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Cellular prion protein dysfunction in a prototypical inherited metabolic myopathy

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Summary

Inherited fatty acid oxidation diseases in their mild forms often present as metabolic myopathies. Carnitine Palmitoyl Transferase 2 (CPT2) deficiency, one such prototypical disorder is associated with compromised myotube differentiation. Here, we show that CPT2-deficient myotubes exhibit defects in focal adhesions and redox balance, exemplified by increased SOD2 expression. We document unprecedented alterations in the cellular prion protein PrP^C, which directly arise from the failure in CPT2 enzymatic activity. We also demonstrate that the loss of PrP^C function in normal myotubes recapitulates the defects in focal adhesion, redox balance and differentiation hallmarks monitored in CPT2-deficient cells. These results are further corroborated by studies performed in muscles from *Prnp*^{-/-} mice. Altogether, our results unveil a molecular scenario whereby PrP^C dysfunction governed by faulty CPT2 activity may drive aberrant focal adhesion turnover and hinder proper myotube differentiation. Our study adds a novel facet to the involvement of PrP^C in diverse physiopathological situations.

Key words: inherited metabolic myopathy, cellular prion protein, muscle differentiation, inherited fatty acid oxidation disorders, focal adhesions, redox balance

Introduction

Skeletal muscle dysfunction represents a frequent hallmark of inborn metabolic disorders [1]. One prototypical metabolic myopathy is the deficiency in Carnitine Palmitoyl Transferase 2 (CPT2), one of the most common inherited fatty acid oxidation (FAO) disorders. Until recently, the pathophysiological mechanisms underlying skeletal muscle defects in CPT2-deficient patients had remained enigmatic. Taking advantage of a unique collection of myoblasts from CPT2-deficient patients harboring “mild” mutations, associated with residual enzyme activity and responsible for the most frequent disease phenotype characterized by myalgia, stiffness, cramps, rhabdomyolysis and exercise intolerance [2], we highlighted for the first time the existence of an impaired differentiation process in CPT2-deficient myotubes [3]. Notably, myotubes from CPT2-deficient patients display reduced fusion index and decreased levels of myotubes differentiation markers such as the myosin heavy chain (MHC) [3]. However, the cascade of molecular events driving the alterations in myotube differentiation in the context of CPT2 deficiency remains to be fully elucidated. In the present work, we sought to assess the impact of CPT2 deficiency on focal adhesions (FA), whose remodeling is mandatory for myoblast fusion (reviewed in [4]). In particular, we focused on focal adhesion kinase (FAK), a pivotal non-receptor protein tyrosine kinase that has been well studied for its pleiotropic role in muscle development and homeostasis [5].

We further evaluated a contribution of the cellular prion protein PrP^C, which, beyond being infamous for its involvement under its scrapie isoform PrP^{Sc} in a group of neurodegenerative diseases [6], is known to play a role in cellular redox balance [7] and was also reported to take part to focal adhesion turnover [8,9]. Interestingly, several studies have shown that different models of mice overexpressing PrP^C [10-12] develop a progressive myopathy sometimes associated with mitochondrial hyperplasia [12]. On another hand, although PrP null mice do not show any obvious muscle abnormality, the absence of PrP^C delays the regeneration of both glycolytic and oxidative fibers after acute muscle damage [13] and affects the profiles of muscle fibers [14,15] as well as the expression of mitochondrial markers in skeletal muscles of aged individuals [16]. Finally, mice lacking PrP show reduced tolerance for physical exercise [16,17]. Altogether, studies showing that the over-expression of PrP^C, or its absence, all have severe consequences on muscle, additionally warrant examining PrP^C in CPT2-deficient myotubes.

Here we show that CPT2 deficiency triggers ROS imbalance and PrP^C loss-of-function, which contribute to impair myotube formation by altering focal adhesions dynamics.

Methods

Cell culture and treatments

Controls (n=3) and CPT2-deficient (n=4) human myoblasts used in this study were obtained from individuals that gave informed consent for research use and have been previously described in details [18,19,3]. Myoblasts were cultured in Ham's F10 with Glutamax (GIBCO) containing 20% FBS, 0.5% Ultrosor G, 0.2% Primocin and 1mM Carnitine. At 80-90% confluence myoblasts were induced to differentiate for 6 days in DMEM with Glutamax (GIBCO) supplemented with 2% horse serum, 0.2% Primocin and 1mM Carnitine. Cells were cultured at 37°C at 5% CO₂. In some experiments, control myoblasts were switched to differentiation medium containing 2mM L-Amino-Carnitine (gift from Sigma-Tau, Italy) for 6 days. For transient siRNA-mediated silencing, myoblasts were transfected with siRNA sequences (30 nM) using the Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen) and directly induced to differentiate. Specific siRNA sequences used were: 5'-CAGUACAGCAACCAGAACATT-3' (sense siPRNP) 5'-AACGAUGACACGAACACACTT-3' (sense non-target, NT),

Animal studies

All animal experiments were carried out in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 2016/63/UE). All efforts were made to minimize suffering. Experiments were approved by the INRAE local animal experiment ethics committee of Jouy-en-Josas (Comethea, Permit Number 02532.01). FVB/NJ *Prnp*^{-/-} mice were established through the CRISPR/Cas9 technology and will be described in detail in an upcoming publication. Hind limb muscles (Gastrocnemius) from four-month-old male FVB/NJ *Prnp*^{-/-} mice and their wild-type isogenic FVB/NJ controls were dissected and immediately frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

Western blotting

Myotubes were lysed in a buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% Nonidet P40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM de phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail, (Complete mini, Roche), 1x Phosphatase inhibitor cocktail

