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Impaired mitochondrial quality control in Rett Syndrome

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ABSTRACT

Rett Syndrome (RTT) is a rare neurodevelopmental disorder caused in the 95% of cases by mutations in the X-linked *MECP2* gene, affecting almost exclusively females. While the genetic basis of RTT is known, the exact pathogenic mechanisms that lead to the broad spectrum of symptoms still remain enigmatic. Alterations in the redox homeostasis have been proposed among the contributing factors to the development and progression of the syndrome. Mitochondria appears to play a central role in RTT oxidative damage and a plethora of mitochondrial defects has already been recognized. However, mitochondrial dynamics and mitophagy, which represent critical pathways in regulating mitochondrial quality control (QC), have not yet been investigated in RTT.

The present work showed that RTT fibroblasts have networks of hyperfused mitochondria with morphological abnormalities and increased mitochondrial volume. Moreover, analysis of mitophagic flux revealed an impaired PINK1/Parkin-mediated mitochondrial removal associated with an increase of mitochondrial fusion proteins Mitofusins 1 and 2 (MFN1 and 2) and a decrease of fission mediators including Dynamin related protein 1 (DRP1) and Mitochondrial fission 1 protein (FIS1). Finally, challenging RTT fibroblasts with FCCP and 2,4-DNP did not trigger a proper apoptotic cell death due to a defective caspase 3/7 activation.

Altogether, our findings shed light on new aspects of mitochondrial dysfunction in RTT that are represented by defective mitochondrial QC pathways, also providing new potential targets for a therapeutic intervention aimed at slowing down clinical course and manifestations in the affected patients.

1. Introduction

Rett Syndrome (OMIM #312750) is a progressive neurodevelopmental disorder caused in 95% of cases by mutations in the X-linked gene encoding Methyl-CpG-binding protein 2 (MeCP2) [1]. Although considered a rare disease, it is the second most prevalent cause of severe mental retardation in the female gender, with a frequency of 1:10,000 live births [2,3]. RTT begins to manifest 6–18 months after birth and is characterized by neurodevelopmental regression that severely affects motor, cognitive and communication skills; eye-hand coordination may be insufficient and excess of repetitive hand washing can be observed [4].

Despite considerable progress made in understanding the functions of MECP2, the pathogenic mechanisms that link its mutation to the clinical manifestations of RTT are not yet fully known. The reason for this complexity lies in the ability of MECP2 to act either as a transcriptional activator or as a transcriptional repressor of a multitude of target genes that cover many cellular functions [5,6]. For instance, MECP2 either directly or indirectly regulates the transcription of a variety of nuclear genes encoding mitochondrial factors and, therefore, its mutation is associated with a perturbed expression of mitochondria related genes which also affects their protein expression as evidenced by proteomics analysis [7,8].

As we have recently proposed, RTT is characterized by a harmful

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vicious cycle between redox imbalance and inflammation, i.e. oxinflammation, in which mitochondria seem play a central contributing role [9,10]. Indeed, mitochondria are the major site of reactive oxygen species (ROS) production in cells, but also the main targets of their damaging effects, thus representing the trigger for several mitochondrial conditions [11].

To counteract the adverse effects resulting from excess amounts of these reactive species and to maintain functional mitochondria, a highly complex and efficient integrated network of mitochondrial quality control (QC) mechanisms operating at molecular, organelle and cellular level has evolved [12]. Thanks to these mechanisms, as dynamic organelles, mitochondria constantly change their number, size, shape, and distribution in response to intra- and extracellular stimuli [13,14]. Through the process called biogenesis mitochondria can proliferate and enter in the cycles of fission and fusion. When they become compromised from various injuries, solitary mitochondria generated *via* fission events are subjected to degradation through a clearance pathway, known as mitophagy [15].

Mitophagy represents a highly specific QC pathway and is a selective bulk degradation process in which, mainly using the macroautophagy machinery, entire mitochondria are enclosed in a double-membrane vesicle called autophagosome to subsequently fuse with lysosomes for their hydrolytic degradation. It has been proven that it is usually accompanied by a characteristic phenotype of fragmented mitochondria [12,14].

Twig et al. showed that in cultured mammalian cells the segregation of damaged mitochondria by fission and subsequent inhibition of the fusion machinery is a prerequisite for mitophagic degradation [12,16]. In this context, defective mitochondrial QC processes can lead to the accumulation of damaged mitochondria that may release more ROS and mitochondrial components into cytosol, also activating inflammatory signaling. Moreover, damaged mitochondria may less efficiently produce ATP and have a lower threshold for cytochrome *c* release, resulting in apoptotic events [17]. In fact, the induction of apoptosis is effectively controlled by mitochondria and is considered the highest level of QC pathway, which acts to remove aberrant cells, thus preventing propagation of functional impairments [12].

Since their implications in differentiation, development, cell reprogramming, cell death and immune response [18–20], both mitophagy and apoptosis are fundamental to maintain homeostasis and to regulate the normal turnover of organelles or cells in the human body. Consequently, altered levels of mitophagy and apoptosis have been associated with many pathologies such as cancer, heart, liver and neurodegenerative diseases [18,21].

In a previous work, we found that the altered redox homeostasis in RTT is related to impaired cellular defense enzymes, increased NADPH oxidase activity as wells as altered mitochondrial bioenergetics [22]. Moreover, in a recent proteomics study, we confirmed the pathological relevance of mitochondrial dysfunction in RTT, demonstrating an altered expression of proteins implicated in mitochondrial structure/function and showing the first evidence of impaired mitochondrial dynamics and mitophagy in RTT cells [8].

Based on these premises, the aim of this study was to conduct a more in-depth investigation of the mitophagic pathway in RTT and its relationship with mitochondrial dynamics and apoptosis.

