Astrocytic reactivity triggered by defective mitophagy
activates NF-κB signaling and causes
neurotoxicity in frontotemporal dementia type 3

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ABSTRACT

Background

Frontotemporal dementia type 3 (FTD3) caused by a point mutation in the charged multivesicular body protein 2B (CHMP2B), affects mitochondrial ultrastructure and function as well as endosomal-lysosomal fusion in neurons. However, there is a critical knowledge gap in understanding how mutations in CHMP2B affect astrocytes. Hence, we investigated the disease mechanisms in astrocytes derived from hiPSC with mutations in CHMP2B and their impact on neurons.

Methods

To dissect the astrocyte-specific impact of mutant CHMP2B expression, we generated astrocytes from human induced pluripotent stem cells (hiPSCs) from FTD3 patients and their CRISPR/Cas 9 gene edited isogenic controls and produced heterozygous and homozygous CHMP2B-mutant hiPSC via CRISPR/Cas 9 knock-in gene editing. Additionally, we confirmed our findings in CHMP2B mutant mice. The hiPSC were subjected to astrocyte differentiation and the mutation dependent effects were investigated using immunocytochemistry, western blot, cytokine assays, transmission electron microscopy, RNA-sequencing and gas chromatography-mass spectrometry. Finally, neurons were exposed to conditioned media of mutant astrocytes and viability, growth and motility were measured.

Results

To dissect the astrocyte-specific impact of mutant CHMP2B expression, we generated astrocytes from human induced pluripotent stem cells (hiPSCs) and confirmed our findings in CHMP2B mutant mice. Our findings include perturbed mitochondrial
dynamics with impaired glycolysis, increased reactive oxygen species and elongated mitochondrial morphology, indicating increased mitochondrial fusion in FTD3 astrocytes. Furthermore, we identified a shift in astrocyte homeostasis triggering a reactive astrocyte phenotype and increased release of toxic cytokines. This cumulates in NF-kB pathway activation with increased production of CHF, LCN2 and C3, which cause neurodegeneration. The neurotoxic effect was investigated by exposing hiPSC-derived neurons to astrocyte-conditioned media, which severely reduced neurite outgrowth capacities. Rescue experiments targeting ROS could restore ROS levels back to normal levels, indicating that the impaired removal of abnormal mitochondria triggers the pathological cascade in CHMP2B mutant astrocytes culminating in the formation of neurotoxic reactive astrocytes.

**Conclusion**

Our data provide mechanistic insights into how defective mitophagy causes impaired mitochondrial fission, leading to the adoption of reactive astrocyte properties with increased cytokine release, NFκB activation and elevated expression of neurotoxic proteins in FTD3.

**KEYWORDS**

CHMP2B FTD3, hiPSC-derived astrocytes, autophagy, mitochondria, cytokines.
BACKGROUND

Frontotemporal Dementia linked to chromosome 3 (FTD3) is a rare heterozygous early-onset form of frontotemporal dementia [1]. The disease is caused by a single nucleotide substitution in the gene encoding the charged multivesicular body protein 2B (CHMP2B) resulting in a C-terminal truncated protein [2]. CHMP2B is a central component of the endosomal-sorting complex required for transport-III (ESCRT-III). The mutation is associated with impairment of the endolysosomal pathway, leading to accumulation of early-stage endosomes and culminates in neuronal death and gliosis of the surrounding tissue [3,4].

Astrocytes are one of the most abundant non-neuronal cell types of the central nervous system, displaying remarkable heterogeneity both in morphology and function [5]. Previous research has shown that astrocytes participate in numerous functions such as the formation of neuronal networks, production of neurotrophic factors, recycling of neurotransmitters, and maintenance of homeostasis via detoxification and nutritional distribution [6,7]. Glial cell pathology, manifested in astrocyte reactivity, has been reported for Alzheimer’s disease, Huntington’s disease and amyotrophic lateral sclerosis (ALS) [8,9].

Macro-autophagy (from here on referred to as autophagy) is intracellular degradation of damaged organelles such as mitochondria, protein aggregates and long-lived proteins for subsequent recycling of usable subunits [10]. The autophagy pathway is highly selective and often subdivided based on its engulfed material, where aggrephagy is the degradation of protein aggregates, while mitophagy facilitates the breakdown of damaged mitochondria (reviewed in [11,12]). Several factors such as starvation, pharmacological treatment and stress can upregulate autophagy to facilitate faster
recycling and release of important nutritional factors, to restore cell homeostasis [13].

An important connection between the endolysosomal pathway and autophagy is the fusion between late endosomes and autophagosomes into amphisomes, which further fuse with lysosomes[14]. This part of the process is an important feature in FTD3 pathology, and impairment of the amphisome formation due to ESCRT-III dysfunction has been demonstrated to result in autophagosome and endosome accumulation [15].

Sequestosome 1 (SQSTM1), also known as P62 is a soluble cytosolic protein, incorporating polyubiquitinated cargo into autophagosomes. Hence, P62 accumulation indicates an increase of ubiquitinylated cargo transfer into autophagosomes. It is assumed that P62 is produced at steady rates and degraded exclusively by autophagy [16]. Several studies have provided evidence that P62 expression is increased in metabolic diseases [17] as well as neurodegenerative diseases such as ALS and Frontotemporal lobar degeneration (FTLD) [18]. Our previous findings revealed accumulation of P62 in the S100 β-positive glial cell population amongst hiPSC-derived FTD3 neurons [4], which triggered the current investigation of autophagic alterations in CHMP2B mutant astrocytes and to understand the mechanism contributing to FTD3 pathology.

MATERIALS AND METHODS

Patient Information

In this study we have used, two unedited FTD3 hiPSC lines carrying CHMP2B mutation (referred to as FTD3 patient 1 and FTD3 patient 2 throughout the study), two corresponding CRISPR/Cas 9 gene edited isogenic control hiPSC lines (referred to as Isogenic Control 1, Isogenic Control 2 throughout the study), one heterozygous mutant CHMP2B CRISPR/Cas 9 induced hiPSC line (referred to as heterozygous thoughout
the study), one homozygous mutant CHMP2B CRISPR/Cas 9 induced hiPSC line (referred to as homozygous throughout the study) and one corresponding healthy wildtype control hiPSC line (referred to as wildtype throughout the study). All patients provided written informed consent. The studies were approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-157) institutional review board.

Method Details

Generation of Cell Lines

hiPSCs were generated via episomal reprogramming (Addgene, 27077, 27078, 27080) from human dermal fibroblasts obtained from skin biopsies as described in [4]. The hiPSC lines were maintained on matrigel (BD Matrigel; 15535739, Stem Cell Technologies) in E8 culture media (05991, Stem Cell Technologies). The media was changed daily, and the cells were passaged every 5-7 days using 0.5 mM EDTA (B52, Thermo Fischer) according to the manufacturer’s instructions. All hiPSC lines used in this study were characterized and published earlier in [4,19]. Karyogram for the CRISPR/Cas 9 induced heterozygous and homozygous CHMP2B hiPSC are presented in Additional Figure S6 d-e.

CHMP2B Mice

Snap frozen brains of $CHMP2B^{\text{wild-type}}$ and $CHMP2B^{\text{intron 5}}$ were kindly provided by Adrian M. Isaacs. The mouse model was published earlier in [20]. Mouse brains were dissected and the cerebellum and brainstem were removed. Subsequently, one cerebral hemisphere was used for WB and the other for qPCR.

Neural Induction/ Generation of Neural Progenitor Cells
Neural progenitor cells (NPCs) were generated from each of the hiPSCs by dual SMAD inhibition, using LDN193189, an inhibitor of the BMP pathway (Selleck, S2618) and SB431542, a small molecule inhibitor of the TGFβ pathway (Selleck, S1067) [21]. The neural differentiation was induced via 3D sphere-based method, according to the previously published method [22].

Astrocyte Generation

We generated an efficient astrocyte differentiation protocol based on a modified version previously published [23]. To generate astrocytes, NPCs were first generated via 3D neural induction, where the neuronal rosettes were selected for astrocyte generation. NPCs at passage 6 were then plated at 70% confluence on matrigel in astrocyte differentiation media (ADM) media supplemented with 10 ng/ml of bFGF, 10 ng/ml of Activin A (Thermofisher, PHG9014) & 10 ng/ml Heregulin 1β (HRGβ1) (Peprotech, 100-03), and 200 ng/ml of Insulin Growth Factor-1 (IGF-1) (Peprotech, 100-11) [23]. Astrocyte maturation was achieved by passaging astrocyte progenitor cells (APCs) few times before plating (at least until passage 5, to switch from neurogenesis to gliogenesis). Enriched astrocyte progenitors were plated at a seeding density of 50,000 cells/cm² in astrocyte maturation media (AMM) medium supplemented with 10 ng/ml of Activin A, 10 ng/ml of HRGβ1, 200 ng/ml of IGF1, 1% sodium pyruvate (Thermofischer, 1136070), 10% heat inactivated and toxin free FBS (Invitrogen, 10100147) and 200 μM L-Ascorbic acid 2-phosphate (Sigma, A8960). The medium was changed every other day and cells were differentiated for 35+ days. Media preparation is mentioned in supplement table S7a-c. The efficiency of terminal differentiation astrocytes was monitored for S100β, AQP4, and SOX9 by ICC (Table S1a). In the current study astrocyte progenitor cells were differentiated up to 7 weeks for astrocyte verification;
and 10 weeks for the remaining assays: western blot (WB), transmission electron microscopy, cytokine profiling, RNA-seq, qPCR and metabolic assay.

**Rapamycin Treatment**

To evaluate the rescuing effect of rapamycin on autophagy impairments, cell cultures were treated with rapamycin (R8781, Sigma) before performing the following assays. At 10 weeks of maturation, cell cultures were induced with 200 and 500 nM rapamycin in AMM medium to visualize any concentration dependencies of rapamycin and incubated at 37°C for 24 hours and subsequently fixed for TEM. For ICC, cell cultures were induced with 200 nM and 500 nM of rapamycin at 10 weeks of maturation and incubated for 37°C for 24 hours followed by fixation. Control cell lines were kept in AMM media as reference.

**Immunocytochemistry and Confocal Microscopy**

Cells were cultured on 0.17 μM glass coverslips coated with matrigel. Cell cultures were fixed in 4% paraformaldehyde (PFA) for 20 min, room temperature (RT), washed 3 times with PBS and permeabilized (0.2% Triton X-100 in PBS; 20 min). After blocking for 30 minutes (at RT in 5% donkey serum) the cells were incubated with primary antibodies (antibody details are listed supplement table S4) overnight at 4°C. On the following day, the isotype specific secondary antibodies (detailed in table S4) were applied (1 hour at RT). Samples were washed in PBS and stained with DAPI (Sigma-Aldrich, D9542) to label the nuclei of the cells. Samples were visualized on a confocal microscope equipped with a Leica TCS SPE microsystem controlled by LAS X software (v 2.0.0.14332).
**Puncta Quantification**

Puncta of LAMP1 (small spherical fluorescence points) were counted by analyzing fluorescent images using the Puncta Analyzer plugin of the Image J software. Fifteen different randomly chosen fields from three independent experiments were counted by an independent investigator in a double blinded approach.