(PhosSTOP, Roche), 10mM Nicotinamide. Protein concentration was determined by the Lowry method. Cytoplasmic and nuclear extracts were obtained using a kit from Thermo Scientific according to the manufacturer's instructions and the same total protein amounts of membrane/cytosolic and nuclear fraction were loaded in the gel. For PrP^C protein, PNGaseF treatment was performed according the manufacturer's protocol (New England Biolabs). Protein samples (15-30µg) were run in Bolt™ 10% Bis-Tris Plus gels (Invitrogen) or 10% or 12% SDS-PAGE and transferred to PVDF membranes (Biorad). Membranes were blocked with 5% milk or 5% BSA in 1x TBS-T for 1 h before incubation with primary antibodies overnight at 4°C. Immunoreactive bands were analyzed with a computerized video densitometer or with a LAS-4000 luminescent image analyzer. The results were expressed as arbitrary units normalized to the amount of an appropriate reference protein: Tubulin for total cell extracts, Hsp90 for the membrane/ cytosolic extracts and Lamin A/C for the nuclear extracts. Most of the time, blots were stripped using Antibody Stripping Buffer (Gene Bio-Application) according to the manufacturer's instructions and probed again. The following antibodies were used: rabbit polyclonal anti-CPT2 (Millipore), mouse monoclonal anti-tubulin (Sigma-Aldrich), mouse monoclonal anti-myosin, Skeletal, Slow (Sigma-Aldrich), rabbit polyclonal anti-FAK (Cell Signaling), rabbit monoclonal anti-paxillin (Abcam), mouse monoclonal anti-SOD2 (Abcam), mouse monoclonal anti-PrP^C (Sha31, SPI-Bio), the Sha31 monoclonal antibody targets the epitope corresponding to amino-acids 145-152 (human numbering), rabbit polyclonal anti-MYF5 (Abcam), rabbit polyclonal anti-Hsp90 (Abcam), mouse monoclonal anti-Lamin A/C (Cell Signaling).

RNA analysis

RNA was isolated by using the RNeasy extraction kit (Qiagen, Limburg, Netherlands), as recommended by the manufacturer's instructions. cDNA was generated from 1µg of total RNA using the High-capacity cDNA Reverse Transcription (Applied Biosystems) and quantified in triplicates on the 7900HT Fast Real-Time PCR system (Applied Biosystems) using Absolute qPCR SYBR Green ROX Mix (Thermo Scientific). The primer sequences are shown below:

PRNP-F: CGAGCTTCTCCTCTCCTCAC; PRNP-R: GTCCATCCTCCAGGCTTC; MYH7-F:
GACATGCTGCTGATCACCAACA; MYH7-R: CGCCTGTCAGCTTATACATGGA; SOD2-F:
GAGTTGCTGGAAGCCATCAAAC; SOD2-R: TGGAATAAGGCCTGTTGTTCT; MYF5-F:
TGCCAGTTCTCACCTTCTGAGT; MYF5-R: TCGCACGTGCTGGTCCTCAT; RPL13A-F:

CCTGGAGGAGAAGAGGAAAGAGA; RPL13A-R: GAGGACCTCTGTGTATTTGTCAA. The results are expressed as the relative quantification of a target gene transcript normalized to RPL13A housekeeping gene, using the $\Delta\Delta C_t$ method.

Immunofluorescence

Myotubes were fixed 20 min in 4% paraformaldehyde and permeabilized for 3 min by 0.2% Triton/PBS. Cells were washed and pre-incubated in PBS/1% BSA/0.3M Glycine for 30 min. The cells were then incubated 1h at room temperature, with the primary antibody diluted in PBS containing 1% BSA. Antibodies used for immunofluorescence experiments are identical to those used for western blotting except for FAK (Abcam). Myotubes were washed three times in PBS/ 0.1% Tween and then incubated 1h at room temperature, with secondary antibody anti-rabbit Alexa Fluor 546 and/or anti-mouse Alexa Fluor 488 (Life Technologies). F-actin fibers were stained with TRITC-Phalloidin (250 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich) diluted in PBS containing 1% BSA for 1h at room temperature. Nuclei were stained with blue-fluorescent TO-PRO-3 (Invitrogen). Coverslips were washed and mounted in Dako mounting medium (Dako corporation). Images were acquired on a Carl-Zeiss LSM 510 META confocal microscope and analyzed with Zeiss LSM Image Browser software.

Statistical analysis

The results are presented as the mean \pm SEM. All statistical analyses were performed using GraphPad Prism software (version 6.0). Data distribution was first checked for normality using the Shapiro-Wilk test, and parametric or non-parametric tests were then applied accordingly. Differences between groups were analyzed by Mann-Whitney test, or paired or unpaired two-tailed Student's *t* test for the comparison of two groups, or by Two-way ANOVA and the Tuckey test for comparison of four groups. The statistical tests used are indicated in the figures' legends. $P < 0.05$ was considered significant.

Results and Discussion

CPT2-deficient myotubes exhibit a remodeling of focal adhesions.

We hypothesized that CPT2 deficiency in myotubes, which is associated with reduced levels of CPT2 and MHC-I proteins (Fig. S1) as previously demonstrated [3], is accompanied by alterations in focal adhesions (FAs). To address this question, we evaluated the distribution of the FA scaffold protein Paxillin (PAX) in myotubes from CPT2-deficient patients and healthy subjects by immunofluorescence. While control cells exhibited a diffuse cytoplasmic staining of PAX associated with a high MHC-I fluorescence intensity, CPT2-deficient myotubes showed intense cytoplasmic PAX-positive dots and a reduced MHC-I staining (Fig. 1A), with no change in PAX protein levels (Fig. 1B), suggesting that the defect in CPT2 is associated with an intracellular redistribution of PAX and increased FA number. In further support of this hypothesis, we found a striking difference in the amount and distribution of the Focal Adhesion Kinase (FAK), a key FA adaptor protein, between patients- and control-derived myotubes. Indeed, control cells showed a strong cytoplasmic signal comparable to that obtained with PAX, while in contrast, we monitored a very weak signal only in CPT2-deficient myotubes (Fig. 1C). The reduction in FAK protein levels in CPT2-deficient cells could be firmly substantiated in western blot experiments (Fig. 1D) and likely accounts for the stabilization of FAs as seen in Figure 1A [20]. This decrease most likely participates to the mechanisms leading to the impaired differentiation process revealed in our previous study [3]. Indeed, FAK inhibition in mouse primary myoblasts was shown to result in a marked impairment of myoblast fusion, a critical step of myogenesis [21]. FAK activation requires ROS-dependent inactivation of phosphatases [22]. Importantly, it has been shown that de-phosphorylation of FAK by phosphatases may be associated with FAK degradation [20]. Thus, the decreased level of FAK observed in CPT2-deficient myotubes might be due to an increased FAK degradation, itself due to more active phosphatases resulting from a reduction in ROS levels. This hypothesis fully fits in with our previous observation that ROS levels are significantly decreased in CPT2-deficient myotubes compared to controls [3]. However, finding that ROS levels were lower in CPT2 cells was first very surprising to us since it is widely admitted that FAO defects, like many others inherited mitochondrial disorders, are rather associated with oxidative stress [23]. This is why we went on assessing the expression of the central antioxidant enzyme SOD2 in CPT2-deficient myotubes. Of note, we found that CPT2-deficient myotubes expressed higher levels of both