2. Materials and methods

2.1. Subjects population

The study population consisted of 3 female patients with classic RTT (mean age ± SD: 20 ± 8) and 3 healthy female controls age-matched (mean age ± SD: 21.8 ± 7.3). All the patients were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Siena, Italy). Diagnosis of RTT and selection criteria (inclusion/exclusion) were set in accordance with revised RTT nomenclature consensus [23]. This study was designed and performed according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Institutional Review Board of University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Siena, Italy. Informed consents were obtained in written form from either the parents or the legal tutors of the participants.

2.2. Fibroblasts isolation from skin biopsy

Control skin biopsies were obtained during routine health checks or by donations, while skin biopsies from RTT patients were carried out during the periodic clinical checks-up. Human skin fibroblasts were isolated from 3 mm skin punch biopsy (n = 3 for RTT and n = 3 for controls), as described in a previous report [24].

2.3. Cell culture

Human primary fibroblasts were grown in DMEM, containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) (Lonza, Milan, Italy) in 100 mm dishes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Only fibroblasts from passage 2–5 were used for the experiments. Cells were seeded onto 24-mm glass coverslips for morphological analysis [24].

2.4. Cell treatments

For mitophagy and apoptosis evaluation, RTT and control fibroblasts were treated with 10 μM FCCP (ab120081, abcam, Cambridge, UK) for 2, 4, 8, 16, 24 h (mitophagy assay) or 20 μM FCCP for 48 h (apoptosis assay) in complete medium.

For 2,4- Dinitrophenol (2,4-DNP) treatment (Merck KGaA, Darmstadt, Germany), RTT and control fibroblasts were treated with 2 mM 2,4-DNP for 48 h in complete medium. After the treatments, cells were collected or processed as described below.

2.5. Transmission electron microscopy (TEM) analysis

Mitochondrial morphology was evaluated by transmission electron microscopy (TEM). Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4 °C. Then were washed with 0.1 M cacodylate buffer (pH 7.4) three times and postfixed in 1% osmium tetroxide and 0.1 M cacodylate buffer at pH 7.4 for 1 h at room temperature. The specimens were dehydrated in graded concentrations of ethanol and embedded in epoxide resin (Agar Scientific, Stanstead, UK). Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60 °C for 48 h. Semithin sections (0.5–1 μm thickness) were cut using an ultramicrotome (Reichert Ultracut S Ultramicrotome, Leica Microsystems, Wien, Austria), stained with toluidine blue and blocks were selected for thinning. Ultrathin sections of approximately 40–60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% citrate for 30 min each and examined under a transmission electron microscope (Hitachi, H-800, Tokyo, Japan) at an accelerating voltage of 100 KV [10].

2.6. Confocal microscopy analysis

Mitochondrial morphology was assessed in basal condition. RTT and control fibroblasts were stained with 100 nM MitoTracker green (Thermo Fisher Scientific Inc., Wilmington, USA) in Krebs Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 20 mM Hepes (pH 7.4 at 37 °C) for 30 min at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Confocal images were collected on a Zeiss LSM510 confocal microscope (Carl Zeiss, Germany) using a 63 × 1.4 NA Plan-Apochromat oil-immersion objective. Three-

dimensional reconstruction of cells and subsequent analysis of mitochondrial parameters such as number and the volume were performed using the deconvolved Z-stacks and ImageJ (Fiji) software, National Institutes of Health.

2.7. Western blot analysis

For Western blot, cells were harvested, washed and pelleted (280×*g* at 4 °C for 4 min) in phosphate-buffered saline (PBS), and then resuspended in RIPA buffer supplemented with protease (#78430, Thermo Fisher Scientific Inc.) and phosphatase inhibitor cocktails (#78420, Thermo Fisher Scientific Inc.). After 30 min of incubation in ice and centrifugation at 12,000×*g* at 4 °C for 15 min, cell lysates were collected and proteins quantified by Bradford assay (Bio-Rad Laboratories, Inc., Milan, Italy). Ten μg of each protein sample were loaded on home-made gels and transferred to nitrocellulose membranes. After a blocking step of 1 h at room temperature with 5% non-fat dry milk in TBS–Tween-20 (0.1%), membranes were incubated overnight at 4 °C with following primary antibodies: *anti*-caspase 3 (bs-0081R; Bioss Antibodies Inc., Woburn, MA, USA), *anti*-Parkin (1:1000; sc-32282; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-PINK1 (1:1000; sc-517353; Santa Cruz Biotechnology, Inc.), *anti*-MFN2 (1:1000; sc-100560; Santa Cruz Biotechnology, Inc.), *anti*-ATP5A (1:1000; sc-136178; Santa Cruz Biotechnology, Inc.), *anti*-MFN1 (1:500; sc50331; Santa Cruz Biotechnology, Inc.), *anti*-DRP1 (1:1000; #5391; Cell Signaling Technology, Inc., Danvers, MA, USA), *anti*-pDRP1 (1:1000; #3455; Cell Signaling Technology, Inc.), *anti*-FIS1 (1:1000; AP1165; Merck KGaA), anti-TOM20 (1:1000; sc-17764; Santa Cruz Biotechnology, Inc.). Anti-GAPDH (1:5000; #5174; Cell Signaling Technology, Inc.) and *anti*-β-tubulin (1:3000; T8328; Sigma-Aldrich Corp., St. Louis, MO, USA) were included as loading controls. After over-night incubation, the nitrocellulose membranes were incubated with appropriate HRP-conjugated secondary antibodies (anti-mouse, #1706515, Bio-Rad Laboratories, Inc., 1:10000; anti-rabbit, #7074, Cell Signaling Technology, Inc., 1:10000). The proteins were detected by chemiluminescence, using ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.) or ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.8. Subcellular fractionation

RTT and controls fibroblasts were harvested, washed with PBS, pelleted by centrifugation at 280×*g* for 4 min, resuspended in homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 2 mM EGTA) and gently disrupted by using a Potter. The homogenate was centrifuged twice at 2400×*g* for 3 min at 4 °C to remove membranes and unbroken cells. Then the supernatant was centrifuged at 10000×*g* at 4 °C for 10 min to pellet crude mitochondria. The pellet containing mitochondria was finally resuspended in lysis buffer with protease and phosphatase inhibitors. The cytosolic fraction was obtained by centrifuge a portion of the homogenate at 16000 g for 30 min at 4 °C and the supernatant was collected and added of lysis buffer with protease and phosphatase inhibitors.

