), **c** HMOX1 (*HO-1*), **d** SOD2 (*Sod2*). Results are presented relative to beta-actin, and normalized to Wt-1. *p* values were calculated by an ANOVA test with a stochastic level for each observation (*Hsp70* *p*=0.60, *Hsp60* *p*=0.30, *HO-1* *p*=0.21, *SOD2* *p*=0.04). *Error bars* reflect standard deviations of the mean values of triplicate cell cultivations performed three times (the day-to-day variation of triplicate measurements). *Vertical bar* reflects the mean value of the three Wt or p.Arg107Cys expressing clones

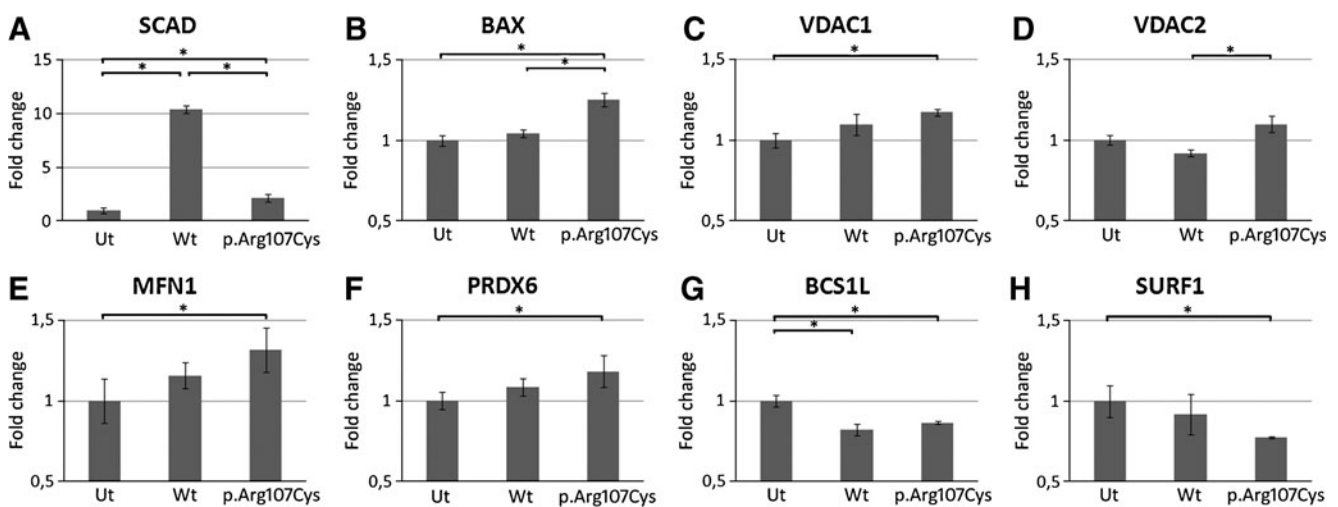


influencing the mitochondrial respiration, BCS1L and SURF1, to be reduced in the transduced cells (Wt and p.Arg107Cys) compared with the Ut cells (Fig. 6g, h).

## Discussion

In the present study, we have investigated the effect of expressing a disease-associated variant of SCAD, by stably

expressing the wild-type (Wt) and the p.Arg107Cys protein in astrocytic cells. This system has the advantage of an identical genetic background compared to patient cells (except for different *ACADS* integration sites), giving the possibility to study the primary effects of the variant SCAD protein. This may help in elucidating if *ACADS* variations are capable of disturbing normal mitochondrial functions, thus having a potential pathological role.



**Fig. 6** Selected differentially expressed proteins, identified by mass spectrometry of iTRAQ labeled peptides. Differences in protein amounts are depicted as fold-changes, relative to the value of untransduced cells (=1). *Error bars* reflect the standard deviation of

three independent experiments. \**p*<0.05, with differences exceeding  $2 \times$  global standard error ( $2 \times 0.058=0.116$ ). *AU1* untransduced cells, *Wt* wild-type



When analyzing the expression of the introduced SCAD proteins (Wt and p.Arg107Cys) in the astrocyte cell model system, a discrepancy was observed between the *ACADS* transcription (Fig. 1a) and the SCAD protein levels (Fig. 1b). Despite approximately equal *ACADS* gene transcription, the p.Arg107Cys protein level was about half the amount of the Wt protein level, indicating an enhanced turnover of the variant protein. Part of the misfolded variant protein may also exist in an insoluble aggregated form, and further studies are needed to determine whether the variant proteins are located as insoluble aggregates or possibly as other types of soluble oligomers or disordered aggregates (Stefani and Dobson 2003; McKenzie et al 2007).