SOD2 mRNA and protein than healthy control cells (Fig. 1E). Thus, as a possible scenario, we propose that the inherited CPT2 deficiency is permanently generating ROS and that a compensatory mechanism to adapt to this high level is to increase the expression of SOD2, which ultimately results in a low level of ROS as measured in the CPT2-deficient cells [3]. Altogether, these results reveal an abnormal distribution of PAX associated with a lower level of FAK likely due to a complex loop in the regulation of ROS levels in CPT2-deficient myotubes. It can be surmised that these changes alter adequate dynamics at FAs in the protrusions of migrating myoblasts and thereby hamper proper migration and fusion.

CPT2-deficient myotubes reveal an alteration in the cleavage of PrP^C

Because the cellular prion protein PrP^C is increased during the differentiation of muscle cells [14] and its deficiency is associated both with impaired FA turnover [8,9] and muscle function [13], we anticipated that alterations in this protein might take part to the defects observed in CPT2-deficient patients. The deficiency in CPT2 had no impact on *PRNP* transcripts encoding PrP^C in myotubes (Fig. 2A). Next, protein extracts were submitted to deglycosylation with PNGaseF, in order to better estimate the amount of PrP^C as well as its cleavage products. The two main processing events described for PrP^C are the so-called alpha- and beta-cleavages, yielding N1/C1 and N2/C2 fragments, respectively (reviewed in [24]) (Fig.S2). Whereas the N1/C1 cleavage of PrP^C mainly depends on proteases, the N2/C2 cleavage is, for its part, primarily triggered by ROS [25,26]. As shown in Fig. 2B, patients exhibited reduced levels of the full-length (FL) PrP^C isoform together with increased levels of the C2 isoform, while the shorter C1 isoform slightly increased without reaching significance. We were initially puzzled by the fact that the FL- PrP^C was not the major PrP^C fraction in control human myotubes, and that C1- and C2-isoforms exhibited higher levels compared to the FL. However, similar profiles of PrP^C isoforms have been recently reported in polarized retinal epithelial cells [27] and in mouse pancreas [28].

To further characterize the expression pattern of PrP^C in CPT2-deficient myotubes, we examined its intracellular distribution in permeabilized cells through immunofluorescence experiments. PrP^C mostly co-localized with PAX in control cells while, strikingly, we observed a strong nuclear PrP^C staining in CPT2-deficient myotubes, which was not present in healthy cells (Fig. 2C). This observation was fully corroborated with western blot analyses of nuclear vs. cytoplasmic/membrane extracts from CPT2-

deficient and control myotubes (Fig. 2D). Indeed, while all isoforms were mostly non-nuclear in control cells, we detected significant levels of C1 and majorly C2 isoforms in the nuclear fraction of CPT2-deficient myotubes. These experiments further confirmed the robust increase in C2-PrP^C in the myotubes of CPT2-deficient patients, as seen in whole cell extracts (Fig. 2B). Thus, these data exemplify strong changes in PrP^C processing and localization in CPT2-deficient myotubes. The nuclear localization of a fraction of PrP^C in CPT2-deficient cells is a puzzling observation and will deserve further investigation. Very little is known on nuclear PrP^C, although it has been described in a few studies. For instance, Bravard et al. reported on the nuclear targeting of PrP^C in response to genotoxic stress, where it interacts with the DNA repair enzyme Apurinic/aprimidinic endonuclease 1 and serves a protective function [29]. In line with this, PrP^C was shown to protect against DNA damage induced by oxidative stress [30]. Indeed, oxidative stress is a well-established inducer of DNA damage, see review [31]. Thus, we may surmise that the nuclear targeting of a fraction of PrP^C in CPT2-deficient cells may be a consequence of oxidative stress, similarly to PrP^C beta-cleavage.

PrP^C alterations are driven by CPT2 enzymatic deficiency

Next, we sought to assess whether the alterations in PrP^C described above directly arise from an impairment of CPT2 enzymatic activity rather than indirectly from a confounding effect of the CPT2 mutation. To this purpose, control myoblasts were induced to differentiate in the presence of 2mM L-Amino-Carnitine (L-Amino-Car), a specific inhibitor of CPT2 [32], for 6 days. We previously demonstrated that this concentration of L-Amino-Car strongly inhibits fatty acid oxidation in human myoblasts [33]. As shown in Fig. 3A, L-Amino-Car-treated myotubes exhibited significantly reduced levels of MHC-I and FAK together with increased levels of SOD2, thus recapitulating the alterations found in genetically CPT2-deficient myotubes. Hence, L-Amino-Car appears to interfere with myotube differentiation, a conclusion that is further supported by the increase in MYF5, a marker of myoblasts lost once committed to differentiation [34], after L-amino-Car treatment (Fig. 3A). Of note, L-amino-Car promoted a decrease in FL-PrP^C accompanied by increases in the C1 and C2 cleaved isoforms (Fig. 3B). We may thus conclude that exposure of healthy myotubes to L-amino-Car reproduces the molecular features observed under genetic CPT2 deficiency, including at the level of PrP^C. As a corollary, these results indicate that impaired CPT2 enzymatic function fosters PrP^C alterations.