2.9. Annexin V/PI staining

For Annexin V/PI staining, RTT and healthy fibroblasts were plated onto 60 mm well dishes. After treatment with 20 μM FCCP or 2 mM 2,4-DNP for 48 h, cells were gently harvested, processed with buffers, and incubated with Annexin V/PI according to manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were then analysed with a flow cytometer (BD FACSCanto™ II Cell Analyzer, BD Biosciences, San Jose, CA, USA) and the several parameters were collected.

2.10. ApoTox-Glo assay

For caspase 3/7 activation, RTT and healthy fibroblasts were plated onto 96 well plate (Corning Incorporated, Corning, NY, USA). After treatment with 20 μM FCCP for 48 h, reagents included in the kit were added according to manufacturer's protocol (Promega Corporation, Madison, WI, USA). Then luminescence was measured by Synergy H1 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and data were analysed.

2.11. Statistical analysis

Statistical analysis was performed using unpaired two-tailed *t*-test (two groups) or one/two-way ANOVA followed by Bonferroni's multiple comparisons test. Normal distribution of data was assessed by applying Shapiro-Wilk normality test. A p-value < 0.05 was considered significant. All data are reported as mean ± SEM. Exact p-values are indicated in the figure legends.

3. Results

3.1. Altered mitochondria morphology in RTT

As depicted in Fig. 1A, mitochondria from control fibroblasts showed a normal tubular morphology with well-defined cristae structure. On the contrary, RTT mitochondria exhibited an altered morphology with a dumbbell-like shape, a structural swelling with cristae disarrangement and a predominantly electron dense matrix deposition (Fig. 1A, right panel). Moreover, by reconstructing 3D images, RTT fibroblasts showed a filamentous interconnected network of elongated and hyperfused mitochondria (Fig. 1B, right panel), while in control cells MitoTracker Green revealed typical mitochondrial structures represented by short lines or dots (Fig. 1B, left panel). Analysis of confocal images indicated in RTT cells a decreased number (almost halved) of mitochondria with a larger volume, compared to control cells (Fig. 1C and D). These ultrastructural changes in RTT fibroblasts suggest a possible failure of normal mitochondrial QC mechanisms.

3.2. Impaired Pink1/Parkin-mediated mitophagy in RTT fibroblasts

A highly specific mitochondrial QC pathway is represented by mitophagy, a process responsible for the degradation of terminally damaged or dysfunctional mitochondria [12,16]. As RTT mitochondria appeared more fused with an aberrant morphology and a greater volume, we were interested in understanding whether an alteration of the mitophagic process could be involved in this aspect. We first evaluated the level and function of key players in mitophagy, such as PINK1 and Parkin [14]. As shown in Fig. 2A, there was a significant decrease of the ubiquitin E3 ligase Parkin protein expression in unstimulated RTT cells compared to the controls, while no differences were found in PINK1 protein levels. However, upon mitophagy induction by FCCP treatment, a strong defective translocation of PINK1 from cytosol to mitochondria was observed in RTT fibroblasts (Fig. 2B).

To further corroborate the evidence of a compromised mitochondrial removal in RTT, we evaluated levels of some mitochondrial proteins that are degraded during mitophagy. As depicted in Fig. 2C, FCCP treatment induced a significant and time-dependent decrease of Parkin, MFN2 and ATP5A in control fibroblasts with a maximum decline at 24 h after the stimulation, suggesting a proper activity of the mitophagic machinery in these cells. In contrast, RTT fibroblasts showed an almost complete absence of Parkin protein. Moreover, no significant changes were appreciable in MFN2 and ATP5A expression after FCCP-induced mitochondrial damage (Fig. 2C). Altogether, these results are consistent with a possible impairment of the mitophagic flux in RTT cells.

