Loss of MICOS complex integrity and mitochondrial damage, but not TDP-43 mitochondrial localisation, are likely associated with severity of CHCHD10-related diseases

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ABSTRACT

Following the involvement of CHCHD10 in FrontoTemporal-Dementia-Amyotrophic Lateral Sclerosis (FTD-ALS) clinical spectrum, a founder mutation (p.Gly66Val) in the same gene was identified in Finnish families with late-onset spinal motor neuropathy (SMAJ). SMAJ is a slowly progressive form of spinal muscular atrophy with a life expectancy within normal range. In order to understand why the p.Ser59Leu mutation, responsible for severe FTD-ALS, and the p.Gly66Val mutation could lead to different levels of severity, we compared their effects in patient cells. Unlike affected individuals bearing the p.Ser59Leu mutation, patients presenting with SMAJ phenotype have neither mitochondrial myopathy nor mtDNA instability. The expression of CHCHD10S59L mutant allele leads to disassembly of mitochondrial contact site and cristae organizing system (MICOS) with mitochondrial dysfunction and loss of cristae in patient fibroblasts. We also show that G66V fibroblasts do not display the loss of MICOS complex integrity and mitochondrial damage found in S59L cells. However, S59L and G66V fibroblasts show comparable accumulation of phosphorylated mitochondrial TDP-43 suggesting that the severity of phenotype and mitochondrial damage do not depend on mitochondrial TDP-43 localization. The expression of the CHCHD10G66V allele is responsible for mitochondrial network fragmentation and decreased sensitivity towards apoptotic stimuli, but with a less severe effect than that found in cells expressing the CHCHD10S59L allele.

Taken together, our data show that cellular phenotypes associated with p.Ser59Leu and p.Gly66Val mutations in CHCHD10 are different; loss of MICOS complex integrity and mitochondrial dysfunction, but not TDP-43 mitochondrial localization, being likely essential to develop a severe motor neuron disease.

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; BN-PAGE, blue native electrophoresis; CHCHD10, Coiled-coil-Helix-Coiled-coil-Helix Domain containing 10; CMT2, Charcot-Marie-Tooth disease type 2; COX, cytochrome c oxidase; FBS, fetal bovine serum; FTD, FrontoTemporal Dementia; FTD-ALS, FrontoTemporal Dementia-Amyotrophic Lateral Sclerosis; MICOS, mitochondrial contact site and cristae organizing system; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; PSF, point spread function; PTD-P-43, phosphorylated TDP-43; RRF, ragged-red fibers; SMA, Spinal Muscular Atrophy; SMAJ, late-onset Spinal Motor Neuropathy; STS, staurosporine; TDP-43, transactivation response element DNA-binding protein 43

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1. Introduction

Recently, we provided genetic evidence that mitochondrial dysfunction can have a causative effect in motor neuron degeneration (Bannwarth et al., 2014). We reported a large family with a late-onset phenotype including myopathy, motor neuron disease, cognitive decline looking like frontotemporal dementia (FTD) and cerebellar ataxia. In all patients, muscle biopsy showed ragged-red fibers (RRF) and cytochrome c oxidase (COX) negative fibers, a hallmark of mitochondrial myopathy, with accumulation of mitochondrial DNA (mtDNA) deletions. Patient fibroblasts presented with respiratory chain deficiency, mitochondrial ultrastructural alterations and fragmentation of the mitochondrial network. We identified a missense mutation (c.176C > T; p.Ser59Leu) in CHCHD10 that encodes a mitochondrial protein located in the intermembrane space and enriched at cristae junctions (Bannwarth et al., 2014). Mitofilin/MIC60, another protein enriched at mitochondrial cristae junctions, is a central component of mitochondrial contact site and cristae organizing system (MICOS) complex, the integrity of which is required for the formation and/or maintenance of mitochondrial cristae (Friedman et al., 2015). We showed that the expression of the CHCHD10p.Ser59Leu mutant allele leads to MICOS complex disassembly and loss of mitochondrial cristae. The abnormalities of the inner membrane found in CHCHD10 mutant fibroblasts are responsible for nucleoid disorganization, likely explaining the accumulation of mtDNA deleted molecules in patient muscle. Interestingly, the expression of CHCHD10 mutant alleles inhibits apoptosis by preventing cytochrome c release (Genin et al., 2016).