PrP^C loss of function recapitulates specific features of CPT2-deficiency

These observations raise the question as to how PrP^C alterations may contribute to altered myotube differentiation in the context of CPT2 deficiency. The reduced levels of FL-PrP^C in patients suggest a loss-of-function phenotype. To address this possibility, we probed the impact of siRNA-mediated PrP^C silencing in control myotubes. Remarkably, PrP^C depletion (Fig. 4A) triggered significant decreases in both MHC-I and FAK protein levels, while it promoted strong upregulations in the protein levels of SOD2 and MYF5 thereby mimicking the changes observed in CPT2-deficient myotubes (Fig. 4B). These defects appear to arise from changes in gene expression since PrP^C-silenced cells expressed lower levels of MYH7 mRNA encoding MHC-I and higher amounts of SOD2 and MYF5 transcripts (Fig. 4C). Finally, we found that the impact of PrP^C knockdown compared that of CPT2 genetic deficiency when examining the cellular distribution of Paxillin and MHC-I in immunofluorescence staining (Fig. 4D). Importantly, PrP^C silencing in control myotubes reproduced the impaired commitment to terminal differentiation of CPT2-deficient myotubes previously described in [3], as indicated by the decreased myogenic fusion index (%of nuclei present in MHC-stained myotubes) (Fig. 4E). Corroborating these results, we monitored reduced MHC-I and FAK proteins together with increased levels of SOD2 in muscles from *Prnp*^{-/-} mice (Fig. 4F and 4G). Altogether, these data strongly support the view that the alterations of PrP^C promoted by the deficit in CPT2 activity induce a loss-of-function phenotype, itself contributing to defective myotube differentiation and FA turnover.

In summary, we have established that the impairment of differentiation is associated with defects in FAs turnover in CPT2 patients' cells and that both genuinely arise from the deficit in CPT2 enzymatic activity. A second important conclusion of this work is that changes in PrP^C appear to exert a central role in the CPT2-dependent phenotype. Altogether, our findings allow proposing a molecular scenario (Fig. 4H) whereby reduced CPT2 activity in patients-derived cells would, in the first place, trigger a situation of oxidative stress, boosting the production of C2-PrP^C via beta-cleavage [26]. C2-PrP^C would, in turn, help cells cope with this oxidative stress situation as depicted for this isoform [30,26] and, in the end, would generate a chronic state of defence against ROS, notably based on elevated SOD2 levels. Therefore, the beta-cleavage of PrP^C can be considered as a surrogate marker of excessive ROS production. Importantly, this scenario might also reconcile the literature data generally indicating variable ROS levels in various inherited muscle diseases [35,23], which could originate from

different capacities of the muscle to cope for excessive ROS production as a compensatory mechanism [36,37].

Furthermore, the loss-of-function phenotype ensuing from PrP^C beta-cleavage would hinder FA turnover and thus disrupt myoblasts fusion and thereby myotubes formation. Noteworthy, this situation is highly reminiscent of the neuronal differentiation defects observed upon PrP^C silencing. Indeed, knock-down of PrP^C in neuronal cells leads to reduced FA turnover, and increased stability of actin microfilament, which inhibits neuritogenesis [8]. Likewise, we observed altered/thicker F-actin fibers in CPT2-deficient myotubes (Fig. S3). Of particular interest, increased mitochondrial SOD2 activity was also reported in brain extracts from PrP^C knockout mice [38]. Furthermore, it was recently shown that exposure of neural stem cells (NSC) to synthetically produced N1 or N2 fragments resulted in reduced migration and neurite outgrowth [39]. These cellular changes were accompanied by a reduction in intracellular ROS production and an increased SOD2 level. The authors also reported enhanced mitochondrial fission in NSC treated with N1/N2, which is reminiscent of the higher mitochondrial fission that we previously reported in CPT2-deficient myotubes [3].

Altogether, the data presented here identify for the first time PrP^C as a novel and crucial protein participating to the physiopathology of a common metabolic myopathy namely the CPT2 deficiency, likely via its role in cellular redox balance and FA dynamics.

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Author contributions

FZB, CTR and VP performed experiments. JC and JLV generated *Prnp*^{-/-} mice. JB analyzed data. FD prepared figures. SMR and FD conceived and designed the experiments; analyzed and interpreted the data and wrote the article.

Conflict of interest

The authors declare no competing interests.

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Figure legends

Figure 1: CPT2-deficient myotubes exhibit FA alterations and increased SOD2 expression.

- A. Representative images of myotubes from healthy subject (top panel) or CPT2-deficient patient (bottom panel) stained with MHC-I and PAX antibodies. Nuclei were stained with TO-PRO-3. Scale bar: 20 μ M
- B. Representative western blot and quantification of PAX protein from control or CPT2-deficient myotubes.
- C. Representative images of myotubes from healthy subject (top panel) or CPT2-deficient patient (bottom panel) stained with MHC-I and FAK antibodies. Nuclei were stained with TO-PRO-3. Scale bar: 20 μ M
- D. Representative western blot and quantification of FAK protein from control or CPT2-deficient myotubes.
- E. *SOD2* mRNA expression in control and CPT2-deficient myotubes (left panel) and representative western blot and quantification of SOD2 protein from control or CPT2-deficient myotubes (right panel).

(A) and (C) Images are representative of three independent experiments carried out on 2 controls and 2 patients. In (B), (D) and (E) results are means of at least three independent experiments, each carried out on 3 controls and 4 patients. For (B), statistical significance was assessed by Mann-Whitney test, for (D) and (E), statistical significance was assessed by unpaired two-tailed Student's *t* test. The P values are indicated in the figure. Data are represented as mean \pm SEM.

Figure 2. CPT2-deficient myotubes display defects in PrP^C.

- A. *PRNP* mRNA expression in control and CPT2-deficient myotubes.
- B. Representative western blot and quantification of the various PrP^C protein isoforms from control or CPT2-deficient myotubes.
- C. Representative images of myotubes from healthy subject (top panel) or CPT2-deficient patient (bottom panel) stained with PrP^C and PAX antibodies. Nuclei were stained with TO-PRO-3. Scale bar: 20 μ M

- D. Representative western blot and quantification of the various PrP^C protein isoforms in the cytoplasmic (C) or nuclear (N) fraction of control or CPT2-deficient myotubes.

In (A), (B) and (D) results are means of at least three independent experiments, each carried out on 3 controls and 4 patients. (C) Images are representative of two independent experiments carried out on 2 controls and 2 patients. For (A) and (B) statistical significance was assessed by unpaired two-tailed Student's *t* test; for (D) statistical significance was assessed by Two-way ANOVA and the Tuckey test. The P values are indicated in the figure. Data are represented as mean ± SEM.