**MitoTracker® Assay**

Cells for MitoTracker® Red CMXRos (Molecular Probes, M7512) analyses were cultured on 0.17 μM glass coverslips coated with matrigel. Cells were incubated with 100 nM MitoTracker® in DMEM/F-12 medium for 15 min at 37 °C, followed by fixation in 4% PFA in PBS for 15 min, permeabilization in 0.5% Triton X-100 in PBS for 15 min and incubation with DAPI for 7 min at RT. In between each step the samples were washed 3 times in PBS. After the last wash, the glass coverslips were mounted with mounting media and sealed onto slides. Images were acquired by laser scanning confocal microscopy Leica TCS SPE microsystems controlled by LAS X software (v 2.0.0.14332). The analysis was performed on 10 weeks matured astrocyte samples.

**Quantitative qPCR**

RNA was extracted using RNeasy® Plus Mini Kit (Qiagen, 74134) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA (from the human samples) in 20 μL reaction using iScript™ cDNA synthesis Kit (BIO-RAD, 1708890). After synthesis, the cDNA was diluted four times with double distilled water and were subjected to PCR amplification with primers listed in supplement table S6. Likewise for mouse brain samples, 1 μg of CHMP2B mouse brain RNA from three mice per genotype at 6 months of age was synthesized. Quantitative real-time PCR (qPCR) reactions were
done in triplicates using the FastStart Lightcycler 480 SYBR Green I Master (Roche, 04707516001) on Lightcycler® 480 real-time PCR system (Roche, Switzerland) for both human and mouse.

**RNA- Sequencing Library Preparation**

For RNA sequencing, two unedited FTD3 hiPSC lines carrying CHMP2B mutation (FTD3 patient 1, FTD3 patient 2), two corresponding CRISPR/Cas 9 gene edited isogenic control hiPSC lines (Control 1, Control 2) and one heterozygous mutant CHMP2B CRISPR/Cas 9 induced hiPSC line (heterozygous) were employed for gene expression analysis. The heterozygous astrocytes were used for variant-aware off-target evaluation. The quality of extracted RNA was checked on Agilent 2100 Bioanalyzer system using RNA 6000 nano chip and reagents. All the RNA samples had RIN value > 6. QC passed RNA samples were further used to construct the RNA libraries with TruSeq RNA Library Prep Kit using manufacturer’s protocol. The libraries were quantified using Qubit 3.0 and Agilent 2100 Bioanalyzer system using DNA HS chips. qPCR was performed for precise concentration of the libraries and all the libraries were pooled in equimolar concentrations. The pooled libraries were sequenced on Illumina HiSeq 2500 sequencing platform with 1X100 single end reads.

**RNA-Sequencing Data Analysis and Functional Association of Differentially Expressed Genes**

Transcript reads were pre-processed by trimming low-quality 3’ ends and adapter sequences with cutadapt v1.13 [24] (phred quality threshold=30). Reads shorter than 90 nt were removed and all remaining reads were trimmed at the 3’ end to a common length of 90 nt. Reads matching rRNAs were extracted with BBduk v38.22 [25]
(minimum covered fraction = 0.5, kmer size = 31) based on the annotations available in SILVA v119.1 [26]. Pre-processed reads were mapped to the human genome (hg38) with HISAT2 v2.1.0 [27], resulting in an average of 33,84 M uniquely mapping reads per sample. Alignments were inspected with Samtools v1.9 mpileup function [28] to confirm the presence/absence of the CHMP2B mutation (Chr3:87,253,711 G>C) and of the silent edits introduced by template-directed Cas9 editing (Chr3:87,253,729 A>C, Chr3:87,253,732 T>G). Non-chimeric reads were assigned to genomic features annotated in GENCODE v29 [29] with feature Counts (Subread v1.6.3) [30]. Differential expression analysis was carried out with DESeq2 v1.22.2 [31] by comparing n = 4 patient-derived CHMP2B mutated cell lines (time points 5 and 10 weeks) with their CRISPR/Cas 9 gene-corrected isogenic controls. Time point information was introduced in the DESeq2 formula as batch effect. Genes with Benjamin-Hochberg adjusted P-value (Wald test) <= 0.05, absolute log2 fold change >= 1 and mean of normalized counts >= 10 were considered significantly differentially expressed in the comparison between the two groups.

Functional and compartmental labeling was done for the differentially expressed genes by grouping them based on functional or subcellular compartment annotation downloaded from AmiGO v2 [32] (GO:0000266, GO:0008053, GO:0000422, GO:0019646, GO:0006119, GO:1900016, GO:1900017, GO:0034351, GO:0034352, GO:0090141), Wikipathways [33] (WP3941, WP408, WP4459, WP534), COMPARTMENTS [34] (GO:0005768, GO:0005764, GO:0005739), KEGG [35] (hsa04064, hsa04668), or from manually curated literature [For details refer: Supplement Table s2 & s3]. For subcellular compartment annotation, only genes with a confidence cut-off of at least 3 were considered. Additional functional associations between differentially expressed genes with a confidence cut-off of at least 0.8 were
retrieved from STRING v11 [36] through the StringApp v1.5 [37] and further visualized and annotated in Cytoscape v3.7.1 [38]. Functional enrichment analysis of differentially expressed genes was computed with the StringApp, setting as background all the genes expressed in our samples (mean of normalized counts >= 10). Results were filtered by excluding annotations showing high redundancy with a more statistically significant annotation (overlap between gene sets >= 50%).

Potential off-target loci of the Cas9/gRNA complex with up to 6 mismatches to the designed gRNA target were identified with CRISPRoff v1.1 [39] on a variant-aware version of the hg38 genome. Short variants were discovered from RNA-seq generated for the wild type line (week 5) using the GATK v4.1.4.1 pipeline, following the latest best practices suggested by the authors on the tool's website [40]. In addition to the base filters proposed in the guidelines, the following filters were applied to the variants: read depth > 10, phred-scaled quality of variant assertion > 200. Short variants were applied to the reference genome hg38 with fBCF tools v1.9 [41]. The top 10 predicted off-targets with highest binding potential (CRISPRoff score) were selected for sequencing. To design primers, sequences comprising 200 nt upstream and downstream the 23 nt gRNA+PAM were extracted from the variant-aware genome using BED tools v2.29.2 [42].

**Metabolic Mapping**

The culture medium was removed and the cells were washed with PBS (37°C). Cell cultures were subsequently incubated for 90 min at 37°C in the presence of 2.5 mM [U-\(^{13}\)C] glucose or 2.5 mM [1,2-\(^{13}\)C] acetate plus 2.5 mM unlabeled glucose [43]. The concentrations of substrates were chosen based on previous studies [44]. After the incubation, the medium was collected and the cells were washed with cold PBS (4°C),
lysed and extracted with 70% ethanol. The cells were scraped off the dish and centrifuged at 20,000 g for 20 min (4°C) to separate the soluble extract (supernatant) from the insoluble components (pellet). Cell extracts were lyophilized and reconstituted in water for subsequent biochemical analyses. The cell extracts were separated and metabolites were mapped using a gas chromatograph (Agilent Technologies 7820A chromatograph, J&W GC column HP-5MS, parts no. 19091S-433) coupled to a mass spectrometer (Agilent Technologies, 5977E). The isotopic enrichment was calculated according to [45]. Data are presented as labelling (%) of M+ X, where M is the mass of the unlabeled molecule and X is the number of labeled C-atoms in a given metabolite.

Transmission Electron Microscopy

For transmission electron microscopy (TEM) the cells were fixed at 10 weeks of maturation with 3% glutaraldehyde (Merck, 1042390250) in 0.1 M mNa-phosphate buffer, pH 7.4, post-fixed in 1% osmium tetroxide in 0.1 M Na phosphate buffer, dehydrated stepwise in a graded ethanol series, and embedded in Epon (TAAB, T031). Semi-thin (2 μM) sections were cut with glass knifes on an ultramicrotome (Leica Ultracut, Leica Microsystems, Wetzlar, Germany), stained with 1% toluidine blue O (Millipore, 1159300025) in 1% Borax (LabChem, LC117101). Ultra-thin (50 nm to 70 nm) sections were sectioned with a diamond knife (Jumdi, 2 mm) on an ultramicrotome (Leica Ultracut), contrasted with 2% uranyl acetate (Polyscience, 21447) and lead citrate (Merck, 1073980100), and examined using a Philips CM100 transmission electron microscope operating at 60 kV. Photographs were taken using Olympus Morada 11-megapixel camera and iTEM software (Olympus).

Multiplex Array
Cytokine profiling was performed using the Meso Scale Discovery (MSD) platform. The commercial human-specific U-PLEX Biomarker Group 1 assay (MSD, K15067L-1) was applied. The measurement procedure was performed according to the manufacturer’s protocol. Cytokine concentrations were measured in duplicates using the MESO QUICKPLEX SQ 120 imager (MSD) with the software DISCOVERY WORKBENCH 4.0. Only values above detection level and with a CV value below 25 were included.

Mitochondrial-derived ROS (mROS)

mROS were detected in cells ($5 \times 10^5$) stained at 37°C for 30 min with 5 µM (cell lines) MitoSOX™ Red. After staining, the cells were washed and analyzed at an emission of 580 nm by plate reader. As a precaution, cells were analyzed within 10–20 min of the completion of staining as MitoSOX tends to accumulate in the nucleus after approximately 30 min (according to manufacturer’s instruction). As a positive control to increase the mROS Antimycin A (20 µM) was added to the control wells.

Co-culture with Astrocyte Condition Media

To assess the effects of neurite outgrowth, condition media obtained from FTD3 related astrocytes and controls were cultured on wild type neurons for 5 days, then cells were fixed using 4% PFA for immunocytochemistry. Wild type neurons were kept in neuronal differentiation media as reference. Neurite length was assessed using neurite tracer software of Image J. The analysis was carried out blinded by an independent investigator.