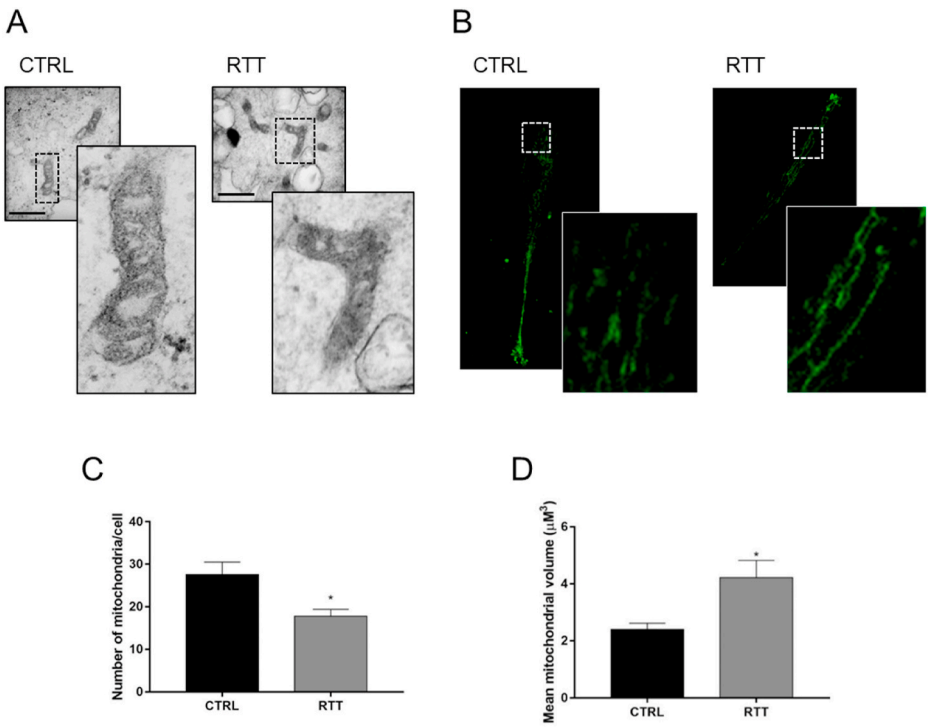

Fig. 1. A. Transmission electron microscopy analysis of mitochondria of RTT and control fibroblasts. Cells of RTT (n = 3) and control (n = 3) fibroblasts were harvested and mitochondria were observed at TEM. Magnification 40000×. Scale bar 2 μm. **B.** Confocal microscopy analysis of RTT and control fibroblasts. MitoTracker (green fluorescence) was used to stain mitochondria in RTT (n = 3) and control (n = 3) fibroblasts prior to confocal microscope analysis. Magnification 40×. Scale bar 10 μm. **C and D.** Quantification of mitochondrial number (C) and mean volume (D) of RTT (n = 3) and control (n = 3) fibroblasts. Data are expressed as mean ± SEM. *p = 0.0031 and * p = 0.0174 for panel C and D, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Increased mitofusins levels in RTT cells

It has been demonstrated that mitophagy could be blocked by an excessive mitochondrial fusion, which together with fission represents key determinants in mitochondrial QC processes [25]. Thus, since RTT mitochondria displayed a hyperfused morphology and an impaired mitophagic process, we subsequently focused our attention on the fusion pathway. Of note, we found that mitochondrial subfractions of RTT fibroblasts exhibited an increased expression of MFN1 and MFN2 proteins in both basal condition and upon FCCP-mediated mitophagy induction when compared to control samples (Fig. 3), supporting an indication for a prevalent mitochondrial fusion activity in RTT.

3.4. Impaired DRP1/FIS1-mediated fission in RTT fibroblasts

To further explore QC mechanisms in RTT, we also evaluated mitochondrial fission which is essential for the physiological degradation of damaged mitochondria *via* mitophagy [16,25]. As shown in Fig. 4A, we observed a significant decrease of more than 50% in protein levels of the fission protein FIS1 in unstimulated RTT fibroblasts compared to the control, while no differences were found in the levels of DRP1 and its phosphorylated form pDRP1 (S616).

It is widely known that, upon mitophagic stimulus, DRP1 translocates from cytosol to mitochondria to activate mitochondrial fission by interaction with various adaptors including FIS1 [16,25]. As depicted in Fig. 4B, upon FCCP-dependent mitophagic induction, RTT cells showed the inability to properly recruit DRP1 at the mitochondrial compartment compared to control fibroblasts (Fig. 4B). In line with previous findings, these data support the picture of unbalanced fusion/fission dynamics, promoting mitochondrial hyperfusion and incomplete mitochondrial clearance after mitophagy induction in RTT.

3.5. Aberrant mitochondrial-dependent apoptosis in RTT fibroblasts

Mitochondria also play a central role in the apoptotic process [19]. Indeed, when the mitochondrial QC pathways are overwhelmed, the programmed cell death which represents the highest order of mitochondrial QC mechanisms can be triggered, thus preventing the propagation of functional impairments [12]. Since RTT mitochondria displayed a defect in mitochondrial fusion/fission dynamics associated with compromised mitophagic flux, we tried to understand whether these alterations could affect also the functionality of mitochondria in triggering apoptosis. As shown in Fig. 5A, after 48h of FCCP and 2, 4-DNP challenge, we observed a significant decrease in the percentage of apoptotic cells in RTT compared to controls, indicating a lower susceptibility or a greater resistance to the apoptotic cell death in RTT.

To better elucidate the impaired mechanism that prevents apoptosis in RTT cells, we further investigated the final step of the intrinsic mitochondrial-dependent apoptotic cascade that implicates activation and cleavage of caspase-3/7 [19]. First, we evaluated caspase enzymatic activity using a luminogenic caspase-3/7 substrate assay. As expected, after 48h of stimulation, FCCP treatment induced a significant increase of caspase-3/7 activity in control cells (Fig. 5B). Nevertheless, RTT fibroblasts didn't show activation of caspase-3/7 in response to FCCP apoptotic stimulus (Fig. 5B). Moreover, we also performed an immunoblotting assay to determine the cleavage of caspase-3. As shown in Fig. 5C, significant decrease in the levels of cleaved caspase-3 in RTT fibroblasts compared to control cells confirmed their inability to properly activate apoptosis in response to a specific intrinsic stimulus.

4. Discussion

It is increasingly recognized that mitochondrial dysfunction is a clear pathological hallmark in RTT. Data from RTT animal models and patient specimens highlighted a plethora of different mitochondrial defects ranging from morphological abnormalities to atypical functions [7,26–28]. In our previous work, we reported a characteristic suppressed bioenergetics associated with increased mitochondrial production of oxidants and impaired antioxidant defense system in primary skin fibroblasts isolated from RTT patients [22]. Moreover, more recently, in a large-scale proteomic analysis we also revealed an altered expression of several proteins related to the mitochondrial network along with the first evidence of aberrant mitochondrial dynamics and mitophagy in RTT fibroblasts [8].