Figure 3. Inhibition of CPT2 enzymatic activity phenocopies CPT2 genetic deficit.

- A. Representative western blot and quantification of MHC-I, SOD2, MYF5 and FAK proteins from vehicle or L-amino-Car-treated (2mM for 6 days) myotubes from healthy subject.
- B. Representative western blot and quantification of the various PrP^C protein isoforms from vehicle or L-amino-Car-treated myotubes from healthy subject

Results are means of at least three independent experiments carried out on 3 controls. Statistical significance was assessed by paired two-tailed Student's *t* test. The P values are indicated in the figure. Data are represented as mean ± SEM.

Figure 4. PrP^C silencing recapitulates the phenotypes of CPT2-deficiency

- A. Representative western blot of PrP^C protein in myotubes from healthy subject treated with siRNA Non target (NT) or against *PRNP* (siPrPc).
- B. Representative western blot and quantification of MHC-I, SOD2, MYF5 and FAK proteins in myotubes from healthy subject treated with siRNA Non target (NT) or against *PRNP* (siPrPc).
- C. *PRNP*, *MYH7*, *SOD2* and *MYF5* mRNA expression in myotubes from healthy subject treated with siRNA Non target (NT) or against *PRNP* (siPrPc).
- D. Representative images of myotubes from healthy subject treated with siRNA Non target (NT) (top panel) or against *PRNP* (siPrPc) (bottom panel) stained with PAX and MHC-I antibodies. nuclei were stained with TO-PRO-3. Scale bar: 20µM
- E. Calculation of the fusion index in myotubes from healthy subject treated with siRNA Non target (NT) or against *PRNP* (siPrPc).
- F. Representative western blot of PrP^C protein in muscles from wild-type (WT) and *Prnp*^{-/-} mice.

G. Representative western blot and quantification of MHC-I, SOD2, and FAK proteins in muscles from WT controls and *Prnp*^{-/-} mice.

H. Proposed schematic model explaining the relationship between CPT2 deficiency, ROS imbalance, PrP^C cleavage, altered FA dynamics and impaired myotubes differentiation.

(A-C) Results are means of at least three independent experiments carried out on 3 controls. (D) Images are representative of two independent experiments on 2 controls. (E) Four fields of each condition were counted at x20 magnification. (F-G) Results are means of at least three independent experiments performed on 4 WT and 4 *Prnp*^{-/-} mice. Data are represented as mean ± SEM. For (B), (C) and (E), statistical significance was assessed by paired two-tailed Student's *t* test. For (G), statistical significance was assessed by unpaired two-tailed Student's *t* test. The P values are indicated in the figure. Data are represented as mean ± SEM.

Appendix Fig S1: CPT2-deficient myotubes exhibit reduced CPT2 and MHC-I protein levels.

A. Representative western blot and quantification of CPT2 protein from control or CPT2-deficient myotubes.

B. Representative western blot and quantification of MHC-I protein from control or CPT2-deficient myotubes

Results are means of at least three independent experiments, each carried out on 3 controls and 4 patients. Statistical significance was assessed by unpaired two-tailed Student's *t* test. The P values are indicated in the figure. Data are represented as mean ± SEM.

Appendix Fig S2: Schematic representation of PrP^C primary sequence and processing

PrP^C is majorly found at the plasma membrane to which it is tethered through its glycosylphosphatidyl inositol (GPI) anchor. The alpha-cleavage yielding N1 and C1 fragments, occurs at position 111/112. The beta-cleavage yielding N2 and C2 fragments, occurs in the vicinity of octapeptides. H1, H2, H3 indicate alpha helices and β1 and β2 indicate beta sheets.

Appendix Fig S3: CPT2-deficient myotubes exhibit thicker actin fibers

Representative images of myotubes from healthy subject (top panel) or CPT2-deficient patient (bottom panel) stained TRITC phalloidin to visualize F-actin (ACT). Nuclei were stained with TO-PRO-3. Scale

bar: 20 μ M. Images are representative of three independent experiments carried out on 2 controls and 2 patients.