Western Blot
CHMP2B mouse brain tissues were lysed in tPER™ tissue protein extraction reagent (Thermo Fisher Scientific, 78510) and cell from FTD3 astrocytes and controls were lysed in mPER™ mammalian protein extraction reagent (Thermo Fisher Scientific, 78501) containing protease inhibitor (Complete tablets, Roche Diagnostics 04693116001) and phosphatase inhibitors (PhosSTOP tablets, Roche Diagnostics 04906845001). 10 μg of protein were separated by NuPAGE™ Novex™ 4-12 % Bis-Tris mini gel (Thermo Fisher Scientific, NP0322BOX) and transferred from gel to membranes by XCell II™ Blot Module (Invitrogen, EI9051). The membranes were incubated with primary antibodies solution overnight at 4°C (refer details and dilutions in supplement table s5). After washing, the membranes were incubated with conjugated secondary antibodies for 1½ hour at RT. All the secondary antibodies are detailed in supplement table S5. Immunoblots were developed with LI-COR Developer Odyssey® FC Imager with 2 minutes at channel 800 and 2 minutes at channel 700 and data was processed using the Image Studio Lite Version 5.2. Expression levels of LC3B, LAMP1, C3, NF-κB and pNF-kB were normalized to GAPDH. In general, we also looked into caspase 3 activity via WB.

**Neurite Length Analysis**

The cells were plated for neurite outgrowth. At the time of plating, the pre-differentiated neurons (Day 0 of terminal differentiation) appeared spherical in shape with no apparent neurite outgrowth. After 24 hours post-plating, thin neurites began to emerge from the cell bodies of the cells. After 5 days of terminal differentiation in ACM, the neurons were quantified via image analysis as described in [46].

**Quantification and Statistical Analysis**
For all experiments, data are presented as mean ± SEM (standard errors of the mean). Statistical analysis was made in GraphPad Prism 7.03 and determined using Student’s t test; by one-way ANOVA with a Tukey’s post-test or by or two-way ANOVA with Bonferroni correction for differences of mean between each group, as indicated. Puncta quantifications were determined using one-way ANOVA with a Tukey’s post-test and qPCR were determined using student t.test. Metabolic labeling were tested using two-way ANOVA with Tukey multiple comparisons test. Statistic significance was labelled in figures as (*p < 0.05, **p<0.01 and ***p<0.001).

RESULTS

Efficient Generation and Characterization of hiPSC-derived FTD3 Astrocytes

Astrocytes were differentiated from hiPSC lines derived from two related patients with CHMP2B mutation (referred to throught the paper as FTD3 Patient 1 and FTD3 Patient 2), and their corresponding isogenic control, in which the mutation was corrected via CRISPR/Cas9 gene editing (referred to throught the paper as Isogenic control 1 and Isogenic Control 2, respectively). Additionally, two CRISPR/Cas9 knock-in cell lines were generated carrying the same CHMP2B mutation (referred to throught the paper as a homozygous and a heterozygous). All FTD3-hiPSCs and control-hiPSCs were successfully differentiated into mature astrocytes with comparable efficiencies and duration of differentiations following modified, previously established protocols [22,23] (Figure 1a). All astrocyte cultures expressed characteristic markers: transcription factor SOX-9 (SOX9), protein S100-B (S100β) and Aquaporin-4 (AQP4) validated by immunocytochemistry (ICC) (Figure 1b) at 7 weeks of astrocyte differentiation. No obvious difference was observed in the ICC analyses comparing FTD3 astrocyte cultures and their respective controls, indicating that the CHMP2B mutation has no
significant effect on the differentiation capacity towards astrocytes (Figure 1c-e). On average 85% of astrocytes were S100β, AQP4 and SOX9 positive in all investigated hiPSC-derived astrocyte differentiations (Figure. 1c-e, Table S1a). Furthermore, transcriptomic analysis was performed by RNA-sequencing (RNA-seq) on differentiated astrocytes. The results consistently separated FTD3 patient lines and control lines based on their gene expression levels via principal component analysis (Additional Figure S1a). Analysis of differential gene expression between patient and control astrocytes, revealed 1,133 genes with a log2 fold change (LFC) of gene expression ≥ 1; and a significant P-value (<=0.05) after adjusting for multiple testing (Additional Figure S1b).

**hiPSC-derived FTD3 Astrocytes Show Increased and Imbalanced Autophagy**

Impaired autophagy has been widely implicated in the pathogenesis of ALS and FTD3 [47]. Due to its classical function of delivering autophagic cargo, P62 localization is generally considered to correlate with the level of autophagic degradation in combination with LC3B [48]. In order to investigate autophagy alterations in CHMP2B mutant astrocytes, ICC was performed for LC3B and P62 (Additional Figure S2a-c & Table S1f) and P62 (Figure 2a, Table S1b, f). Our results, uncovered high expression of both in FTD3 patients, heterozygous and homozygous astrocytes compared to their respective controls, indicative of impaired autophagy (Figure 2b-c, Additional Figure S2, S3), in accordance with previously published data [49]. The results were quantitatively confirmed via western blot (WB) (Figure 2d-e, Table S1b). Rapamycin, a potent inducer of autophagy [50] was implemented to rescue the autophagic failure in FTD3, heterozygous and homozygous astrocytes at two different concentrations (200 nM and 500 nM). This rescue was unsuccessful and neither changed the abundance of P62 and
LC3B puncta (Additional Figure S2, S3, and S4 & Table S1f) nor abundance of accumulated endosomes and autophagosome (Additional Figure S5).

TEM analyses of FTD3 patients, heterozygous and homozygous astrocytes displayed autophagosomes with increased electron-dense content of engulfed cellular components and organelles, indicating accumulation of un-processed material, reflecting impaired autophagosome fusion with endosomes into amphisomes. In contrary isogenic control lines presented electron-lucent autophagosomes, potentially representing early autophagosomes, and secondary lysosomes (Figure 2g, Additional Figure S4). Consequently, we investigated if this impairment of autophagy mediated clearance is caused by the inability to recruit lysosomes to the amphisomes. Co-labelling for P62 and RAB7 (Figure 2a, middle column) revealed a significant increase in co-localization between RAB7 and P62 in FTD3 patients, heterozygous and homozygous astrocytes compared to their respective controls (Figure 2h-i). These findings confirm an increase in RAB7 recruitment, binding and transport to the autophagosomes. To further examine the potentially defective amphisome-lysosome fusion processes, we performed ICC for P62 and Lysosomal Associated Membrane Protein 1 (LAMP1) [51] (Figure 2a, right column). Co-localization of P62 and LAMP1 demonstrates the ability for amphisomes to fuse with lysosomes to form functional autolysosomes [52]. Image analyses via puncta quantifications revealed a specific increase in LAMP1 co-localization with P62 in FTD3 patients, heterozygous and homozygous astrocytes, compared to their respective controls, indicating efficient and enhanced formation of autolysosomes in the mutated astrocytes (Figure 2j-k), further validated by WB (Figure 2l-m and Table S1b).

To further substantiate the autophagy abnormalities in human FTD3 astrocytes, we analyzed autophagy related genes in brains obtained from a transgenic mouse model
overexpressing human CHMP2B Intron5 mutation (CHMP2B<sup>intron5</sup> mice) [20]. The results showed an increase in the expression of autophagy genes <i>p62</i>, <i>Rab7</i> and <i>Lamp1</i> in CHMP2B<sup>intron5</sup> mice (Figure 2f), resembling the increased autophagy activity in iPSC derived astrocytes.

**FTD3 hiPSC-derived Astrocytes Display Impaired Mitochondrial Dynamics**

Next we investigated if the autophagic defects in our FTD3 astrocytes, leads to accumulation of dysfunctional mitochondria and enhanced production of reactive oxidative species (ROS). Assessment of mitochondrial ROS (mROS) revealed significantly increased levels in homozygous mutant astrocytes compared to control astrocytes, substantiating that mitochondria function and turnover is affected. (Figure 3a). Furthermore, our RNA-seq analyses revealed altered expression levels of genes related to oxidative stress and damage in FTD3 mutant astrocytes vs. controls (Table S2). For instance Thioredoxin Reductase 2 (<i>TXNRD2</i>) (LFC = -2.05, adj.P-value = 1.29E-05), known to scavenge ROS in mitochondria [53] was significantly down-regulated in FTD3 mutant astrocytes. This is in agreement with a recent study reporting that TXNRD2 deficiency causes increased ROS levels, affecting the OXPHOS system and results in reduced ATP availability [54]. Similarly, Mitogen-Activated Protein Kinase 9 (<i>MAP3K9</i>) (LFC = -2.21, adj.P-value = 7.53E-22), an essential component of the MAP kinase signal transduction pathway, was down-regulated in FTD3 mutant astrocytes. MAP3K9 is known to be involved in mitochondrial death signaling pathway, leading to apoptosis [55]. Next, we investigated whether the increased oxidative stress affects cell viability. WB analysis for cleaved caspase 3 (CASP3), did not reveal increased apoptotic activity in FTD3 patient, heterozygous, homozygous astrocytes (Figure 3b). Even though astrocyte survival is not affected at this point our data indicates an impairment
of handling oxidative stress in FTD3 patient, heterozygous, homozygous astrocytes. Oxidative stress has been linked to the formation of stress granules (SGs) [56], and indeed the number of SGs was increased and more scattered in FTD3 patients, heterozygous and homozygous astrocytes, compared to their controls, indicating increased SGs formation as a direct result of impaired autophagy [57] (Figure 3c). Disruption of mitochondrial fission and fusion processes have been implicated in neurodegenerative diseases such as Parkinson’s Disease [58]. Mitochondrial dynamics in astrocytes are far less studied compared to neurons but could play an important role in the FTD3 disease mechanisms. We examined mitochondrial networks using MitoTracker® Red CMXRos, followed by high-resolution morphology studies via TEM of FTD3 patient, heterozygous, homozygous astrocytes. MitoTracker® experiments revealed that FTD3 patient, heterozygous and homozygous astrocytes display an interconnected meshwork of elongated mitochondria compared to their controls, indicative of decreased fission and increased fusion (Figure 3d-e, Table S1c). Our TEM results confirmed excessively elongated mitochondria in FTD3 patients, heterozygous and homozygous astrocytes compared to their controls (Figure 3f, Table S1c). Strikingly, RNA-seq analysis revealed differential expression of genes functionally linked to mitochondrial fission/fusion processes (Table S2). Dynamin 3 (DNM3) a member of the dynamin superfamily was upregulated in FTD3 patient astrocytes (LFC = 1.01, adj. P-value = 1.26E-02, Table S2, validated by quantitative real-time PCR, qPCR, Figure 3g). The dynamin-related GTPase DNM3 controls mitochondrial morphology by recruiting dynamin-related protein 1 (DRP1) and mutation in DNM3 interferes with the mitochondria morphology [59]. In addition BCL2 Interacting Protein 3 (BNIP3), a regulator of mitochondrial fission/fusion dynamics [60], was significantly reduced in FTD3 patient astrocytes (LFC = -1.04, adj. P-value = 6.67E-05, Table S2).
Furthermore, expression of the Kinase insert domain receptor (KDR), whose silencing was shown to increase the mitochondrial membrane permeability [61], was significantly reduced (LFC = -1.09, adj. P-value = 2.59E-02, Table S2) in FTD3 patient astrocytes, further supporting unbalanced fission-fusion events. Next we investigated if key regulators of mitochondrial fission and fusion processes are affected. Overexpression of either optic atrophy 1 (OPA1) or mitofusin 1 (MFN1) induces perinuclear mitochondrial clustering, whereas MFN1 or OPA1 deficiencies lead to severely fragmented mitochondria [62]. Fission protein 1 (FIS1) facilitates recruitment of DRP1 to mitochondria and thereby promoting fission. Whilst overexpression of FIS1 results in extensive mitochondrial fragmentation ablation of FIS1 has the opposite effect and causes mitochondrial elongation [63]. In accordance with the observed mitochondria elongation phenotype we observed increased OPA1 and MFN1 expression levels in FTD3 patient astrocytes and CHMP2B\textsuperscript{intron5} mice brains. A similar trend in the expression level changes of FIS1 was observed in both FTD3 patient astrocytes and in CHMP2B\textsuperscript{intron5} mouse brain (Figure 3h-i). The evidence of mitochondria dysfunction is further supported by our RNA-seq data showing significant down-regulation of additional mitochondrial related genes in FTD3 patient astrocytes (Figure 3j, Table S2). Collectively, our results suggest that the impaired mitochondrial function and antioxidant capacity of CHMP2B astrocytes contribute to both metabolic and oxidative stress.