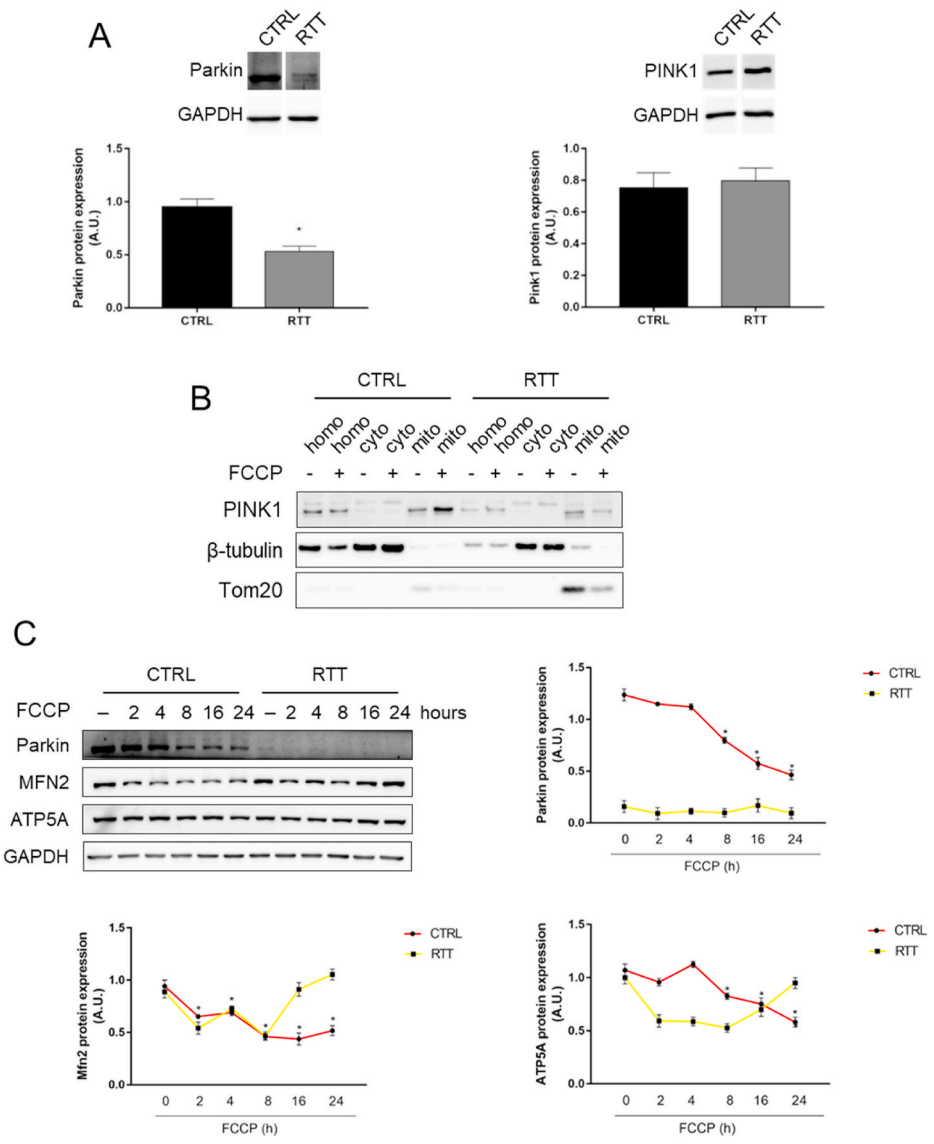

Fig. 2. A. Parkin and PINK1 protein expression in RTT and control fibroblasts. Cells of RTT (n = 3) and control (n = 3) fibroblasts were harvested and protein expression of Parkin and PINK1 was measured by Western blot (upper panels). Quantification of Parkin and PINK1 bands is shown in the histograms. Data are expressed as mean ± SEM. *p = 0,0021. GAPDH was used as a loading control. **B.** PINK1 protein expression on mitochondria of RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were treated with 10 μM FCCP for 16h, harvested and subjected to subcellular fractionation (homo, homogenate; cyto, cytosol; mito, mitochondria) prior to protein expression measurement by Western blot. β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial subfractions, respectively. **C.** Parkin, MFN2, and ATP5A protein levels in RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were treated with 10 μM FCCP for the time points indicated above, harvested and the protein expression was measured by Western blot. Quantification of Parkin, MFN2 and ATP5A bands is depicted in the right and in the bottom panels. Data are represented as mean ± SEM. *p < 0.05. GAPDH was used as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

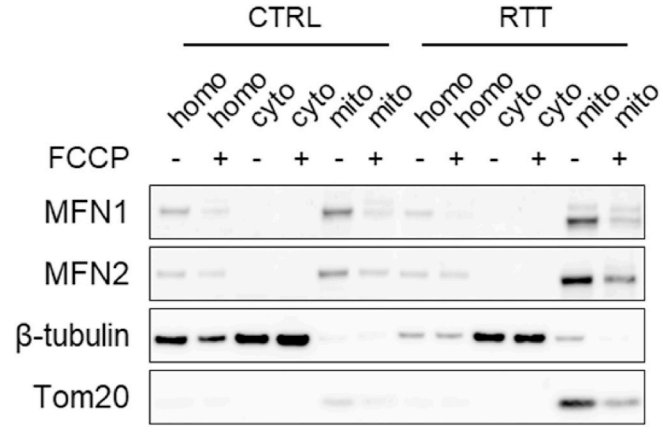

Fig. 3. MFN1 and MFN2 protein expression in mitochondria of RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were treated with 10 μM FCCP for 16h, harvested and subjected to subcellular fractionation (homo, homogenate; cyto, cytosol; mito, mitochondria) prior to protein expression analysis by Western blot. β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial subfractions, respectively.

Taking into account the critical significance of mitochondrial functional state for multiple cellular processes such as energy and ROS production, cell signaling, and cell fate decision, in this study, we wanted to more deeply investigate QC mechanisms, essential to ensure healthy mitochondrial networks, focusing mainly on mitophagy and its relationship with mitochondrial dynamics (i.e. fusion and fission) and apoptotic cell death.