The observation of a frontotemporal dementia-amytrophic lateral sclerosis (FTD-ALS) phenotype in a mitochondrial disease led us to analyze CHCHD10 in a cohort of patients with pathologically proven FTD-ALS. Rapidly, our group and others reported CHCHD10 mutations in patients with FTD-ALS and familial or sporadic pure ALS leading thus secondarily to the identification of a novel gene associated with FTD-ALS clinical spectrum (Chausenot et al., 2014) (for review see (Cozzolino et al., 2015)). Furthermore, Penttilä and colleagues identified a founder mutation in CHCHD10 (c.197G > T; p.Gly66Val) in 17 Finnish families with late-onset spinal motor neuronopathy (SMAJ) (Penttilä et al., 2015), and this variant was later reported also in some Finnish patients that clinically had been diagnosed as Charcot-Marie-Tooth disease type 2 (CMT2) (Auranen et al., 2015; Jokela et al., 2016; Penttilä et al., 2017). SMAJ, also called spinal muscular atrophy, is a relatively benign autosomal dominant form of spinal muscular atrophy (SMA). Symptoms commonly appear after the age of 30–40. The disease is slowly progressive and the patients remain ambulant for several decades after onset of symptoms with a life expectancy within normal range.

A key pathological feature in the vast majority of ALS and FTD patients is the accumulation of cytoplasmic TDP-43 inclusions (Arai et al., 2006; Neumann et al., 2006). Increasing evidence indicates that TDP-43 accumulates in the mitochondria of neurons from subjects with ALS or FTD and induces mitochondrial dysfunction resulting in synaptic damage (Wang et al., 2013, 2016; Woo et al., 2017). However, the pathogenic mechanisms by which mitochondrial TDP-43 leads to mitochondrial dysfunction remain largely unknown. Recent results have tied together the activities of CHCHD10 to TDP-43 cytoplasmic inclusions by showing that the overexpression of CHCHD10 mutations resulted in mislocalization of TDP-43 in the cytoplasm (Woo et al., 2017). In order to understand why the p.Ser59Leu and p.Gly66Val mutations, both located in the α-hydrophobic helix of the CHCHD10 protein, respectively lead to severe and mild motor neuron disease, we compared their respective effects on mitochondrial functions, TDP-43 metabolism and cell death in human cellular models.

2. Materials and methods

2.1. Patients

Clinical features of the seven patients carrying the heterozygous p.Gly66Val mutation are listed in Supplementary Table 1. Age at onset ranged from 25 to 60 years frequently with painful cramping. All patients presented with a slowly progressive, proximal and distal motor neuronopathy with reduced tendon reflexes. P1, P3, P4 and P5 patients respectively correspond to P5, P10, P9 and P2 individuals in (Penttilä et al., 2015). Blood and tissue samples were obtained after patients had given informed consent.

2.2. mtDNA molecular analysis

Total DNA was extracted using standard phenol chloroform procedure. Southern blot analysis was performed as previously described (Moraes et al., 1989).

2.3. Cell culture

Skin punches were obtained from patients after informed consent. Primary fibroblast cultures were established using standard procedures in RPMI supplemented with 10% Fetal Bovine Serum (FBS), 45 μg/ml uridine and 275 μg/ml sodium pyruvate. Cultures were incubated at 37 °C with 5% CO₂. For galactose conditions, medium was replaced 24 h before experiments by glucose-free medium containing 5 mM galactose and 5 mM pyruvate (Bannwarth et al., 2014).

HeLa cells were maintained in DMEM supplemented with penicillin (100 U/ml)/streptomycin (0.1 mg/ml), 10% FBS, at 37 °C in a humidified atmosphere with 5% CO₂ in air. For transient transfections, HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