**Ursodeoxycholic Acid Rescues Reactive Oxygen Species and Mitochondrial Superoxide Levels in hiPSC-derived FTD3 Astrocytes**

Ursodeoxycholic Acid (UDCA) has been proven to rescue apoptotic cascades downstream of autophagic and endosomal perturbations in CHMP2B\textsuperscript{intron5} expressing Drosophila [64]. In addition it has proven to rescue mitochondrial defects in *parkin-
deficient neurons in iPSC derived models [65]. Former studies have revealed that UDCA acts as a translocation inhibitor of BCL2 Associated X, Apoptosis Regulator (BAX) from the cytosol to mitochondria and prevention of ROS formation [66]. Therefore we investigated if UDCA could alleviate pathological CHMP2B driven phenotypes in our homozygous astrocytes. Treatment with 50nM UDCA was sufficient to significantly rescue mROS in homozygous astrocytes compared to its control (Figure 3k, Table S1e), suggesting that UDCA has a rescue effect on oxygen species. However, there was no effect of UDCA on mitochondrial respiration nor on ATP coupled respiration (data not shown). On the contrary when treated with 100nM or 500nM of UDCA, we observed elevated ROS levels causing cell death (data not shown). Overall our data demonstrate that UDCA acts as an antioxidant in CHMP2B astrocytes when applied at low dose concentrations, similar to the effects observed in drosophila CHMP2B^{intron5} models of FTD [64].

hiPSC-FTD3 Astrocytes Display Glucose and Acetate Hypometabolism

The observed functional impairments of mitochondria in FTD3 astrocytes led us to investigate whether these cause metabolic deficiencies. Glucose and acetate oxidative metabolism is closely linked to mitochondria function via the tricarboxylic acid cycle (TCA), which provides high-energy intermediates for the electron transport chain (ETC) leading to ATP production [67]. To provide detailed insights into relevant pathways of glucose and acetate metabolism, we used $^{13}$C-labeled energy substrates and mass spectrometry (GC-MS) analysis to quantify $^{13}$C incorporation in cellular metabolites. Figure 4a-f shows the percentage distribution of $^{13}$C- labeling in fully-labeled (M+3) lactate and alanine arising from direct metabolism of [U-$^{13}$C] glucose (2.5mM) in hiPSC-derived astrocytes. We observed a significant decrease in labeled lactate and alanine
in FTD3 patients, heterozygous and homozygous astrocytes compared to their respective controls (Table S1d). However, the difference in labeled alanine found in FTD3 patient 2 did not reach statistical significance compared to the respective isogenic control 2 (Figure 4e). Interestingly, the $^{13}$C labeling (%) of most of the amino acids and metabolites except for glutamate (FTD3 patient 1, Figure 4g), obtained from a first turn of the TCA cycle in FTD3 astrocytes was significantly lower than their controls, suggesting an overall decreased TCA cycle activity in FTD3 astrocytes (Figure 4g-i & Table S1d). It can be speculated that the increase in labeled glutamate in FTD3 patient 1 compared to the isogenic control 1 may be derived from a small population of metabolically active neurons within the culture that takes up the $^{13}$C-labeled glutamine derived from the astrocytes and synthesizes $^{13}$C-glutamate via glutaminase. In line with our observation of hampered mitochondrial respiration, differential expression analysis based on RNA-seq revealed that numerous genes related to energy production processes such as TCA and ETC are down-regulated in FTD3 patients compared to its control (Table S2, Figure 4m). Specifically, genes of the mitochondrial NADH-ubiquinone oxidoreductase chain family ($MT$-$ND1$, $MT$-$ND2$, and $MT$-$ND3$) and mitochondrial encoded cytochrome C oxidases ($MT$-$CO2$ and $MT$-$CO3$) which are implicated in oxidative phosphorylation were down-regulated in FTD3 patients. This indicates an impaired glucose metabolism resulting in decreased ATP synthesis, which is in line with our observation of hampered mitochondrial respiration.

Acetate has been regarded as an energy substrate preferentially used by astrocytes [68]. To further elucidate alterations in astrocyte metabolism caused by mutations in CHMP2B, hiPSC-derived astrocytes were incubated in medium containing [1, 2-$^{13}$C]acetate (2.5 mM) and unlabeled glucose (2.5mM). In astrocytes, [1, 2-$^{13}$C] acetate (M+2) enters the TCA cycle as acetyl-CoA, which in a first turn of the TCA cycle condenses
with oxaloacetate to form double-labeled citrate (M+2). This TCA cycle intermediate is further metabolized giving rise to α-ketoglutarate, fumarate and malate (M+2). Aspartate (M+2) is formed from oxaloacetate (M+2). The labeled metabolites were determined and summarized in Figure 4j-l (and Table S1d). All metabolites evaluated were decreased in FTD3 patients, heterozygous and homozygous astrocytes compared to respective controls, which supports the observed energy hypometabolism. Reduced expression of MT-ND genes could explain the observed dysfunction of mitochondrial respiratory chain with subsequent reduced ATP production [69]. In addition, we found mitochondrial uncoupling protein 2&3 (UCP2 and UCP3) genes which code for transporter proteins that create proton leaks across the inner mitochondrial membrane, to be down-regulated in FTD3 patient astrocytes. Collectively, these results indicate decreased glucose and acetate metabolism associated to decreased TCA cycle activity in FTD3 astrocytes.

hiPSC-derived FTD3 Astrocytes Demonstrate Increased Reactivity

The observed structural and functional impairment of FTD3 astrocytes could trigger conversion of resting astrocytes to reactive astrocytes contributing to the observed neurodegeneration and gliosis in FTD3 patients [70]. To investigate astrocyte reactivity, we performed ICC using neutrophil gelatinase-associated lipocalin (LCN2) as a marker for reactive astrocytes [71]. LCN2 is a member of the lipocalin family and functions as an iron-trafficking protein, produced by many organs and cells in response to injury [72,73]. Image analyses via puncta quantifications revealed a significant increase in LCN2 expression in FTD3 patients, heterozygous and homozygous astrocytes compared to their controls, indicating increased numbers of reactive astrocytes (Figure 5a, k & Table S1e). In addition, we stained for glial fibrillary acidic protein (GFAP),
extensively used as an astrocyte marker, and additionally used as an indicator of astrocyte reactivity [74,75]. Based on qualitative ICC analysis we observed an increase in GFAP expression in FTD3 patients, heterozygous and homozygous astrocytes compared to their respective control, which further supporting that mutation in CHMP2B trigger astrocyte reactivity (Figure 5a, right-column). Consistent with this, the differential expression analysis based on RNA-seq revealed up-regulation of mitogen-activated protein kinase 1 (MAP3K1) in FTD3 patient astrocytes (LFC = 1.32 adj. P-value = 3.79E-13, Table S2). MAP3K1 is a serine/threonine kinase which activates conserved helix-loop-helix ubiquitous kinase (CHUK) and inhibitor of nuclear factor kappa b kinase subunit beta (IKBKB), triggering phosphorylation and activation of NF-κB [76], which regulates genes implicated in immune responses [77]. Supporting the transcriptome profile, the presence of phosphorylated NF-κB was validated by WB (Figure 5b, d, e Table S1e). Even more intriguing we found a significant upregulation of complement C3 (C3), fibulin 5 (FBLN5) and serpin family G member 1 (SERPING1) in FTD3 patients (Table S2) via RNA-seq analysis. C3 and SERPING1 are regulators of the complement system, important for the innate immune response and mutations in these genes have been implicated to induce toxic reactive astrocytes phenotypes in neurological diseases [78]. As previously reported [79] astrocytes are the main producers of C3 protein in the diseased brain. To validate the RNA-seq expression changes on a protein level, we analyzed astrocyte reactivity via WB. Our results revealed increased protein expression of C3 in FTD3 patients, heterozygous and homozygous astrocytes compared to their controls (Figure 5c, f-g, Table S1e), indicating complement expression and activation of reactive astrocytes phenotype in our CHMP2B disease model. Additionally, we performed WB (Figure 5h, i, l, m) and qPCR analysis (Figure 5j) of CHMP2B intron5 mice
brains, revealing robust increase of C3 and Lcn2 and thereby confirming the astroglial phenotypes both in vivo and in vitro.