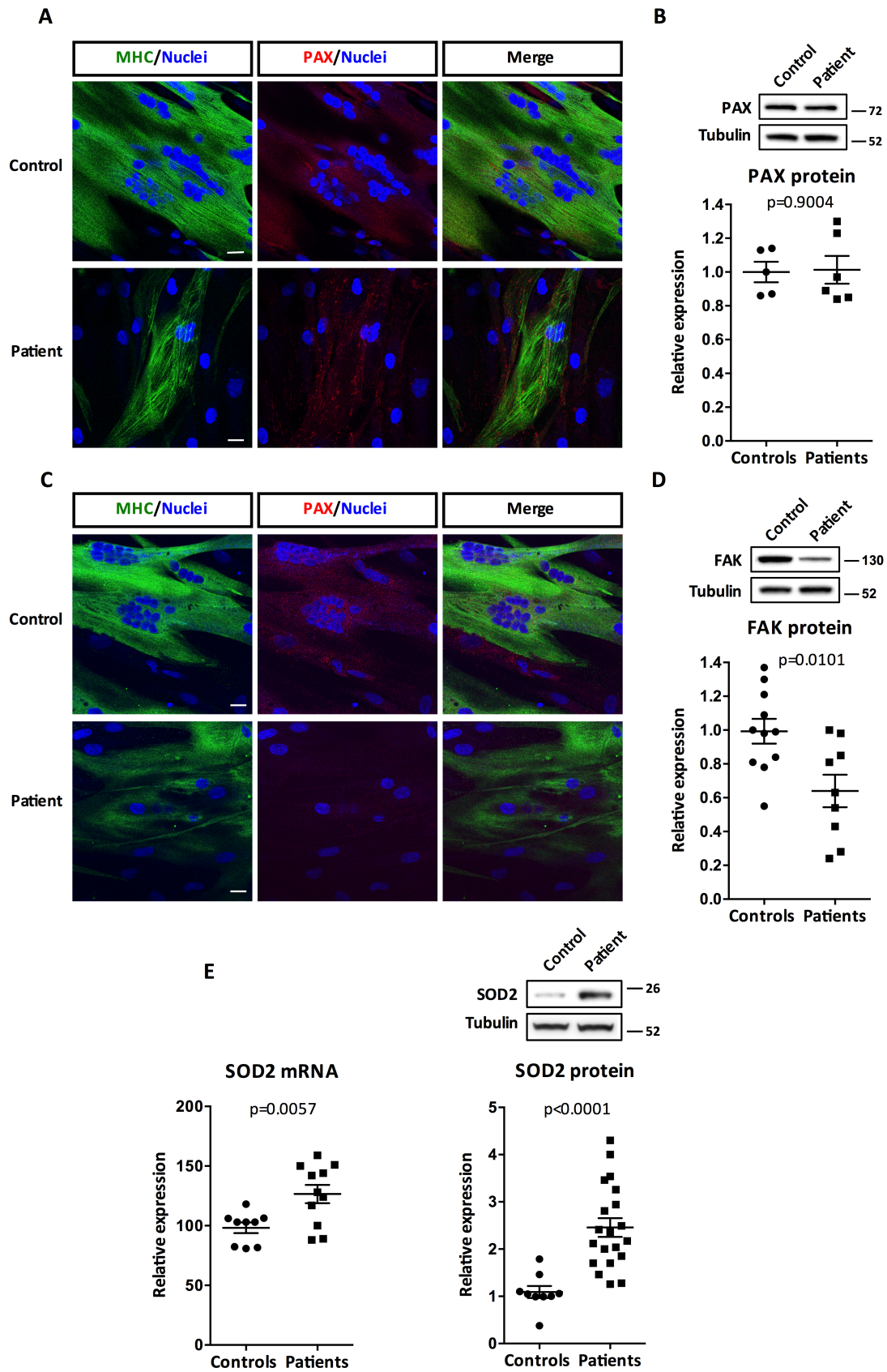


Figure 1

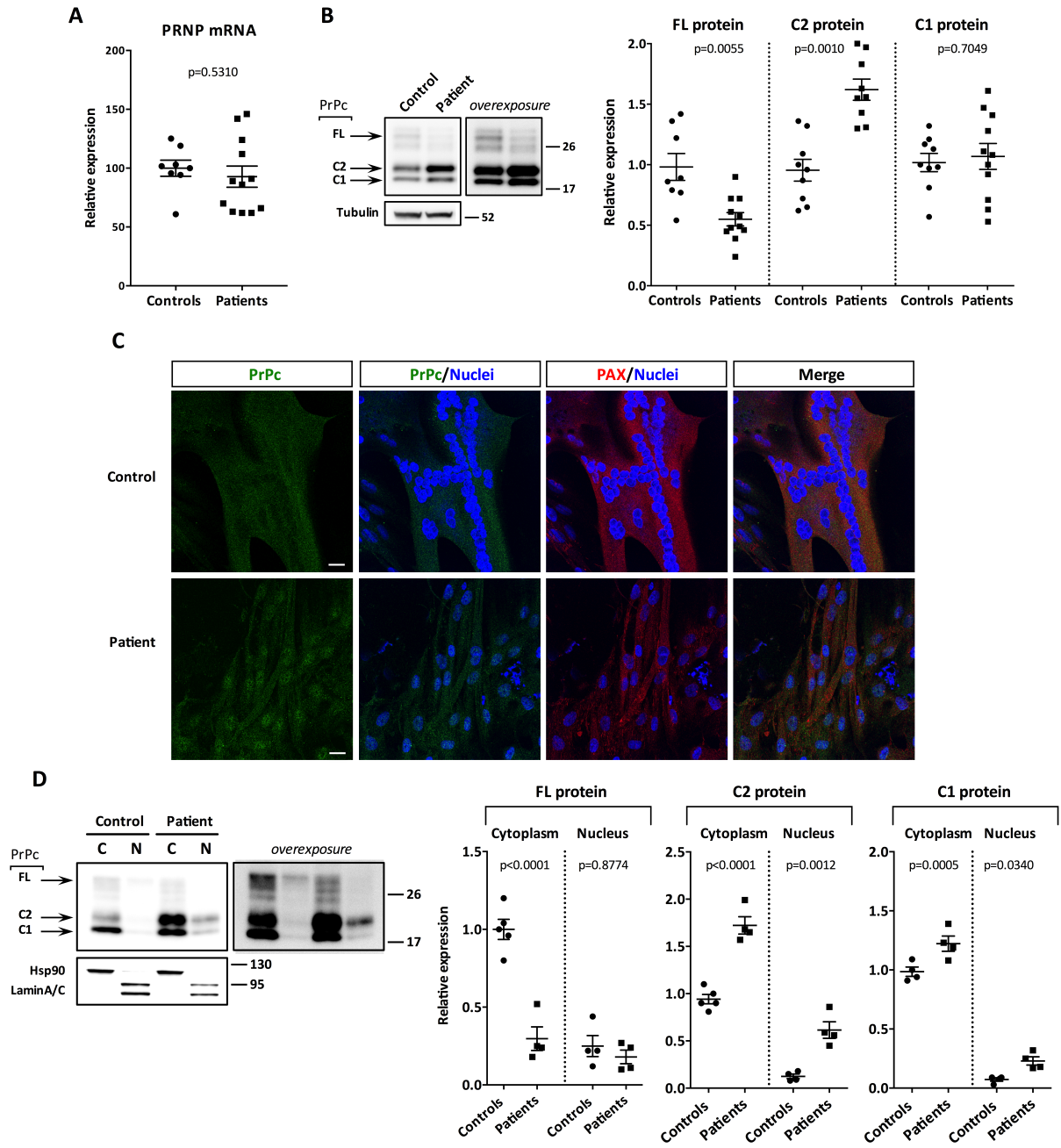


Figure 2