The first approach was to evaluate mitochondrial morphology in our cell model represented by primary fibroblasts isolated from skin biopsies which proved to be a good model to study this pathology [29,30]. Previous studies on *postmortem* muscle and brain specimens from RTT patients revealed several ultrastructural anomalies such as swollen mitochondria with vacuolization, granular inclusions and membranous changes [31–35]. Also Belichenko et al. confirmed these morphological changes in cortical and hippocampal mitochondria of Mecp2$^{-/y}$ mice [36]. Furthermore, our group was also able to show altered organelle morphology in both peripheral blood mononuclear cells (PBMCs) and fibroblasts isolated from RTT patients [8,10]. In line with the results obtained from our and other groups, by electron transmission microscopy we corroborated the evidence of altered morphology in RTT mitochondria characterized by dumbbell-like shape with swollen and disorganized cristae and a predominantly electron dense matrix deposition.

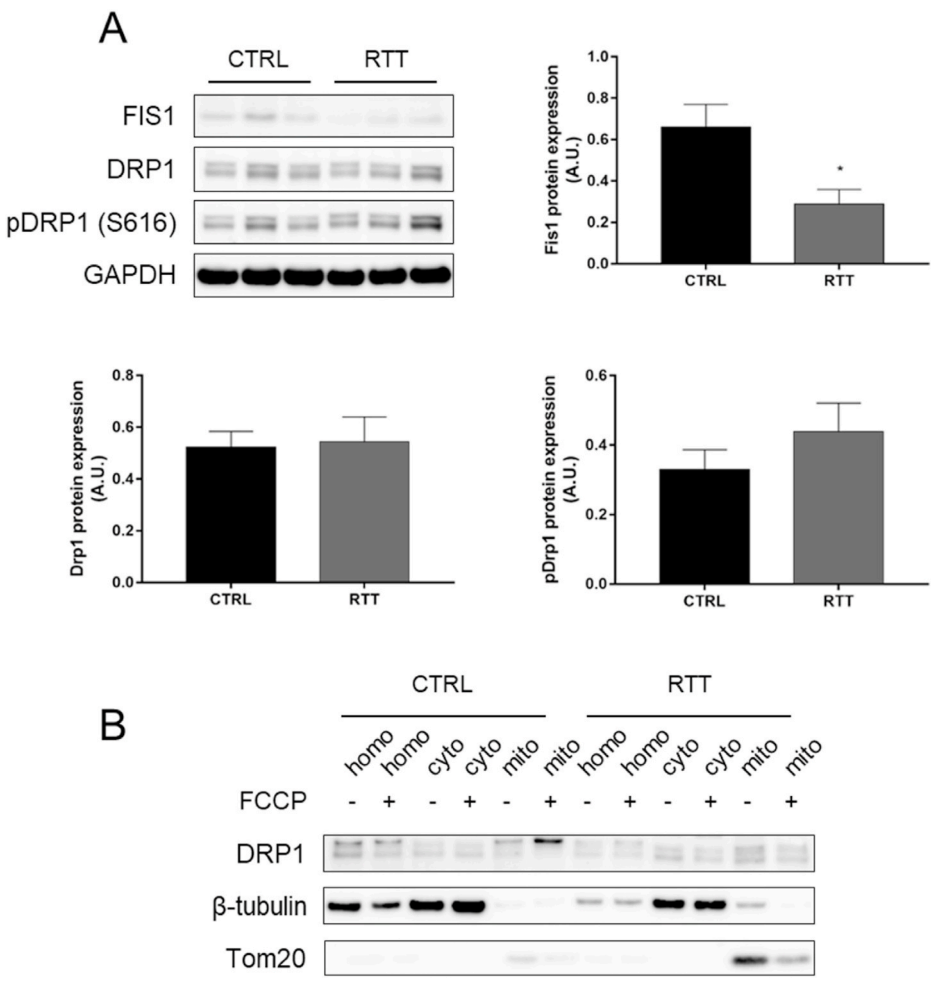

Fig. 4. A. FIS1, DRP1, and pDRP1 protein expression in RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were harvested and the protein expression was measured by Western blot. Quantification of FIS1, DRP1, and pDRP1 is shown in the right and bottom panels. Data are represented as mean ± SEM. *p = 0.0463. GAPDH was used as a loading control. **B.** DRP1 protein expression on mitochondrial subfraction after FCCP treatment. RTT (n = 3) and control (n = 3) fibroblasts were treated with 10 μM FCCP for 16h, harvested and subjected to subcellular fractionation (homo, homogenate; cyto, cytosol; mito, mitochondria) prior to protein expression analysis by Western blot. β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial subfractions, respectively.

Mitochondria are highly dynamic organelles that fuse and divide to form constantly changing tubular networks being modulated by a complex set of cytosolic and mitochondrial proteins which, by orchestrating an intricate system of mitochondrial QC pathways, operate to maintain mitochondria structurally and functionally healthy in response to different stimuli [37]. Interestingly, in our investigation, MitoTracker-stained RTT cells showed the presence of a more filamentous interconnected network of elongated and hyperfused mitochondria compared to control cells in which, instead, typical mitochondrial structures represented by short lines or dots were observable. Moreover, analysis of 3D confocal images also revealed a dramatic decrease in the number of mitochondria in RTT. This specific result could be in part explained by our previous data indicating a decrease in the levels of transcription factors involved in mitochondrial biogenesis including PCG-1α and its downstream target NRF1 (nuclear respiratory factor 1) [22]. Although less in number, RTT mitochondria showed a significant increase in their mean volume that, together with the hyperfused aspect, raises several questions. This data is in line with the recent paper by Bebensee et al. in which the authors observed an increased mitochondrial mass in hippocampal astrocytes of Mecp2$^{-/y}$ mice, [38].