2.4. Blue native electrophoresis (BN-PAGE)

Mitochondrial membranes were isolated from 2.5 × 10⁶ cells or from 200 μg of pure mitochondria as described previously (Nijtmans et al., 2002). Cells were solubilized with 3% digitonin (wt/vol) (Sigma-Aldrich) and 0.4% (wt/vol) lauryl maltoside (Sigma-Aldrich). Ten microliters of samples were electrophoresed on a 5–13% gradient polyacylamide gel as described previously (Nijtmans et al., 2002). Transfer of proteins onto a PVDF membrane (Bio-Rad Laboratories) was carried out overnight at 30 V at 4 °C. For second-dimension gel electrophoresis, cells were solubilized with two rounds of digitonin (3% and 1%, wt/ vol). A lane excised from the first dimension native gel was first treated for 30 min with denaturing buffer containing 15 mM β-mercaptoethanol and 1% SDS and then washed in 1% SDS for 1 h. The gel strip was electrophoresed on a tricine–SDS–polyacrylamide gel as described previously (Ballinger et al., 1999).

For supercomplex analysis, enriched mitochondrial fraction was solubilized with 6 g/g digitonin. Thirty μg proteins were loaded to a 4–13% Bis-Tris Native gel. Electrophoresis run at 4 mA during 23 h. Proteins were transferred at 63 mA for 24 h to a PVDF membrane.

2.5. Purification of mitochondria from fibroblasts

High-purity mitochondria were obtained following manufacturer’s recommendations (Qproteome Mitochondria Isolation Kit, Qiagen). Briefly, fibroblasts were disrupted with ice-cold Lysis Buffer (10 min, 4 °C). After centrifugation (1000 × g, 10 min, 4 °C), cytosolic proteins were removed, and cell pellet was disrupted with ice-cold Disruption Buffer. Complete cell disruption was performed by using a blunt-ended needle and a seringe. After centrifugation (1000 × g, 10 min, 4 °C), pellet containing nuclei, cell debris and unbroken cells were removed. High-purity mitochondria were obtained following centrifugation.
for fibroblasts and HeLa cells. The quantitative data were further analysed in Microsoft Excel and GraphPad Prism 5 (GraphPad Software). Mitochondrial network length was quantified for 35 randomly-selected individual cells. Data are represented as mean ± S.E.M. Statistical analyses were performed by Student’s unpaired t-test using GraphPad Prism 5 (GraphPad Software).

2.13. Immunofluorescence of cultured cells

For immunofluorescence studies, cells were treated as previously described (Genin et al., 2016) and antibodies are described in Supplementary Table 2. To analyze the number of Drp1 puncta, images were first converted to binary images with the default threshold setting by ImageJ. The Drp1 puncta on the mitochondria were selected using colocalization analysis of Drp1 and Tom20 signals (mitochondria) by Imaris software. The puncta number and mitochondrial area were quantified by ImageJ, following the puncta density was calculated by dividing the number of Drp1 puncta on mitochondria by the total mitochondrial area.

2.14. Lentivirus

Lentiviruses were generated as described previously (Kageyama et al., 2011; Kim et al., 2007). Briefly, pHR-SIN-CSGW expressing Drp1K38A or empty vector was co-transduced into HEK293T cells with two other constructs, pHR-CMV8.2ΔR and pCMV-VSVG, using Lipopectamine 2000 (Invitrogen). Two days after transfection, the supernatant of transduced cells containing released viruses was collected. The viruses were quick-frozen in liquid nitrogen and stored at −80 °C.

Fibroblasts from patients were infected along with 8 μg/ml polybrene (Millipore), and with either viral supernatant expressing Drp1K38A or empty vector (as control) for 24 h. Then, the medium was replaced and fibroblasts were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Following 8–12 days of culture, fibroblasts were harvested for immunofluorescence and mitochondrial network analysis.

2.15. Cell death measurement

Cells were treated either with 1 μM Staurosporine (Sigma-Aldrich) or with 1 μM Actinomycin D (Sigma-Aldrich) as indicated, re-suspended in 200 μl of buffer (150 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) and incubated with annexin V-FITC (BD Biosciences) for 10 min at RT. A volume of 0.5 μg/ml of DAPI (Molecular Probes) was then added, and samples were analysed immediately by flow cytometry using a MACS-Quanti Analyzer (Miltenyi Biotec). Antibodies are listed in Supplementary Table 2.

2.16. DEVDase activity

A total of 20 μg of protein (in triplicate) was incubated with 0.2 mM of Ac-DEVD-AMC in 50 mM HEPES pH 8; 150 mM NaCl; 20 mM ethylenediaminetetraacetic acid ± 1 μM Ac-DEVD-CHO. Caspase activity was determined at 460 nm, and specific activities were expressed in ΔOD per minute and per milligram of protein.