**Cytokine Secretion Triggers Reactive Astrocytes to Further Secrete Cytokines Through an Autoregulative Loop**

Cytokines are considered to be effectors of reactivity [80]. Specific cytokines such as interleukin 1 alpha (IL-1α), tumor necrosis factor alpha (TNFα), and complement component 1q subcomponent (C1q) are capable of inducing a reactive phenotype in astrocytes, similar to lipopolysaccharide induced microglia mediated toxic phenotypes [74]. Comparative analyses of secreted cytokines revealed that FTD3 patients, heterozygous and homozygous astrocytes secreted significantly higher levels of inflammatory related proteins IL-6, IL-8, IL-13, TNFα, and IL-2 compared to their respective controls (Figure 5n-p, Additional Figure S6, Table S1e & g). Likewise, we observed a similar pattern of expression for IL6 via qPCR in CHMP2B<sup>intron5</sup> mice brains demonstrating a coordinated immune response [81] (Figure 5j). Additionally, the overrepresentation analysis, based on the RNA-seq showed significant enrichment of differentially expressed genes involved in inflammatory response process (according to GO:0006954, FDR=1.9E-3) in the FTD3 patient astrocytes (Table S3). A number of these genes are engaged in the NF-kB and TNF signaling pathways (KEGG hsa04064 and hsa04668) such as Vascular Cell Adhesion Molecule 1 (VCAM1), CD40 molecule (CD40), Lymphocyte Antigen 96 (LY96), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta (PIK3CD), C-X-C Motif Chemokine Ligand 5 (CXCL5) and Interleukin 6 (IL-6). These data suggest an increased inflammatory response in the FTD3 patient astrocytes, similar to reports in ALS [82]. Functional and pathway enrichment analysis highlighted cytokine activity and cytokine-cytokine receptor
interaction within the most significant enriched terms (Additional Figure S1c). Most notably, apolipoprotein D (APOD) was found to be upregulated in the FTD3 patient astrocytes (LFC = 1.55, adj. P-value = 1.41E-02, Table S2). This gene was previously reported to be upregulated in aging and degenerative brains (reviewed in Dassati, Waldner and Schweigreiter, 2014). Furthermore, we observed a significant up-regulation of Interleukin 17D (IL-17D) (LFC=1.34, adj. P-value=6.45E-4, Table S2), a cytokine reported to increase the expression of IL-8 in NF-κB-dependent manner [84], whilst Signal Regulatory Protein Alpha (SIRP-α) (LFC=-1.73, adj. P-value=3.60E-19), reported as a negative regulator of cytokines production involved in inflammatory response (GO:1900016) was down-regulated. We further observed an upregulation of Proteoglycans (PGs) such as Biglycan (BGN), Syndecan 2 (SDC2), Aggrecan (ACAN) and Lumican (LUM) (Figure 5q). PGs participate in neuroinflammation and were previously linked to neurodegenerative diseases [reviewed in [85]]. Of particular interest is the upregulation of SDC2 (LFC = 1.36, adj.P-value = 8.87E-07) in the FTD3 patients. SDC2 is known to control a large number of cytokines through proteoglycan mediated inflammatory responses [86]. In addition, we observed a strong decrease in the expression of the cystic fibrosis transmembrane conductance regulator (CFTR), (LFC = -6.20, adj. P-value = 2.53E-06) in FTD3 astrocytes (Figure 5q). Mutation in this gene leads to impaired immune cell functions and exaggerated proinflammatory responses in AD [87]. Knockdown of CFTR causes a significant increase in basal secretion of IL-8 as well as in IL-1β-induced secretion of IL-6 and −8 [88] reflected in our dataset. Collectively our findings indicate that conversion of resting astrocytes to reactive astrocytes via mitochondrial deficiencies and NF-κB activation leads to increased pro-inflammatory cytokine release, which triggers an autoregulatory loop reinforcing the
toxic reactive astrocyte phenotype and further enhances cytokine production that poses a toxic threat to neurons.

**Reactive Astrocyte Media inhibit Axonal Outgrowth**

Previous research has demonstrated that secreted factors from reactive astrocytes negatively impact neurite outgrowth rates [89]. To investigate the toxic effect of reactive astrocytes on neurons, we cultured healthy neurons with astrocyte conditioned medium (ACM) from FTD3 astrocytes and control astrocytes. A significant decrease (p<0.05) in neurite length was observed in neurons cultured with FTD3 ACM compared to control ACM (Figure 5r-s). This demonstrates the inhibiting impact on neuronal outgrowth via secreted factors from FTD3 astrocytes. Moreover, we analyzed the expression levels of genes associated with the positive and negative regulation of the glial cell apoptotic process according to GO: 0034352 and GO: 0034351 via RNA-seq analysis. We observe up-regulation of A-Kinase Anchoring Protein 12 (AKAP12) and down-regulation of Growth Arrest Specific 6 (GAS6) in FTD3 patient astrocytes (LFC = 1.40 and -1.20; adj. P-value = 9.00E-18 and 1.42E-05 respectively). AKAP12 is involved in oligodendrocyte apoptosis and in the production of TNF-α as well as nitric oxide in astrocytes with inflammatory stimulation [90]. GAS6 promotes cell survival in the brain [91,92]. These results further underline the overall toxic effect of astrocytes in FTD3 patients and their contribution to neuron apoptosis (Table S2), reflected in the pathology of cortical neurodegeneration.

**DISCUSSION**

It has previously been reported that excess accumulation of autophagosomes has a negative impact on neuronal survival, and dysfunctional ESCRT-III appears to cause
neurodegeneration through numerous mechanisms [93]. However, the exact contribution of autophagy to neurodegeneration is largely unknown. In this study, we report that astrocytes generated from FTD3 patient hiPSC or with introduced CHMP2B mutation, display interrupted amphisomes-lysosomal fusion degradation. In relation to this, we found an increased P62 expression in our CHMP2B mutant astrocytes, as previously observed in our glial population, which is a key sign of increased/impaired autophagy [4]. The ICC results, together with the accumulation of cargo filled vesicles visualized with TEM, are reliable indications that the autophagy process is affected and that FTD3 astrocytes are unsuccessfully attempting to degrade large amount of material in diseased conditions. These results were confirmed in the CHMP2B<sup>intron5</sup> mice model [94].

Defective recycling of organelles is the earliest impairment event, which clearly links autophagy to mitochondria turnover [95]. Abnormal mitochondrial function and aberrant morphology was previously reported by our group in hiPSC-derived FTD3 neurons [4]. Consistent with this, we here present mitochondrial dysfunctions with hypometabolism, increased mitochondrial fusion and release of ROS as important components of FTD3 astrocyte pathology. Increased mitochondrial fusion is an important mechanism in metabolically active cells in order to provide a mitochondrial network, which can sustain and accommodate the need for large amount of energy [94]. In quiescent cells, where the energy demand is less, the mitochondria often appear smaller and spherical due to increased fission activity [96]. Since the CHMP2B astrocytes demonstrate hypometabolism with decreases glycolysis and TCA cycle activity, we propose an increased fusion of functional and damaged mitochondria as a regulatory mitochondrial attempt to accommodate the energy demand in the cells. This could explain the increased and elongated network of mitochondria evident in the mutant astrocytes,
despite the lack of energy production [94]. Furthermore, it has been reported that oxidative stress induces autophagy impairments, driving the accumulation of dysfunctional mitochondria [97]. Similarly, we showed that oxidative stress causes defects in the autophagy-mitophagy pathway, as it directly connects cellular clearance mechanisms with mitochondrial function. Our TEM findings suggests that mitochondrial dynamics are altered in FTD3 patient, heterozygous and homozygous astrocytes in order to compensate the functional deficit. We suggest that autophagy imbalances are increased by an accumulation of dysfunctional mitochondria. This result together with increased ROS levels consequently affect mitophagy, resulting in further accumulation of damaged organelles.

Reactive astrocytes of toxic A1 type promote the loss of beneficial astrocytic properties such as outgrowth, synaptogenesis and phagocytosis, thereby resulting in neuronal cell death [74]. Strikingly, our study also reveals that FTD3 astrocytes significantly upregulate reactive A1 toxic astrocyte genes SERPING1 and FBLN5. Astrocyte expressing SERPING1 may lead to recruitment of large numbers of T-lymphocytes, exacerbating neuroinflammation via complement cascade in the brain [98]. In addition to the up-regulation of A1 genes, we also observed an up-regulation of A2 genes in our astrocytes cultures thereby indicating additional signals may be present which likely increases the expression of A1 and A2 genes. Nowadays glial cell reactivity is increasingly connected to neurodegenerative diseases [75], especially in AD an up-regulation of astrocytic pro-inflammatory cytokines such as TNFα and IL-1β has been shown to cause astrocyte reactivity [99]. Likewise, in ALS increased levels of TGF-β1, produced by SOD1 G93A reactive astrocytes, induces cytoplasmic aggregation and impaired autophagy [100]. Here we showed that mutations in CHMP2B causing FTD3 contribute to astrocyte reactivity by up-regulation of LCN2. These findings are in line
with a recent study investigating the role of reactive astrocytes in ALS [100]. Supporting our findings, studies suggest that increased levels of LCN2 were detected in the cerebrospinal fluid of patients with AD and in the frontal cortex of patients with FTLD, correlating with reactive astrocyte pathology [72,101]. These results point to a potential dynamic regulation of LCN2 in various neurodegeneration. In accordance, we observed altered cytokine profile in patient astrocytes compared to controls, thus proving that the pro-inflammatory response is related to FTD3 pathology. The results are consistent with previous findings where increases in IL-6 and IL-8 in the brain tissues were associated with poor cognitive performance [102]. It should be noted that these cytokines when secreted by activated microglia could induce the formation of A1 astrocytes, which causes a decrease in neuronal neurite length[103]; However, that is not the case with our cultures system, we hypothesize that an autoregulatory cytokine loop triggers the A1 phenotype in CHMP2B astrocytes thereby changing the normal homeostasis of the system by NF-kB activation. Finally, we investigated the presence of astrocyte reactivity in CHMP2B mutant mice brains. Our findings revealed complement protein C3 as an astroglial target of NFkB to be upregulated in CHMP2B^{intron5} mice, resulting in disruption of NFkB signaling pathway [104]. Strikingly our results provide strong support that C3 is a critical mediator of NFkB activity in astroglia. In addition we made an attempt to rescue mitochondrial ROS levels in CHMP2B mutated astrocytes via UDCA as documented in [65]. We were able to show that UCDA reduced the mitochondrial ROS levels but did not rescue mitochondrial phenotypes in CHMP2B mutant astrocytes. Intriguingly, we observed throughout our study a more profound phenotype in the heterozygote compared to the homozygous. Consequently, we carefully investigated the top 10 off-target loci for the sgRNA employed in this study using CRISPRoff [39].
No off-target effects could be detected (Additional Figure S5), indicating a dominant negative effect of the heterozygous CHMP2B mutation [20].

In summary, our findings reveal that CHMP2B astrocytes have perturbed autophagy with accumulation of autophagosomes independent of rapamycin treatment. The impairment of autophagy leads to accumulation of dysfunctional mitochondria with impaired glycolysis, increased ROS and elongated mitochondrial morphology. This indicates discordance between the fission and fusion processes with increased fusion in FTD3 astrocytes. This shift in astrocyte homeostasis results in a switch to a reactive astrocyte phenotype with subsequent increased release of neurotoxic cytokines. This phenotype is restricted to cells with mutant CHMP2B and absent in controls, leading to NF-kB pathway activation with increased production of GFAP, LCN2 and C3, implicated in neurodegeneration. Finally, we were able to validate that the reactive astrocyte phenotype is present in vivo and in vitro and correlated to improper mitophagy (proposed in Figure 6). In a nutshell our data provides mechanistic insights into how defective mitophagy causes impaired fission, leading to the adoption of reactive astrocyte properties with increased cytokine release, NF-kB activation and elevated expression of neurotoxic proteins, indicating how CHMP2B mutant astrocytes contribute to neurodegeneration and disease progression in FTD3.