It has been established that mitochondrial function is intrinsically linked to its morphology and changes of organelle volume may strongly modulate mitochondrial physiology in response to various triggers [39]. In particular, mitochondrial QC mechanisms, operating at the molecular, organelle and cellular level in an efficient integrated network, play a major part in regulating mitochondrial shape, number, function and distribution under different physiological and pathological conditions [12,40]. In the context of RTT, multiple lines of evidence have identified mitochondria as one of the most important sources of redox imbalance in the disorder [7,26,27]. Therefore, it is possible that the structural alterations observed here may be a consequence of the damage resulting from an excess of oxidants and, at the same time, an attempt to counteract the altered redox homeostasis. Nevertheless, the molecular events regulating this phenomenon still remain to be elucidated in RTT making mitochondrial QC processes a candidate involved in RTT pathogenesis.

For example, an emerging hypothesis is that swelling may trigger mitophagy, a process through which aged or damaged organelles are degraded *via* the lysosomal pathway [37]. We have previously reported that autophagy, which also works in part as a QC mechanism, is impaired in RTT fibroblasts under nutrient starvation and also that mature red blood cells of RTT patients retain mitochondria [41]. Furthermore, recently, we demonstrated an altered gene expression of key components of the mitochondrial fusion and mitophagy machineries in RTT fibroblasts [8]. In addition, in an imaging assay to qualitatively assess mitophagy dynamics, RTT cells showed a possible defect in the mitophagic flux, as indicated by absent co-localization between mitochondrial and lysosomal dyes after FCCP treatment [8]. However, since no other data are present in literature regarding the exact molecular mechanisms responsible for the impaired mitophagy in RTT, we were interested in investigating this aspect more in depth and in understanding whether hyperfused RTT mitochondria were able to be degraded by this QC process.

Although several mechanisms regulating mitophagy have been described in mammalian cells, the more understood is the PINK1/Parkin-mediated process which, after Parkin recruitment to mitochondria and its activation by PINK1-mediated phosphorylation, implicates

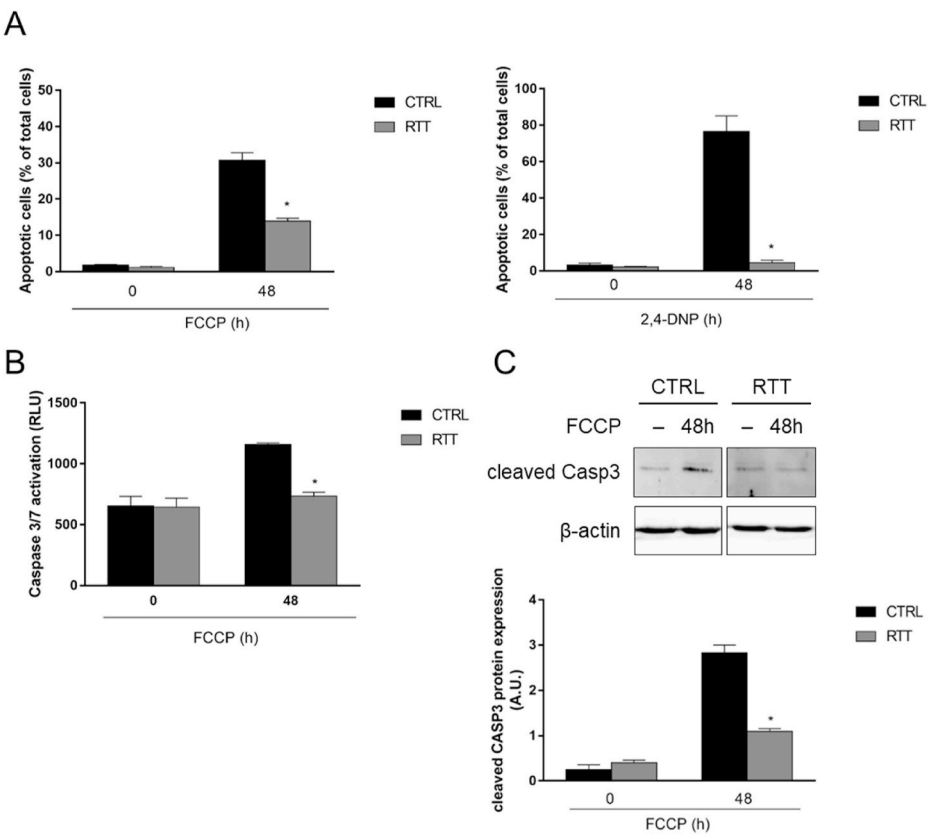

Fig. 5. A. Apoptotic cells in RTT and control fibroblasts after FCCP and 2,4 DNP treatment. RTT (n = 3) and control (n = 3) fibroblasts were treated with 20 μM FCCP and 2 mM 2,4-DNP for 48h, harvested, stained with Annexin V and PI and analysed by flow cytometry. The number of apoptotic cells is reported as percentage (%) of total cells. Data are expressed as mean ± SEM. *p < 0.0001. **B.** Caspase 3/7 enzyme activity in RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were treated with 20 μM FCCP and 2 mM 2,4-DNP for 48h, harvested and the signal of caspase 3/7 was measured by luminescence. Data are expressed as RLU. *p = 0.04. **C.** Cleaved caspase 3 protein expression in RTT and control fibroblasts. RTT (n = 3) and control (n = 3) fibroblasts were treated with 20 μM FCCP and 2 mM 2,4-DNP for 48h, harvested and protein expression of cleaved caspase 3 was measured by Western blot. Quantification of the bands is shown in the bottom panel. Data are expressed as mean ± SEM. *p < 0.0001. β-actin was used as a loading control.

cycles of ubiquitylation of various outer mitochondrial membrane proteins (e.g. mitofusins), triggering autophagosome formation, autophagosome-lysosome fusion and, finally, clearance of the damaged or depolarized organelle [40,42].