3. Results

3.1. Unlike the p.Ser59Leu mutation, the p.Gly66Val variant is not responsible for mitochondrial myopathy with mtDNA instability

Muscle biopsies available from patients carrying the p.Gly66Val mutation did not show a typical mitochondrial myopathy (Auranen et al., 2015; Jokela et al., 2016; Pasanen et al., 2016; Penttilä et al., 2015, 2017). We analysed the mitochondrial genome in the muscle of 5 patients (P1–P5), from 39 to 82 years (Supplementary Table 1). One
patient only (P1) had a few COX-negative fibers (3%) with no RBF at age 70 years (not shown). We found no accumulation of mtDNA deletions by Southern blot analysis and the small amount of deleted molecules observed in some patients was probably age-related (Fig. 1A). These results confirm that the p.Gly66Val variant is not associated with mitochondrial myopathy and mtDNA instability, unlike what is observed with the p.Ser59Leu mutation (Bannwarth et al., 2014).

3.2. A different phenotype in fibroblasts also, with neither MICOS nor OXPHOS assembly defect in cells from patients bearing the p.Gly66Val mutation

To analyze the effects of the p.Gly66Val mutation on MICOS assembly and mitochondrial functions, we studied fibroblast cells from 2 other patients (P6 and P7) because no skin biopsy was available from affected individuals previously described (P1-P5) (Supplementary Table 1). Blue Native (BN)-PAGE analysis revealed no impairment of assembly of MICOS and OXPHOS complexes (Fig. 1B). BN-PAGE followed by 2D western blotting showed that the steady-state levels of assembled CHCHD10 in MICOS complex are not affected in patient fibroblasts (Fig. 1C). BN-PAGE analysis also revealed no impairment of OXPHOS supercomplex formation (Fig. 1D). We next examined the abundance of several MICOS and OXPHOS proteins in patient-derived fibroblasts and we found normal levels compared to control fibroblasts (Fig. 1E–F and Supplementary Fig. 1). These results show that, in fibroblasts also, phenotypes associated with p.Ser59Leu and p.Gly66Val mutations are different.

3.3. No ultrastructural alterations in cells expressing the CHCHD10G66Val allele, no respiratory chain deficiency and no defect of ATP production in patient-derived fibroblasts

We performed ultrastructural analysis of patient fibroblasts. Electron microscopy did not reveal marked ultrastructural abnormalities in patient mitochondria, which demonstrated numerous, thin, well-defined cristae running perpendicularly to the mitochondrial longitudinal axis, and with a regular pattern of parallel organization (Fig. 2A–C). The p.Gly66Val mutation was then expressed in HeLa cells. We previously shown that the expression of the CHCHD10G66Val allele led to a marked defect of the mitochondrial cristae maintenance characterized by loss and desorganization of cristae morphology (Bannwarth et al., 2014). Contrary to overexpression of the CHCHD10G559 allele, the one of the CHCHD10G66Val mutant did not lead to abnormal morphology of mitochondria (Supplementary Fig. 2). Only a few abnormal cristae were observed similar to those found in HeLa cells overexpressing the CHCHD10 wild-type allele.

Spectrophotometric analysis of fibroblasts from P6 and P7 cultivated in glucose medium revealed no respiratory chain deficiency and polarographic analysis showed normal oxygen consumption and mitochondrial substrate oxidation (Table 1A, B). Identical experiments were performed on fibroblasts grown in a glucose-free medium containing galactose as previously described (Rouzier et al., 2012).