CONCLUSION

Taken together, our data clearly links autophagic deficiencies to dysfunctional mitochondria exhibiting oxidative stress and induction of astrocyte reactivity affecting neurite outgrowth and survival in FTD3 hiPSCs-derived astrocyte models. This study reveals the unique role of astrocytes in neurodegeneration caused by CHMP2B mutations in FTD3.
LIMITATION OF THE STUDY

The aim of this study was to investigate the role of astrocytes in FTD3 disease pathology. It is well acknowledged in the field of disease modeling using hiPSC that the number of lines derived from individuals is a limiting factor. We have included two patient hiPSC, their respective CRISPR/Cas9 isogenic controls as well as one control hiPSC line with introduced mutation (a heterozygous and a homozygous). This allows for statistical relevant analyses of the data and identification of potential disease contributing factors besides the CHMP2B mutation. Nevertheless, additional hiPSC would allow for increased confidence. Furthermore, we have validated key *in vitro* data in the transgenic mouse model, which allows us to exclude cell culture effects to some extent and indicates the relevance of our astrocyte pathology in an *in vivo* setting. Ideally, patient data would be added to further support our findings, but due to the limitations in accessing those we can only indicate that overall gliosis is present in FTD3 patients. More complex co-culture systems such as brain organoids and co-cultures with microglia will be implemented in the future to validate our findings.

LIST OF ABBREVIATIONS

A, Human Activin A; AA, L-Ascorbic Acid; ACM, Astrocyte conditioned media; AD, Alzheimer’s disease; ADM, Astrocyte Differentiation Media; Adj, adjusted; ALS, Amyotrophic Lateral Sclerosis; AMM, Astrocyte Maturation Media; APC, Astrocyte Progenitor Cell; AQP4, Aquaporin 4; bFGF, Basic Fibroblast Growth Factor; CHMP2B, Charged Multivesicular Body Protein 2B; CNS, Central Nervous System; D, Day; DE, Differentially Expressed; DMSO, Dimethyl Sulfoxide; EB, Embryonic Bodies; EGF, Epidermal Growth Factor; ESCRT-III, Endosome Sorting Complex required for
Transport-III; FDR, False discovery rate; FTD3, Frontotemporal Dementia linked to Chromosome 3; FTLD, Frontotemporal Lobar degeneration; GDH, Glutamate Dehydrogenase; GFAP, Glial Fibrillary Acidic Protein; GO, Gene Ontology; HRGβ1, Recombinant Human Heregulin β1; hiPSC, Human Induced Pluripotent Stem Cell; ICC, Immunocytochemistry; IGF1, Recombinant Human Insulin-like Growth Factor-1; iPSC, induced Pluripotent Stem Cell; LFC, Logarithm Fold Change (base 2); MPR, Mannose 6 Phosphate Receptor; ND, Neurodegenerative Disease; NEP, Neuroepithelial Cells; NIM, Neural Induction Media; NMM, Neural Maintenance Media; NPC, Neural Progenitor Cell; PC, Principal Component; PFA, Paraformaldehyde; PSC, Pluripotent Stem Cells; RT, Room Temperature; RT-PCR, Reverse Transcription Polymerase chain Reaction; SEM, Standard Error of the Mean; SG, Stress Granules; TCA, Tricarboxylic Acid; TEM, Transmission Electron Microscopy; WB, Western Blot.

DECLARATIONS

Ethics approval and consent to participate
Written informed consent had been obtained from the subjects who provided their samples for iPSC derivation. The Ethics Committee of the Capital Region of Denmark (H-4-2011-157) approved the study, and written informed consent was obtained from each participant before enrollment.

Consent for publication
Not applicable

Lead Contact
Further information and requests for resources and reagents should be directed to Kristine Freude (kkf@sund.ku.dk).

Availability of data
The dataset generated for RNA-seq have been deposited in the NCBI GEO database under the accession number GEO: GSE141388. All other data generated in this study are included in this published article.

Availability of materials
The CRISPR/Cas9 introduced iPSC CHMP2B lines generated in this study will be made available on request through Material Transfer Agreement. The patient derived hiPSC cannot be shared due to specific regional restrictions implemented on patient material by Region Hovedstaden, Denmark.

Competing interests
The authors declare that they are no competing interests.

Funding
This work was supported by awards from: Independent Research Fund Denmark (FTP, grant NO. 109799) (KF), Innovation Fund Denmark (BrainStem, 4108-00008B & NeuroStem, 4096-00001B) (KF), Alzheimer Foundation Denmark (KF), Novo Nordisk Foundation (GliAD—NNF18OC0052369 & NNF19OC0058399) (KF). (AD) have been supported by EUH2020 JPCO-Fund call for Personalized Medicine JPND2019-466-037 / 2019-2.1.7-ERA-NET-2020-00007 (ADAIR).
Acknowledgement

We are grateful to Dr. Adrian M. Isaacs for providing CHMP2B\textsuperscript{intron5} and control mouse brain samples. Tina Christoffersen, Irina Vardia and Zehra Abay Norgaard from the University of Copenhagen for expert technical assistance. The authors are grateful to Philip Seymour for proof-reading the paper.

Author's Contribution

A.C., K.K.F., performed experimental design. A.C., generated hiPSC derived astrocytes and A.C., K.S.D performed most of the experiments. B.I.A., C.A., performed metabolic profiling. A.C., K.S.D. and K.K.F. interpreted the results and wrote the manuscript. G.I.C., N.T.D., analyzed RNA-seq data and made gene network analysis under supervision of J.G. A.C., S.A., were involved in the analyses of RNA-seq data. SC helped in the design, performance and data analysis of qPCR experiment. M.P., prepared samples for TEM, H.H., analyzed TEM samples. S.I.S and M.M, performed cytokine assays. J.K., P.H., A.D., edited and approved the paper. All authors read and approved the final version of the paper.

FOOTNOTES N/A

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17  FIGURES AND FIGURE LEGENDS
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19  Figure 1: Generation and Characterization Of hiPSC-derived Astrocytes. a)
20  Schematic of astrocyte differentiation protocol. b) Representative ICC images of AQP4,
21  S100 β and SOX9. Scale bar= 25 μm. c-e) Quantifications of AQP4, S100 β and SOX9
22  via ICC. Data reported as mean ± SEM, (one-way ANOVA, Tukey multiple comparisons
23  test; *p<0.05). N=3, n=3, N indicates the number of experimental repetitions; n indicates
24  the number of technical replicates per cell line.
Figure 2: FTD3 Astrocytes Display Autophagy Changes. a) Representative images of LC3B and S100β co-labelling of astrocytes (left column), P62 and RAB7 co-labelling of astrocytes (middle column) and P62 and LAMP1 co-labelling of astrocytes (right column) Scale bar= 25 µm. b,c) LC3B quantification. d-e) Western blot of LC3B. f) Validation of CHMP2B intron5 mice vs. Control mice brain for Autophagy. g) Qualitative ultrastructural visualization of FTD3-specific accumulation of electron-dense autophagosomes by TEM indicated with black arrows. (A= autophagosomes and L= autolysosomes h-i) Puncta quantifications of P62 and RAB7 colocalization. j-k) Puncta quantifications of P62 and LAMP1 colocalization l-m) Western blot of LAMP1. Data reported as mean ± SEM, (one-way ANOVA, Tukey multiple comparisons test; *p<0.05, **p <0.01 and ***p <0.001, N=3, n=3). N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line. See also Additional Figure S2, S3, S4.

Figure 3: FTD3 Astrocytes Displays Impaired Mitochondrial Dynamics a) MitoSOX assay b) Caspase 3 activity, GAPDH as control. c) Representative T1A1 labelling, Scale bar= 25 µm. d) Representative mitochondrial distribution, Scale bar= 25 µm. e) Running average length of mitochondria. f) Qualitative analysis of mitochondria visualized with TEM. Mitochondria are marked by black arrows. g) RNA-seq validated by qPCR h-i) qPCR determining the fission and fusion genes (OPA1, MFN1, and FIS1). j) Top 20 significantly differentially expressed genes associated with mitochondria. k) Rescue of mitochondrial function by treatment with 50 nM UDCA for 24 h. Data are reported as mean ± SEM. Statistics for RNAseq used Benjamin-Hochberg adjusted P-value (Wald test) <= 0.05, absolute log2 fold change >= 1 and mean of normalized counts >= 10, qPCR used unpaired student t-test and for remaining analyses one-way ANOVA, Tukey
multiple comparisons test, (*p<0.05, **p <0.01 and ***p <0.001). Significant differences are indicated by *p< 0.05). N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line. See also Additional Figure S1.

Figure 4: Energy Hypometabolism in FTD3 Astrocytes. a-i) Glucose metabolism: 13C-enrichment in metabolites was determined via GC-MS after 90-min incubation with [U-13C] glucose. j-l) Acetate metabolism. m) Networks of down-regulated genes involved in energy-production pathways. Node colors represent expression fold changes in a blue-white-red logarithmic scale. Results are reported as mean ± SEM, (two-way ANOVA with Tukey multiple comparisons test; *p<0.05, **p <0.01). N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line. See also Additional Figure S1.

Figure 5: FTD3 Pathology Contributes To Reactive Astrocyte. a) Representative images of LCN2 and S100 β co-labelling of astrocytes (left column) and GFAP labelling of astrocytes (right column). Scale bar= 25 µm. b, d, e) Western blot of Nf-KB and pNf-KB. c, f, g) Western blot of C3 (human). h, i) Western blot of C3 for CHMP2B\textsuperscript{intron5} mice brain I, m) Western blot of Gfap (mouse). j) Validation of CHMP2B\textsuperscript{intron5} mice brain for reactive astrocytes. k) LCN2 puncta quantification. n-p) Cytokine profiling. q) Top 20 significantly differentially expressed genes associated with lysosomes (left) and endosomes (right). r) Representative image of neurite outgrowth. s) Data analysis of neurite outgrowth. Data are reported as mean ± SEM, (one-way ANOVA, Tukey multiple comparisons test; *p<0.05, **p <0.01 and ***p <0.001). N=3, n=3, N indicates the
number of experimental repetitions; n indicates the number of technical replicates per cell line. See also Additional Figure S1, S6.