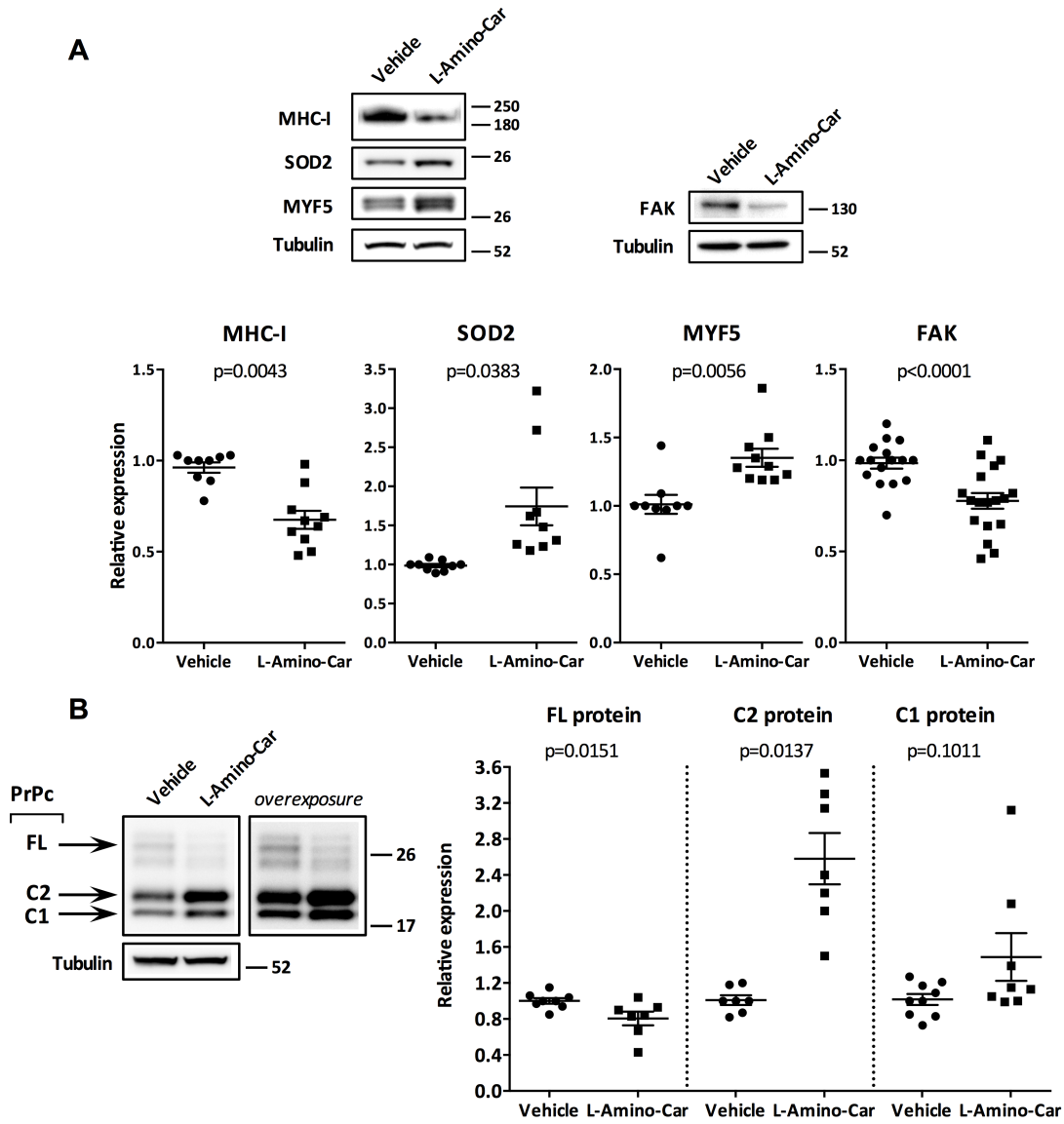


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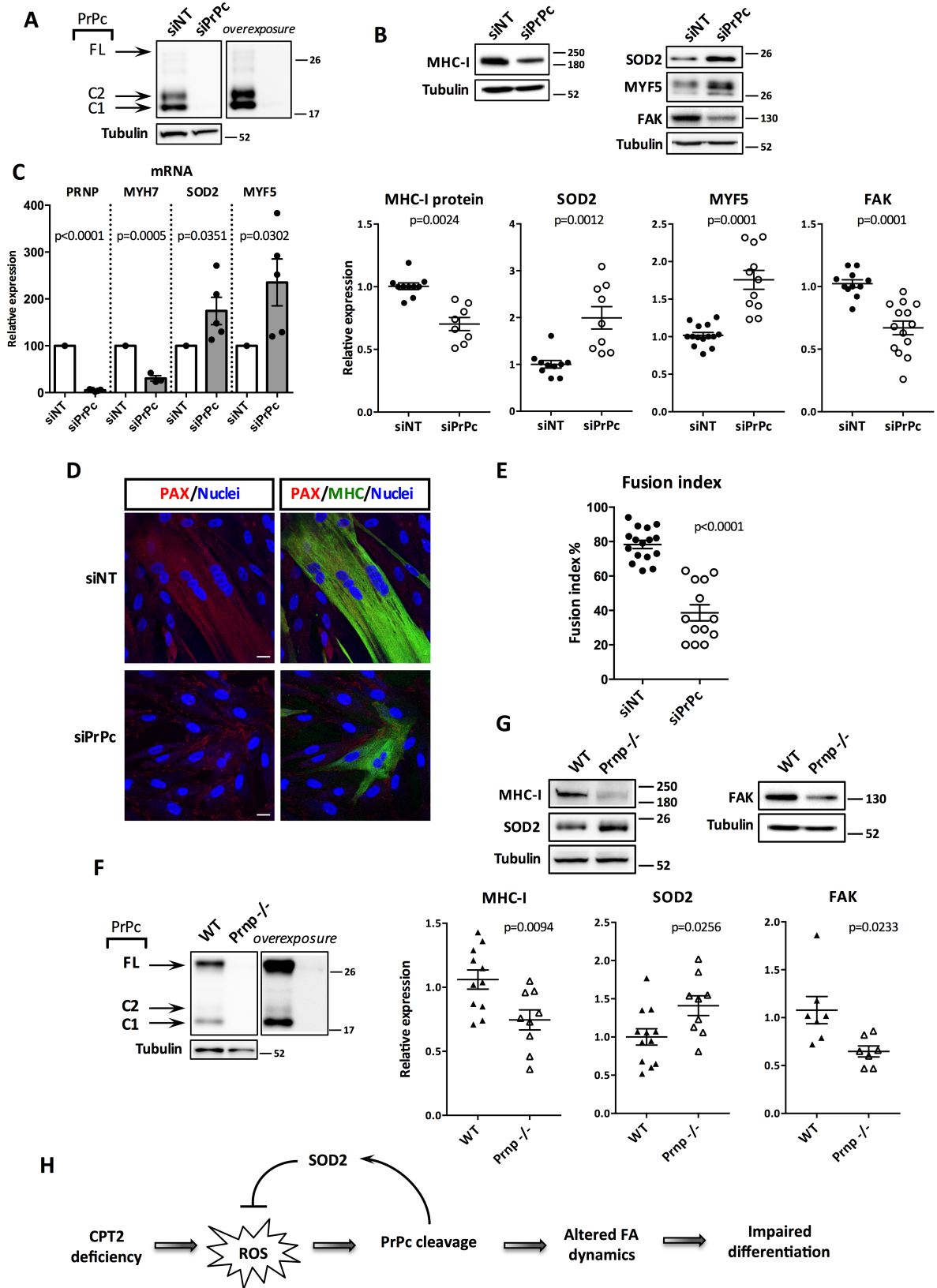