![Fig. 1. Analysis of muscle and fibroblasts from patients bearing the p.Gly66Val mutation. A. Southern blot analysis from patient muscle (P1-P5). Comparatively, an accumulation of mtDNA deletions (A) was observed in the muscle of a patient (III-2) bearing the p.Ser59Leu mutation (Bannwarth et al., 2014). C: control individual. B. BN-PAGE of the MICOS and OXPHOS complexes in control (C) and patient (P6, P7) fibroblasts. Patients P6 and P7 carry the p.Gly66Val mutation. Complexes I to IV (CI-CIV) of OXPHOS were detected with an anti-NDUFS9 antibody (CI), an anti-SDHA antibody (CII), an anti-core I antibody (CIII) and an anti-cytochrome c oxidase subunit I antibody (CIV). MICOS and MIB complexes were detected with an antibody anti-MIC60/mito lin. MIB: Mitochondrial Intermembrane space Bridging complex. C. Second dimension of the BN-PAGE showing that the steady-state levels of assembled CHCHD10 in MICOS complex are not affected in patient fibroblasts. D. Analysis of OXPHOS supercomplexes in control and patient fibroblasts. BN-PAGE from isolated mitochondria permeabilized with 6 g/g (w/v) of digitonin immunoblotted on PVDF membrane and incubated with the indicated antibodies. SC, supercomplexes I + II + IV + Vn. Experiments corresponding to panels B-D, performed with fibroblasts bearing the p.Ser59Leu mutation, are found in Genin et al., 2016. E. Representative western blot of MICOS proteins, including mitofilin (MIC60), CHCHD6 (MIC25) and CHCHD3 (MIC19) performed with fibroblast lysates obtained from controls (C1, C2) and patients (P6, P7). Three isoforms of mitofilin (89, 87 and 80KD) exist due to alternative splicing and can be detected by immunoblot analysis. HS6P60 was used as a loading control. F. Representative western blot of OXPHOS proteins, including ATP5A (CV), UQCR2 (CIII), SDHB (CII), COXII (CV) and NDUF8 (C) performed with fibroblast lysates obtained from controls (C1, C2) and patients (P6, P7). HS6P60 was used as a loading control. Quantitative analysis of blots E and F is found in Supplementary Fig. 1.]

Galactose is a carbon source that feeds the glycolytic pathway with low efficiency and as such cells are forced to rely predominantly on OXPHOS for ATP production leading to unmask defects compensated in glucose medium (Robinson et al., 1992). Culture in glucose-free medium only allowed to identify a slight decrease of complex IV/citrate synthase ratio in the fibroblasts of both patients by spectrophotometry while polarography was normal (Table 1C, D). We also found that the pGly66Val mutation does not impair ATP synthesis in patient fibroblasts grown in glucose or in galactose medium (Fig. 2D).

3.4. Comparable accumulation of phosphorylated mitochondrial TDP-43 in fibroblasts from patients bearing the CHCHD10 p.Ser59Leu or p.Gly66Val mutation

A recent study provided a pathological link between CHCHD10 dysfunction and cytoplasmic TDP-43 inclusions (Woo et al., 2017). We therefore examined TDP-43 expression in fibroblasts from patients bearing the p.Ser59Leu or p.Gly66Val mutation (S59L or G66V fibroblasts) or from a control individual. All fibroblasts showed comparable expression of total TDP-43 (Fig. 3A). Interestingly, S59L and G66V fibroblasts displayed a different pattern of mitochondrial TDP-43 than the one found in control cells. Indeed, only phosphorylated TDP-43 was present in the mitochondria of fibroblasts from patients, contrary to control cells which also presented non phosphorylated TDP-43 (Fig.3B–C). In fibroblasts from ALS patients bearing TDP-43 mutations, mitochondrial TDP-43 protein inhibits translation of ND3 and ND6 mRNAs leading to disassembly and dysfunction of complex I (Wang et al., 2016). In S59L and G66V fibroblasts, the mitochondrial location of TDP-43 was not associated with complex I disassembly (Supplementary Fig. 3A, Fig. 1B). However, the expression level of ND3 was decreased both in S59L and G66V fibroblasts, grown in glucose or galactose medium, compared to control cells (Supplementary Fig. 3B). Decreased expression level of ND3 was not sufficient to induce a complex I activity defect in G66V fibroblasts grown in glucose and galactose medium (Table 1). In S59L cells, the activity of complex I was normal in glucose medium (Bannwarth et al., 2014). In galactose medium, S59L fibroblasts displayed a multiple respiratory chain deficiency which was not limited to complex I, suggesting that mitochondrial TDP-43 toxicity might participate to the respiratory chain defect that we observed (Bannwarth et al., 2014).