**Figure 6: Proposed model: Role of Astrocytes in FTD3 pathology**

Mutant CHMP2B impairs normal mitophagy, leading to accumulation of elongated and dysfunctional mitochondria triggering oxidative stress. These cascades of events culminate in conversion of resting astrocytes to reactive astrocytes releasing neurotoxic cytokines, contributing to neuronal dysfunction and apoptosis in FTD3.
### Table S1a. Statistics Data (Related to Figure 2)

| Cell line          | (Mean ± SEM)(%) |
|--------------------|-----------------|
|                    |                 |
| **SOX9 (ICC)**     |                 |
| FTD3 patient 1     | 91.75±0.8764    |
| Isogenic control 1 | 92.57±0.6373    |
| FTD3 patient 2     | 86.69±2.394     |
| Isogenic control 2 | 93.33±4.216     |
| Homozygous         | 88.14±4.707     |
| Heterozygous       | 88.21±1.267     |
| Wildtype           | 91.33±2.657     |
| **AQP4 (ICC)**     |                 |
| FTD3 patient 1     | 88.67±6.418     |
| Isogenic control 1 | 94.73±0.1618    |
| FTD3 patient 2     | 94.07±3.229     |
| Isogenic control 2 | 97.5±2.5        |
| Homozygous         | 96.43±3.573     |
| Heterozygous       | 95.00±0.00      |
| Wildtype           | 100.00±0.00     |
| **S100β (ICC)**    |                 |
| FTD3 patient 1     | 75.03±3.746     |
| Isogenic control 1 | 86.22±4.288     |
| FTD3 patient 2     | 86.74±2.649     |
| Isogenic control 2 | 97.22±2.778     |
| Homozygous         | 91.03±3.179     |
| Heterozygous       | 100.00±0.00     |
| Wildtype           | 100.00±0.00     |

Significant differences are indicated by *p < 0.05.
| Table S1b. Statistics Data (Related to Figure 3) |
|-----------------------------------------------|
| **Cell line** | **Control (Mean ± SEM)** | **200nM rapamycin (Mean ± SEM)** | **500nM rapamycin (Mean ± SEM)** |
| FTD3 patient 1 | 14,4±2,668* | 20,7±3,62** | 20,57±3,41** |
| Isogenic control 1 | 7,25±1,11 | 4,74±0,43 | 4,74±0,27 |
| FTD3 patient 2 | 10,21±1,714** | 8,54±1,09** | 5,52±0,74* |
| Isogenic control 2 | 2,53±0,08 | 4,08±0,27 | 3,71±0,14 |
| Homozygous | 74,06±10,26** | 82,18±7,82** | 63,45±5,46** |
| Heterozygous | 28,73±3,59 | 16,47±2,24** | 13,77±1,75** |
| Wildtype | 18,92±6,67 | 9,93±1,95 | 9,77±1,74 |
| FTD3 patient (pooled) | 0,1005±0,0034**** | Na | Na |
| Isogenic control (pooled) | 0,423±0,0339 | Na | Na |
| Introduced mutations (pooled) | 0,201±0,0137*** | Na | Na |
| Wildtype | 0,945±0,2107 | Na | Na |
| FTD3 patient 1 | 482,3±71,26** | 949,5±109,1** | 725,4±97,29** |
| Isogenic control 1 | 38,6±8,48 | 78,8±14,98 | 69,8±18,19 |
| FTD3 patient 2 | 471,2±86,63** | 530,6±77,07** | 313,8±84,61** |
| Isogenic control 2 | 36,1±8,4 | 134,3±36,41 | 59,2±10,31 |
| Homozygous | 1227±222,4** | 1660±163** | 1190±114,1** |
| Heterozygous | 1199±150,9** | 1283±157,6** | 1395±165,4** |
| Wildtype | 310,4±50,9 | 355,9±59,6 | 381,9±56,19 |
| FTD3 patient 1 | 368,7±40,2** | 321,3±32,83** | 273,1±31,3** |
| Isogenic control 1 | 81,8±35,81 | 67±30,66 | 8,3±1,95 |
| FTD3 patient 2 | 637,2±128** | 732±106,5* | 848,6±113,2** |
| Isogenic control 2 | 192,7±60,59 | 376,7±75,58 | 227,1±34,46 |
| Homozygous | 588,9±140,5 | 413,3±87,32 | 244,6±64,41** |
| Heterozygous | 781,2±126,4 | 915,4±143,4** | 885,5±106 |
| Wildtype | 497,5±99,51 | 512,8±70,41 | 675,6±102,5 |
| LAMP1 (Based on WB) | 2,596±0,0286 | Na | Na |
| Isogenic control (pooled) | 0,938±0,1166 | Na | Na |
|                     | Introduced mutations (pooled) | Na  | Na  |
|---------------------|-------------------------------|-----|-----|
| Wildtype            | 0,506±0,4918                  | Na  | Na  |
| **P62 (Mouse)**     | Control mouse 1,000±0,0306 Na  | Na  | Na  |
|                     | CHMP2B mouse 1,487±0,183 Na   | Na  | Na  |
| **Rab7 (Mouse)**    | Control mouse 1,000±0,0103 Na  | Na  | Na  |
|                     | CHMP2B mouse 1,323±0,1200 Na   | Na  | Na  |
| **Lamp1 (Mouse)**   | Control mouse 1,000±0,0474 Na  |     |     |
|                     | CHMP2B mouse 1,338±0,1477 Na   |     |     |

Significant differences are indicated by *p < 0.05, **p <0.01 and ***p <0.001.
### Table S1c. Statistics Data (Related to Figure 4)

| Cell line   | (Mean ± SEM) |    |
|-------------|--------------|----|
| ROS         | Homozygous   | 87332±847* |
|             | Wildtype     | 78406±1994 |
| Mitotracker | FTD3 patient 1 | 42,56±1,605** |
|             | Isogenic control 1 | 28,46±0,8091 |
|             | FTD3 patient 2 | 47,91±2,601** |
|             | Isogenic control 2 | 33,2±1,016 |
|             | Homozygous   | 66,05±2,44** |
|             | Heterozygous | 42,51±2,008** |
|             | Wildtype     | 39,28±1,351 |
| RT-qPCR (OPA1) | Homozygous   | 1,37±0,109 |
|             | Heterozygous | 2,15±0,173* |
|             | Wildtype     | 1±0,294 |
| RT-qPCR (MFN1) | Homozygous   | 3,32±0,252* |
|             | Heterozygous | 1,219±0,075 |
|             | Wildtype     | 1±0,032 |
| RT-qPCR (FIS1) | Homozygous   | 14,21±0,751* |
|             | Heterozygous | 6,37±0,828* |
|             | Wildtype     | 1±0,150 |
| RT-qPCR (mOPA1) | Control mouse | 0,83±0,114 |
|             | CHMP2B mouse | 1,09±0,072 |
| RT-qPCR (mMFN1) | Control mouse | 0,189±0,042 |
|             | CHMP2B mouse | 0,39±0,0931 |
| RT-qPCR (mFIS1) | Control mouse | 0,165±0,002 |
|             | CHMP2B mouse | 0,181±0,0074 |

Significant differences are indicated by *p < 0.05 and **p <0.01.
Table S1d. Statistics Data (Relative to Figure 5)

| Metabolic Labeling | Cell line              | (Mean±SEM) |
|--------------------|------------------------|------------|
|                    | FTD3 patient 1         | 14,0±1,4**** |
|                    | Isogenic control 1      | 29,2±1,74  |
|                    | FTD3 patient 2         | 11,3±3,4**** |
| Lactate            | Isogenic control 2      | 51,6±2,7   |
|                    | Homozygous             | 21,6±4,2*  |
|                    | Heterozygous           | 24,8±5,0*  |
|                    | Wildtype               | 47,3±4,4   |
|                    | FTD3 patient 1         | 5,3±1,7***  |
|                    | Isogenic control 1      | 15,4±0,3   |
|                    | FTD3 patient 2         | 9,6±3,1    |
| Alanine            | Isogenic control 2      | 14,3±0,1   |
|                    | Homozygous             | 15,4±2,9** |
|                    | Heterozygous           | 11,5±0,6*  |
|                    | Wildtype               | 27,3±2,9   |

Significant differences are indicated by *p < 0.05, **p <0.01, ***p <0.001 and ****p <0.0001.
| Cell line | (Mean±SEM) |
|-----------|------------|
| LCN2      |            |
| FTD3 patient 1 | 1666±523,5** |
| Isogenic control 1 | 96,8±16,54   |
| FTD3 patient 2 | 856,1±316,7* |
| Isogenic control 2 | 92±22,74     |
| Homozygous | 1405±345,9** |
| Heterozygous | 1613±548,7*  |
| Wildtype   | 89,38±46,87 |

| C3 (Based on WB) |             |
|------------------|-------------|
| FTD3 patient (pooled) | 0,0095±0,0016* |
| Isogenic control (pooled) | 0,0046±0,0004 |
| Introduced mutations (pooled) | 0,0793±0,0178 |
| Wildtype | 0,0384±0,0281 |

| Ratio of p-Nf-Kb/Nf-KB (Based on WB) |             |
|-------------------------------------|-------------|
| FTD3 patient (pooled) | 0,073±0,0737 |
| Isogenic control (pooled) | 0,0457±0,0004 |
| Introduced mutations (pooled) | 2,393±1,327 |
| Wildtype | 1,191±1,151 |

| Cytokine Analysis (IL-6) |             |
|-------------------------|-------------|
| FTD3 patient 1 | 3721.64±173.89** |
| Isogenic control 1 | 571.71±59.17 |
| FTD3 patient 2 | 3006.64±78.80** |
| Isogenic control 2 | 134.72±7.69 |
| Heterozygous | 1099.92±4.63** |
| Wildtype | 26.87±0.183 |

| Cytokine Analysis (IL-8) |             |
|-------------------------|-------------|
| FTD3 patient 1 | 707.15±12.10* |
| Isogenic control 1 | 393.87±52.75 |
| FTD3 patient 2 | 769.51±11.532** |
| Isogenic control 2 | 64.59±1.23 |
| Heterozygous | 348.16±3.31** |
| Wildtype | 44.65±0.865 |

| Cytokine Analysis (IL-13) |             |
|--------------------------|-------------|
| FTD3 patient 1 | 79.90±1.69** |
| Isogenic control 1 | 25.63±0.419 |
| FTD3 patient 2 | 42.29±12.17* |
| Isogenic control 2 | 4.48±1.55 |
| Heterozygous | 22.71±0.77* |
| Wildtype | 3.22±0.434 |

| Neurite Length |             |
|---------------|-------------|
| FTD3 patient (Pooled) | 6951±913 |
| Isogenic Control (Pooled) | 1,054±007** |

| MitoSox (mROS) Fluorescence Percentage |             |
|----------------------------------------|-------------|
| Homozygous (Before Treatment) | 100%±2.5% |
| Homozygous (After UDCA Treatment) | 64.32±1.70% * |

| RT-qPCR (mc3) |             |
|---------------|-------------|
| Control mouse | 1,00±0,002 |
| CHMP2B mouse  | 2,42±0,566 |

| RT-qPCR (mLcn2) |             |
|-----------------|-------------|
| Control mouse   | 1,00±0,01  |
| CHMP2B mouse    | 1,55±0,791 |

| RT-qPCR (ml16) |             |
|----------------|-------------|
| Control mouse  | 1,00±0,0004 |
| CHMP2B mouse   | 2,018±0,923 |
| WB (mc3)          | Control mouse | 0.116±0.036 |
|------------------|---------------|-------------|
|                  | CHMP2B mouse  | 0.215±0.0098|
| WB (mGFAP)       | Control mouse | 0.19±0.103  |
|                  | CHMP2B mouse  | 0.095±0.007 |