By CLSM, we confirmed that the p.Arg107Cys protein has a normal mitochondrial localization (Fig. 2). Previous studies using transient transfection of astrocytes with a plasmid encoding the p.Arg107Cys-SCAD protein has shown that presence of misfolded p.Arg107Cys SCAD protein can lead to a disturbance of the dynamic mitochondrial reticulum. This disturbance was represented by a dot-like mitochondrial pool, resulting from fission of the mitochondrial network (Schmidt et al 2010). In the present study, no clear and complete mitochondrial fission was observed, yet the p.Arg107Cys expression showed a tendency to provoke mitochondrial fission (Fig. 2).

Normally, SCAD functions as a homotetramer, and to further evaluate the folding abilities of the remaining undegraded p.Arg107Cys variant protein, we investigated the tetramerization ability as well as SCAD enzyme activity in the three selected Wt and p.Arg107Cys clones (Fig. 3). These investigations demonstrated that the variant p.Arg107Cys proteins are expressed in the astrocytic cell model system, but appear to be unable to form catalytically active tetramers. This points to a severe misfolding of these proteins, a tendency which has not previously been reported in a human cell model system.

We discovered that cells carrying the SCAD p.Arg107Cys misfolding variant displayed impairment in cell proliferation, indicating a complication of normal physiology and cell cycle (Fig. 4). In order to investigate factors contributing to the decreased proliferation-rates, stress response markers were measured following exposure to 40°C for 24 h. The cytosolic inducible chaperone Hsp70 (Hsp70-1/Hsp72) is induced by various protein unfolding stressors and acts in recognizing unfolded/damaged proteins with subsequent aid in the refolding process (Hartl 1996). Hsp70 mRNA was induced by the heat stress (approximately 3-fold compared with 37°C, data not shown) but no significant difference ( $p=0.60$ ) was observed between Wt and the variant cell lines (Fig. 5a), indicating that the p.Arg107Cys protein did not induce cytosolic unfolded protein stress responses. The folding of complex and aggregation-prone mitochondrial proteins, like SCAD,

is assisted by the chaperonin system Hsp60/Hsp10 (Houry et al 1999; Corydon et al 2005). Furthermore, misfolded SCAD proteins have previously shown to interact with the Hsp60 chaperone for a longer time than Wt SCAD proteins (Pedersen et al 2003). However, no significant change in Hsp60 was observed between Wt and p.Arg107Cys expressing cells either ( $p=0.30$ ) (Fig. 5b). These results indicate sufficient basal levels of these chaperones for p.Arg107Cys protein processing and delivery to the degradation systems, though the results do not add to information about the interaction period between the misfolded SCAD protein and Hsp60.

To test whether the misfolded proteins could participate in the development of oxidative stress, we also tested the gene expression of two markers of oxidative stress, heme oxygenase-1 (HO-1) and superoxide dismutase 2 (SOD2). HO-1 is induced by oxidative stressors (Bauer and Bauer 2002), and is present in only limited amounts under normal unstressed conditions. The SOD2 antioxidant (manganese superoxide dismutase/MnSOD) catalyzes the conversion of superoxide radicals to hydrogen peroxide in the mitochondria, and is also induced by mitochondrial oxidative stress (Fridovich 1997). Despite the fact that the monomeric p.Arg107Cys protein is present in amounts which are only half as compared with the Wt protein, the SOD2 expression was significantly changed in the p.Arg107Cys-expressing clones ( $p<0.05$ ). This indicates a state of oxidative stress in cells expressing the variant SCAD proteins.

By performing a quantitative proteomic study of the cell lines, we found several interesting mitochondrial proteins to be differentially expressed. Wt SCAD protein was found to be five times more abundant than the p.Arg107Cys protein (Fig. 6), though the immunoblots in Fig. 1b showed only twice as high Wt protein. This is presumably due to the fact that the immunoblot was performed on whole cell lysates, whereas the proteomic study was performed on mitochondria-enriched extracts. If the variant SCAD proteins are more prone to aggregate the Wt SCAD, this tendency could be enhanced in the preparation of the mitochondrial extracts.