In order to also check the general mRNA processing ability of TDP-43 in patient fibroblasts, we performed RT-PCR experiments to analyze splicing efficiency of TDP-43 targets: BIM, SKAR, STAG2 and MADD (De Conti et al., 2015). We found no difference in the patterns of splicing between control and patients cells, suggesting that there is no alteration in the level of different isoform expression (Supplementary Fig. 3C–F).

3.5. Cells expressing the CHCHD10<sub>G66V</sub> allele display mitochondrial fragmentation

Others and we have shown that the expression of CHCHD10 mutations, responsible for severe phenotypes (S59L and R15L), in human cells leads to mitochondrial network fragmentation (Bannwarth et al., 2014; Woo et al., 2017) and S59L fibroblasts do not display fusion deficiency (Genin et al., 2016). We compared the mitochondrial network of G66V fibroblasts (P6 and P7) with that obtained from control cells. After staining with Mitotracker and examination by confocal microscopy, control fibroblasts in glucose medium displayed a typical filamentous interconnected network. Patient fibroblasts presented with a fragmentation of the mitochondrial network and less connected mitochondria (Fig. 4A). However, G66V fibroblasts were less fragmented than those harbouring the p.Ser59Leu variant responsible for a severe clinical phenotype (Fig. 4B). The overexpression of the CHCHD10<sub>G66V</sub> allele in HeLa cells confirmed its effect on mitochondrial network fragmentation (Fig. 4C–D).

TDP-43 can form physical complexes with CHCHD10 (Woo et al., 2017). TDP-43 overexpression in transgenic mice induces mitochondrial fragmentation by promoting mitochondrial fission with increased...
levels of Fis1 and Drp1, key components of fission machinery (Wang et al., 2013; Xu et al., 2010). The overexpression of wild-type and CHCHD10 mutant (S59L and G66V) alleles did not lead to enhanced levels of Fis1 and Drp1 (Fig. 4E). In S59L and G66V fibroblasts, we found no difference regarding Fis1 levels compared to control cells but Drp1 expression was increased in patient fibroblasts compared to control (Fig. 4F). We therefore studied Drp1 recruitment in patient cells. We used immunofluorescence to visualize Drp1 puncta on mitochondria. Drp1 staining was observed throughout the cytosol, but a portion can be found in punctuate structure in mitochondria. We performed
image analysis and quantified the density of Drp1 puncta on mitochondria by dividing the number of puncta by the total mitochondrial area (Fig. 5A–B). The density of mitochondrial Drp1 puncta was similar in G66V and control cells. We also analysed S59L fibroblasts and found no difference compared with control cells (Fig. 5A–B). These data suggest that CHCHD10 mutations have no impact on Drp1 recruitment into patient fibroblasts.

Last, we expressed a dominant negative mutant of the fission protein Drp1 (Drp1K38A) (Smirnova et al., 1998) in S59L, G66V and control fibroblasts and found that control and patient cells similarly elongate mitochondria confirming that fusion process is not impaired by the expression of CHCHD10 mutations (Fig. 5C–D).

3.6. Resistance to apoptosis in cells expressing the CHCHD10G66V allele

We have previously shown that the expression of the CHCHD10S59L mutant allele inhibits apoptosis by preventing cytochrome c release (Genin et al., 2016). In order to determine whether the p.Gly66Val mutation leads or not to resistance to apoptosis, we analysed caspase activity in patient fibroblasts. Cells were treated with 1 μM staurosporine (STS) for 6 or 8 h, and caspase activation was determined by DEVDase activity measurement (Fig. 6A). Patient-derived fibroblasts were significantly less sensitive to staurosporine-induced apoptosis compared with control cells. However, they were significantly more sensitive than those bearing the p.Ser59Leu variant, responsible for a severe clinical phenotype. HeLa cells expressing the CHCHD10G66V mutant form were also less sensitive to apoptotic cell death than those overexpressing the wild-type CHCHD10 protein (Fig. 6B–D), as observed by the decrease in cleaved caspase 3, the decrease in SMAC degradation (that is a readout of mitochondrial outer membrane permeabilization) and of caspase activity. Taken together our experimental data indicate that expression of the CHCHD10G66V allele is able to decrease the sensitivity of the cells towards apoptotic stimuli.