In our study, we found a marked decrease of the ubiquitin E3 ligase Parkin protein expression, whereas no differences were found in PINK1 levels in the total protein lysates. Under basal conditions, PINK1 is maintained at very low levels by ubiquitin proteasome degradation, but loss of mitochondrial membrane potential leads to rapid stabilization and accumulation of full-length PINK1 on the damaged mitochondrial membrane [14]. Surprisingly, after FCCP-mediated mitochondrial uncoupling, PINK1 failed to accumulate in RTT mitochondrial subfraction, suggesting a possible defect in the induction of the mitophagic process. This was also confirmed by evaluating the decrease in the levels of mitochondrial proteins such as MFN2 and ATP5A following FCCP insult, a method well accepted to demonstrate a proper mitochondrial removal by mitophagy. In our model, FCCP induced a significant and time-dependent decrease of Parkin, MFN2 and ATP5A in healthy fibroblasts with a maximum loss at 24h after the treatment, indicating an efficient activity of the mitophagic machinery. Interestingly, RTT displayed almost completely absence of Parkin protein and any significant change was appreciable in MFN2 and ATP5A expression after FCCP-induced mitochondrial damage. Altogether, these results are consistent with our previous findings, confirming the inability of RTT mitochondria to be correctly degraded [8]. Moreover, the loss of the PINK1/Parkin-mediated QC pathway can lead to a possible aberrant accumulation of dysfunctional mitochondria, a dysregulated mechanisms that can further exacerbate ROS production and redox imbalance in RTT. On the other hand, mutations of Parkin and PINK1 are mainly associated with Parkinson's disease, a condition also characterized by increased oxidative stress due to the accumulation of impaired mitochondria generating ROS [43].

To better understand the molecular mechanisms underlying the impairment of the mitophagic flux in RTT, we focused our attention on the fusion and fission processes functionality. Indeed, during mitophagy, entire parts of the same organelles are removed, but to be more disposed to segregation and clearance, the damaged mitochondria are first directed towards a fragmented phenotype through a shift of the mitochondrial dynamics towards fission [16,25]. The analysis of fusion process revealed increased levels of MFN1 and MFN2, two main markers of mitochondrial fusion, in RTT mitochondrial subfractions in both basal condition and after FCCP treatment, molecularly explaining the hyperfused morphology of RTT mitochondria. One of the main pathways leading to mitochondrial fission involves the binding of DRP1 to FIS1. Early studies revealed that, when DRP1 activity is inhibited, wild-type mitochondria are transformed into long and interconnected organelles and, conversely, overexpression of DRP1 in cells results in mitochondrial fragmentation [13]. In parallel, FIS1 overexpression in cultured cells results in mitochondrial fragmentation while its depletion leads to elongated mitochondria [13]. Our results showed a marked decrease of FIS1 in the total lysate of RTT fibroblasts, whereas no differences were found in DRP1 and its phosphorylated form pDRP1 (S616), which is known to enhance DRP1-mediated mitochondrial fission activity. However, RTT cells showed the inability to properly recruit DRP1 at mitochondrial surface upon FCCP-mediated mitophagic stimulus. These results suggest that the mitophagic defect observed in RTT could be possibly related to an impairment of the physiological ability to shift between mitochondrial fusion and fission to meet the physiological needs of the cell.

Although essential determinants of cellular survival, paradoxically, mitochondria are also key players in initiating apoptosis which is also controlled by the fusion/fission dynamics [44]. Usually, mitochondrial swelling, as observed in RTT fibroblasts, is one of the fundamental features of pathological states of mitochondria, leading to the final result to activate downstream cascades, mostly life-or-death decisions including apoptosis [40]. During this process, the mitochondrial

network disintegrates, promoting the release of cytochrome *c* prior to caspase activation and leading to more numerous and smaller mitochondria [44]. Since RTT mitochondria displayed an altered morphology (i.e. hyperfusion) together with a defective mitophagy, we tried to understand whether these impairments could affect also its ability in activating apoptosis [12].

Previous paper reported a low susceptibility to apoptosis in lymphoblastoid cell lines as well as in bone marrow mesenchymal stem cells from RTT patients [45,46]. In line with these previous reports, we found that RTT fibroblasts had a lower percentage of apoptotic cells after insult with FCCP. The induction of apoptosis with an alternative synthetic lipid-soluble uncoupler of oxidative phosphorylation, 2,4-DNP, led to the same conclusion, further validating our results. Because the cleavage of caspase 3 represents the final step of the mitochondria-dependent apoptotic cascade, playing an essential role as an executor in apoptosis, we determined whether caspase 3 could be activated under our experimental conditions. Interestingly, using a luminescence-based assay, we found that RTT fibroblasts didn't show any activation of caspase 3/7, both operating at the same level of the caspase cascade. As a proof of concept, we performed an immunoblotting analysis, obtaining the same result and thus confirming that no cleavage of caspase 3 occurred in RTT fibroblasts, suggesting a possible inability of RTT cells to activate this crucial stress response. A recent paper published by Sbardella et al. showed that RTT fibroblasts did not tolerate growing in a starving medium, rapidly undergoing apoptosis [41]. This divergence from our results could be linked to the different stimulus used in our experimental conditions.

In conclusion, we provided first evidence of defective mitochondrial-regulated QC mechanisms such as mitophagy and apoptosis *via* the loss of the physiological interplay between fusion and fission dynamics. It is likely to hypothesize that dysfunctional and damaged mitochondria, remaining within RTT cells, may be detrimental to cellular homeostasis, probably contributing to the vicious circle of oxinflammation already proposed for this disorder. Although further studies are needed to better understand the pathophysiological significance of impaired mitochondrial function in RTT, our study identify new possible targets for the development of novel pharmacological therapies.

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