Nevertheless, the proteomic study showed a statistically significant increased amount of the proapoptotic protein BAX in the p.Arg107Cys-expressing cells, compared with both the untransduced cells and the SCAD Wt-expressing cells. Accumulated mitochondrial BAX indicates initiation of apoptosis, as BAX mediates opening of the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane (Shimizu et al 1999). Opening of VDAC releases cytochrome c and other proapoptotic factors to the cytosol, leading to activation of the caspases in the apoptotic pathway. Interestingly, we found both VDAC1 and VDAC2 up-regulated in the p.Arg107Cys-expressing cells. Whether this finding is linked to the indications of

apoptosis remains to be investigated. An indication of oxidative stress in the p.Arg107Cys-expressing cells was shown by the presence of increased amounts of the antioxidant peroxiredoxin 6 (PRDX6), compared with the untransduced cells. Peroxiredoxin 6 has been reported to be induced under oxidative stress, and is capable of reducing phospholipid hydroperoxides, and is therefore proposed to be involved in the repair of oxidatively damaged membranes (Chowdhury et al 2009). The increased amount of SOD2 mRNA expression found in the p.Arg107Cys-expressing cells after 24 h at 40°C was not found in the proteomic study performed at 37°C. We did see a subtle increase of SOD2 at mRNA level at 37°C (data not shown), but small concentration changes are not as evident when investigating proteins as with transcripts.

A member of the mitochondrial fission/fusion machinery (reviewed Youle and Karbowski 2005), MFN1, was also found to be differentially expressed. Previously, we have observed fission of the mitochondrial network when SCAD proteins are overexpressed. This fission was significantly more prevalent in cells transfected with the p.Arg107Cys variant. Surprisingly, we found the mitochondrial fusion protein, MFN1 (Chen et al 2003), to be significantly up-regulated in the p.Arg107Cys-expressing cells compared with the untransduced cells and the Wt-expressing cell line. This finding could be explained as a compensatory response to a presumed increased fission of the mitochondrial network in these cells. Interestingly, we found two assembly factors of members of the respiratory chain to be down-regulated in the transduced cells, compared with the untransduced cells; BCS11 (involved in the assembly of Complex III; Cruciat et al 1999) and SURF1 (involved in the assembly of Complex IV; Barrientos et al 2009). Whether these factors, presumably inhibiting respiration, reflects a consequence of overexpression or if it is a direct consequence of the increased SCAD expression remains to be investigated.

In summary, in the present paper, we have reported the development of an astrocytic cell model system, expressing wild-type SCAD or the disease-associated variant SCAD protein, p.Arg107Cys. We found that misfolded inactive p.Arg107Cys protein localizes to the mitochondria, inhibiting cellular proliferation, increasing the mRNA expression of the antioxidant SOD2. Additionally, we found signs of increased apoptosis and oxidative stress in the cells expressing the misfolded SCAD variant protein, as well as a response in the mitochondrial fission/fusion machinery. These observed changes are found despite the fact that the misfolded SCAD variant protein was found present in low amounts, compared with wild-type SCAD protein. We hypothesize that misfolded SCAD variant proteins elicit a toxic response, partly conveyed by a mild production of oxidative stress, leading to apoptosis. We therefore propose

that this toxic response of the misfolded protein may be pathophysiologically relevant in patients. Asymptomatic carriers of p.Arg107Cys may upregulate competent compensatory mechanisms, such as the antioxidant system, to cope with the cellular stress. On the other hand, symptomatic individuals could arise if this variant protein is present together with susceptibility variations in the genes of pathways involved in the handling of the misfolded proteins and their consequences (e.g. the protein quality control system or the antioxidant system). These are therefore suitable candidates, when investigating susceptibility factors for SCADD in the future. It is furthermore interesting to elucidate whether these findings are specific for the misfolded SCAD variant studied in the present paper, or if we are dealing with a more general mechanism for misfolded proteins in the mitochondria. The cellular system employed in this study has enabled us to isolated investigate the effects of expressing a misfolding mitochondrial variant of SCAD. To investigate the relevance of these observed changes, in concert with a genetic variable background, the next step in the process of evaluating the consequences of expressing a mitochondrial misfolded SCAD protein is to investigate patient cells.

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