Fig. 3. Accumulation of phosphorylated mitochondrial TDP-43 in S59L and G66V fibroblasts. A. Representative western blot and quantification of TDP-43 protein performed with total fibroblast lysates obtained from a control (C) and patients bearing the p.Ser59Leu (S59L) or Gly66Val (P6, P7) mutations. Bars represent the mean ± SD of 3 independent experiments. HSP60 was used as a loading control. B. Representative immunoblot of TDP-43 and phosphorylated TDP-43 (pTDP-43) in mitochondria (right panel) and total lysates (left panel) from S59L, G66V (P6, P7) and control fibroblasts. Quantifications of TDP-43 and phosphorylated TDP-43 are performed in mitochondria. TDP-43 antibodies recognize total TDP-43 (including phosphorylated forms) whereas pTDP-43 is specifically directed against phosphorylated TDP-43. After treatment by CIP (Calf Intestinal Phosphatase), probing with pTDP-43 Ab does not allow to reveal any band, thus confirming the specificity of the Ab (not shown). Five micrograms were loaded for mitochondria extracts and total lysates. Bars represent the mean ± SD of 3 independent experiments. Differences between the control and patient fibroblasts were analysed by two-way ANOVA: extremely significant (**P < 0.001). HSP60 was used as a loading control. GAPDH and PCNA were used as controls for mitochondrial fraction purity. C. Representative images, at confocal microscopy after Huygens deconvolution (Huygens Essential Software™), using Mitotracker and an anti-phosphorylated TDP-43 antibody (pTDP-43), showing partial localization of pTDP-43 in mitochondria of patient cells (overlay in yellow). Enlarged details of the area are indicated. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

Over the past years, numerous data provided clear molecular evidence that ALS and FTD represent a clinicopathological spectrum of diseases (DeJesus-Hernandez et al., 2011; Janssens and Van Broeckhoven, 2013; Renton et al., 2011; Weishaupt et al., 2016). In addition to overlapping clinical symptoms, the common pathological hallmark found in most ALS and FTD patients is the accumulation of TAR DNA-binding protein 43 (TDP-43) inclusions (Arai et al., 2006; Neumann et al., 2006). TDP-43 plays a major role in the regulation of RNA metabolism and is mainly localized in the nucleus in normal conditions (Buratti et al., 2001). In patient neurons, TDP-43 is depleted from the nucleus and accumulates within cytoplasmic ubiquitinated inclusions. Mutations in TARDBP, which encodes TDP-43, are
associated with familial and sporadic ALS but TDP-43 inclusions are observed in ALS, FTD and several other neurodegenerative diseases without TARDBP mutations (Chanson et al., 2010; Josephs et al., 2015; Kabashi et al., 2008; Sreedharan et al., 2008). It is still unknown whether TDP-43 pathology is caused by nuclear depletion, cytoplasmic accumulation of TDP-43, or both. However, recent studies suggest that the neuronal toxicity of TDP-43 seems, at least in part, due to its mitochondrial localization (Wang et al., 2016). In spinal cord and cortical tissues of ALS or FTD patients, there is a higher expression of TDP-43 in mitochondria than in those from controls (Wang et al., 2016). Fibroblasts from ALS patients bearing TARDDBP mutations show higher levels of mitochondrial TDP-43 than control cells and overexpression of both wild-type and mutant TDP-43 impairs the expression of complex I ND3 and ND6 subunits and causes complex I disassembly (Wang et al., 2016). Transgenic mouse expressing the disease-causing human TDP-43 M337V mutant (TDP-43M337V mice) show increased levels of mitochondrial localization of TDP-43 (Wang et al., 2017).

Among all factors involved in ALS pathogenesis, mitochondrial dysfunction has always been recognized as a major player because the p.Ser59Leu mutation despite their proximity within the N-terminus of CHCHD10 (Banwarth et al., 2014). A recent work showed that CHCHD10 interacts with TDP-43 (Woo et al., 2017). Overexpression of CHCHD10 mutations (S59L and R15L) causing ALS-FTD disease induced cytoplasmic TDP-43 accumulation, which often co-localized with mitochondria, associated with mitochondrial dysfunction and neuronal loss; the mouse phenotype being reversed by inhibiting the mitochondrial localization of TDP-43 (Wang et al., 2017).