Figure 4

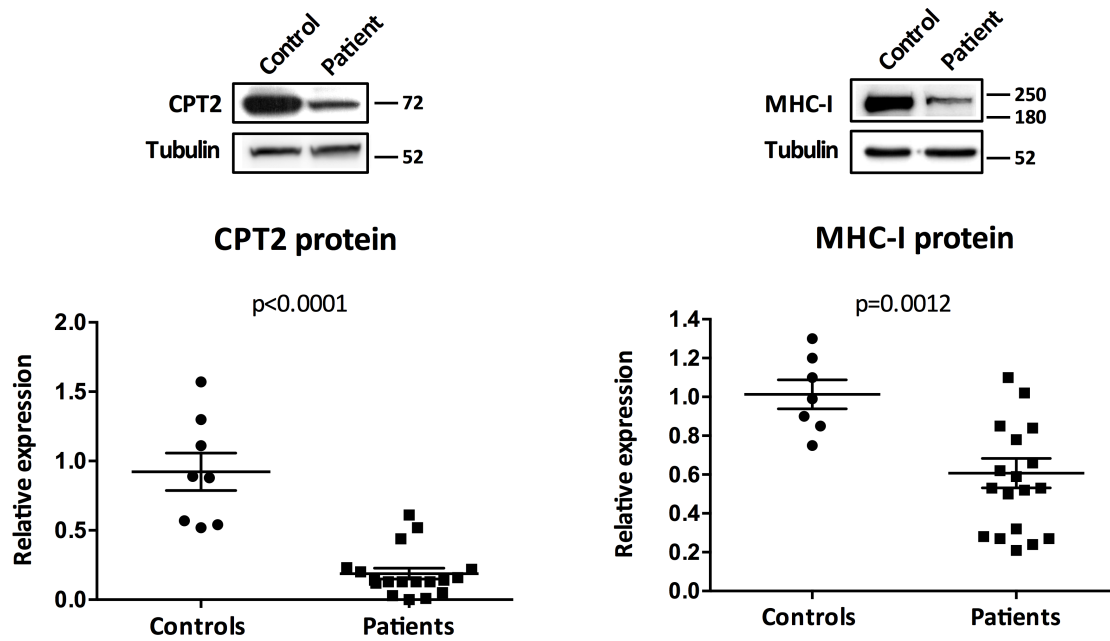


Figure S1

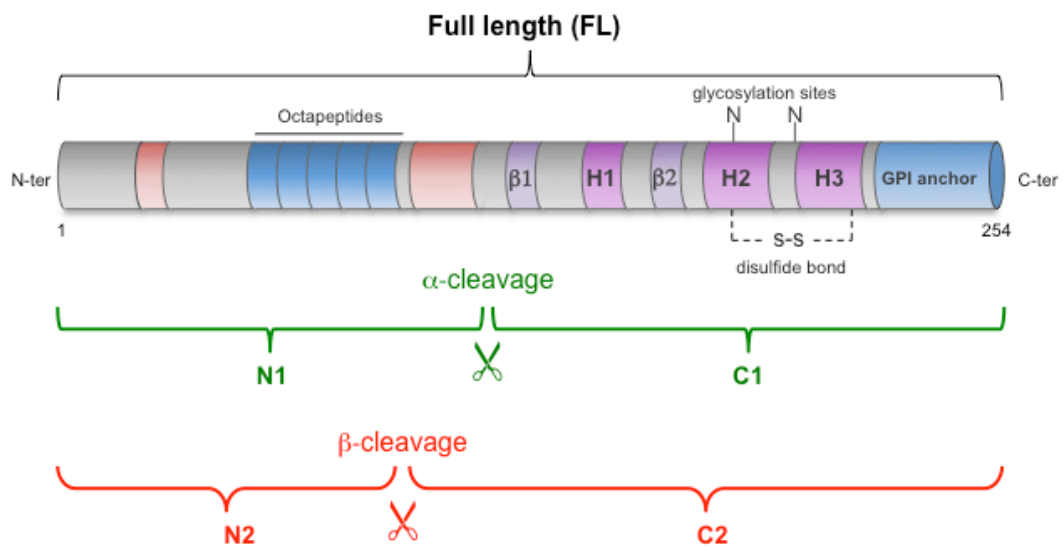


Figure S2

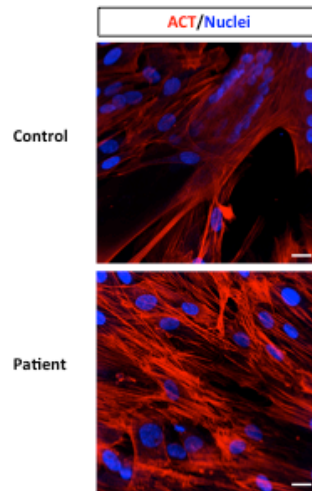


Figure S3