We previously showed that the expression of wild-type CHCHD10 does not protect HeLa cells from apoptosis induced by actinomycin D (Genin et al., 2016). Surprisingly, the expression of the CHCHD10S59L allele led to a reduction in cell death and S59L fibroblasts were significantly less sensitive to staurosporine-induced apoptosis compared with control cells (Genin et al., 2016). We show that the p.Gly66Val mutation also reduces the sensitivity to actinomycin D-induced death, albeit to a lesser extend that in S59L cells. Our results also confirm the surprising anti-apoptotic tendency found in fibroblasts from patients bearing CHCHD10 mutations leading to motor neuron disease. The recent study describing the functional link between CHCHD10 and TDP-43 showed that overexpression of wild-type CHCHD10 protected HT22 cells from TDP-43 induced apoptosis, whereas R15L and S59L mutations synergized with the pro-apoptotic effect of TDP-43 (Woo et al., 2017). We agree with the authors that this potential discrepancy might be explained by the use of overexpressed proteins in different cellular models and of TDP-43 as a specific stressor. Our data show that, in absence of overexpression and despite an accumulation of mitochondrial phosphorylated TDP-43, S59L and G66V fibroblasts are significantly less sensitive to staurosporine-induced apoptosis compared with control cells.

Woo and colleagues reported that the effects of the S59L mutation...
Fig. 5. Analysis of mitochondrial dynamics in patient fibroblasts. A. Control and patient cells were subjected to immunofluorescence with antibodies to Drp1 (green) and Tom20 (mitochondria, red). Converted binary images are shown. Drp1 puncta on mitochondria were defined using colocalization analysis of Drp1 and Tom20 signals. Scale bar: 20 μm. B. The Drp1 puncta numbers on the mitochondria were quantified. Values are mean ± SEM (n = 10 cells). C. Control and patient cells were infected with lentiviruses carrying the empty vector (EV) or a dominant negative Drp1K38A. Cells were subjected to MitoTracker staining (red) and Drp1 immunolabelling (green). The expression of Drp1K38A blocked mitochondrial division and elongated mitochondrial tubules in the control and patient cells. Boxed regions show magnified images. Scale bar: 10 μm. D. Quantification of the images shown in C for 25 randomly-selected individual cells per each studied fibroblast cell line (from 2 independent experiments). Values are mean ± SEM. Differences between the control and patient fibroblasts were analysed by one-way ANOVA: extremely significant (***P < 0.001). NS: non significant. E. Western blot on control and patient fibroblasts, infected with lentiviruses carrying the empty vector (+EV) or a dominant negative Drp1K38A (+Drp1K38A), using antibodies against Drp1 or HSP60. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
were more severe than that of the R15L mutation (Woo et al., 2017). Because the S59L mutation was found in patients with severe ALS-FTD phenotype whereas the R15L mutation was carried by patients with ALS phenotype only, they suggested that differences observed may reflect the severity of the phenotypes. Our findings are in agreement with this hypothesis. The p.Gly66Val mutation is responsible for a benign form of lower motor neuron disease and the cellular phenotypes found in S59L and G66V fibroblasts are different. G66V fibroblasts do not display...
mitochondrial dysfunction and MICOs assembly defect compared to SS9L cells. Both SS9L and G66V fibroblasts show comparable mitochondrial acc stomalulation of phosphorylated TDP-43 but the effects on mitochondrial fragmentation and cell death are significantly less pronounced in G66V than in SS9L cells.

5. Conclusions and significance

MICO disassembly has also been described in early-onset familial mitochondrial encephalopathy with liver disorder (Guarani et al., 2016). In this disease, affected children carry a homozygous loss-of-function mutation in QTL1 encoding a subunit of MICOs. Patient fibroblasts exhibited MICOs assembly defect with respiratory chain deficiency and abnormal mitochondrial cristae. The rapidly fatal evolution does not make possible to say if these children could have developed a motor neuron disease. Our findings suggest that loss of MICOs complex integrity and mitochondrial dysfunction are likely essential to develop a severe CHCHD10-related motor neuron disease whereas accumulation of phosphorylated TDP-43 within mitochondria would not be a criteria of severity. However, these experiments need to be performed in neuron cells to understand the respective role of MICOs complex and TDP-43 in this disease.

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