Significant differences are indicated by *p < 0.05 and **p < 0.01.
| Cell line | Control (Mean ± SEM) | 200nM rapamycin (Mean ± SEM) | 500nM rapamycin (Mean ± SEM) |
|-----------|-----------------------|-----------------------------|-----------------------------|
| LC3B      |                       |                             |                             |
| FTD3 patient 1 | 297.4±35.85**          | 209.7±17.61                 | 272.8±34.01*                |
| Isogenic control 1 | 139.4±24.01          | 202.4±17.07                 | 147±29.24                  |
| FTD3 patient 2 | 301.4±47.25**          | 419.1±60.76**               | 582.8±76.06**              |
| Isogenic control 2 | 31.2±4.70            | 55.5±12.26                  | 95±18.02                   |
| Homozygous | 1758±333.8**            | 2643±432**                   | 2393±279**                 |
| Heterozygous | 609.3±166.2*          | 543.3±39.86*                 | 386.9±70.33*              |
| Wildtype   | 223±70.68             | 266.3±97.67                  | 183.8±37.99               |
| P62 puncta numbers |                   |                             |                             |
| FTD3 patient 1 | 2685±357.5**          | 2877±351.7**                 | 2220±223.4**               |
| Isogenic control 1 | 349.5±45.21          | 528.5±55.91                 | 387.9±87.43               |
| FTD3 patient 2 | 2196±278.8**          | 2234±200.4**                 | 1818±278.8**              |
| Isogenic control 2 | 913.1±191.2          | 840±170.7                   | 725.7±164.8               |
| Homozygous | 1138±136.4            | 1279±152.7                   | 1333±169.2                |
| Heterozygous | 2606±311.5**          | 3071±350.2**                  | 3361±218.6**             |
| Wildtype   | 1065±248.6            | 1144±201.2                   | 1223±142.4                |
| RAB7 puncta numbers |                   |                             |                             |
| FTD3 patient 1 | 961.1±118.4**          | 1723±174.4**                 | 1272±124.6**              |
| Isogenic control 1 | 183.1±51.86          | 399.2±70.19                 | 536.8±120.2               |
| FTD3 patient 2 | 1449±285.7*           | 1440±180.1                   | 1243±312                  |
| Isogenic control 2 | 729.3±144.1          | 997.1±184.3                  | 549.7±123.8               |
| Homozygous | 2023±366.5**          | 2279±201.4*                 | 1848±200.1                 |
| Heterozygous | 2164±243.4**          | 2279±332.5**                 | 2951±288**                |
| Wildtype   | 882.8±123.2           | 1290±312.9                   | 1303±175.4                |
| LAMP1 puncta numbers |                   |                             |                             |
| FTD3 patient 1 | 692.6±122**           | 563.2±74.6**                 | 596.7±60.51**             |
| Isogenic control 1 | 95.4±19.98           | 200.1±73.82                  | 137±43.23                 |
| FTD3 patient 2 | 1242±207.5**          | 1190±229.8**                 | 965.3±166.6**             |
| Isogenic control 2 | 392±41.2             | 253.6±25.73                  | 235±26.84                 |
| Homozygous | 878.5±174.5           | 1021±211.7                   | 553±135.6**               |
| Heterozygous | 1279±251.6            | 1381±153.5                   | 1608±208.8                |
| Wildtype   | 1315±307              | 1528±250.1                   | 1909±347.2                |

Significant differences are indicated by *p < 0.05, **p <0.01 and ***p <0.001.
Table S1g. Statistics Data (Related to Figure S6)

| Cytokine Analysis       | Cell line          | Control (Mean ± SEM) |
|-------------------------|--------------------|----------------------|
|                         | FTD3 patient 1     | 9,49±1,82            |
|                         | Isogenic control 1 | 7,33±0,639           |
|                         | FTD3 patient 2     | 10,04±2,26*          |
|                         | Isogenic control 2 | 0,45±0,003           |
|                         | Heterozygous       | 3,89±0,475           |
|                         | Wildtype           | 0,535±0,074          |

| Cytokine Analysis       | Cell line          | Control (Mean ± SEM) |
|-------------------------|--------------------|----------------------|
|                         | FTD3 patient 1     | 9,54±2,99            |
|                         | Isogenic control 1 | 2,003±0,62           |
|                         | FTD3 patient 2     | 6,17±1,24            |
|                         | Isogenic control 2 | 1,00±0,18            |
|                         | Heterozygous       | 5,26±0,76            |
|                         | Wildtype           | 1,1±0,04             |

| Cytokine Analysis       | Cell line          | Control (Mean ± SEM) |
|-------------------------|--------------------|----------------------|
|                         | FTD3 patient 1     | 5,94±0,162           |
|                         | FTD3 patient 2     | 6,373±0,154          |
|                         | Heterozygous       | 2,05±0,082           |

Significant differences are indicated by *p < 0.05.

Table S2

Subset of significantly differentially expressed genes detected by comparing patient-derived CHMP2B-mutant cell lines with Cas9-corrected controls. For a full list of significantly DE genes, see Table S3. Genes are grouped based on their association with processes, functions or characteristics of interest for the disease. Lists of genes related to each annotation were retrieved from one of the following sources as indicated: Gene Ontology (GO); WikiPathways; COMPARTMENTS; KEGG: literature (manually curated). For each gene, we report its name, Ensembl identifier, log2 fold change and adjusted P-value. Only significantly differentially expressed genes are reported (adjusted p-value <= 0.05, absolute log2 fold change >=1 and average of normalized read counts >=10). For genes grouped by associated compartment, we also provide the confidence score assigned in COMPARTMENTS; only genes with a confidence score >=3 is shown.
**Table S3**

List of significantly differentially expressed genes detected by comparing patient-derived CHMP2B-mutant cell lines with Cas9-corrected controls, and overrepresented terms obtained by comparing differentially expressed genes with expressed genes. For each differentially expressed gene, we report its name, Ensembl identifier, log2 fold change and adjusted P-value. Gene counts normalized by DeSeq2 in the sample are also provided. Only significantly differentially expressed genes are reported (adjusted p-value <= 0.05, absolute log2 fold change >=1 and average of normalized read counts >=10). Overrepresented terms were filtered by excluding redundant annotations (overlap between gene sets >= 50%).

**Table S4. Antibodies used for Immunocytochemistry**

| Antibody                      | Antibody Registry* Identifier | Dilution | Company     |
|-------------------------------|-------------------------------|----------|-------------|
| **Astroglial differentiation** |                               |          |             |
| rabbit anti-SOX9             | AB_2665492                    | 1:400    | CST         |
| rabbit anti-GFAP             | AB_10013382                   | 1:1000   | Dako        |
| rabbit anti-aquaporin4       | AB_2274338                    | 1:50     | Abcam       |
| mouse anti- S100β            | AB_882426                     | 1:500    | Sigma       |
| **Reactive Astrocyte**       |                               |          |             |
| rabbit anti-LCN2             | AB_10618739                   | 1:200    | Millipore   |
| mouse anti-TIA1              | AB_2201439                    | 1:100    | Abcam       |
| rabbit anti-C3               | AB_1240642                    | 1:500    | GenTex      |
| **Autophagy-Endolysosomal pathway** |                           |          |             |
| rabbit anti-LC3B             | AB_881433                     | 1:1000   | Abcam       |
| rabbit anti-LC3B             | AB_881429                     | 1:2000   | Abcam       |
| mouse anti-RAB7              | AB_882241                     | 1:1000   | Abcam       |
| guinea pig anti-P62          | AB_2687531                    | 1:100    | Progen      |
| mouse anti-LAMP1             | AB_2296838                    | 1:400    | HB          |
| **Secondary antibodies**     |                               |          |             |
| AF 488 donkey anti-rabbit IgG| AB_2534015                    | 1:1000   | TFS         |
| AF 488 donkey anti-guinea IgG| AB_2535788                    | 1:2000   | TFS         |
| AF 594 donkey anti-rabbit IgG| AB_2556547                    | 1:2000   | TFS         |
| AF 594 donkey anti-goat IgG  | AB_2534105                    | 1:1000   | TFS         |
| Antibody Description                          | Catalog Number   | Dilution | Supplier       |
|----------------------------------------------|------------------|----------|----------------|
| AF 594 donkey anti-mouse IgG                 | AB_253578        | 1:1000   | TFS            |
| AF 647 donkey anti-mouse IgG                 | AB_162542        | 1:1000   | TFS            |

AF, Alexa Fluor; TFS, Thermo Fisher Scientific Inc; HB, Hybridoma bank; CST, Cell Signalling Technology, [http://antibodyregistry.org/](http://antibodyregistry.org/)
Table S5. Antibodies used for Western blot

| Antibody                        | Antibody Registry* Identifier | Dilution | Company |
|---------------------------------|-------------------------------|----------|---------|
| **Autophagy Proteins**          |                               |          |         |
| *rabbit anti-GAPDH              | AB_9485, AB_307275            | 1:3000   | Abcam   |
| *mouse anti-GAPDH               | AB_627678                     | 1:4000   | SCT     |
| rabbit anti-LC3B                | AB_881429                     | 1:3000   | Abcam   |
| rabbit anti-C3                  | AB_1240642                    | 1:10000  | GeneTex |
| mouse- LAMP1                    | AB_2296838                    | 1:4000   | HB      |
| rabbit-Phospho NF-Kb           | AB_10827881                   | 1:1000   | CST     |
| rabbit- NF-Kb                   | AB_10859369                   | 1:1000   | CST     |
| mouse- Caspase 3                | AB_781826                     | 1:500    | SCT     |
| **Secondary antibodies**        |                               |          |         |
| IRDye® 800CW Donkey anti-Rabbit IgG (H + L) | AB_621848      | 1:15,000 | LI-COR  |
| IRDye® 800CW Donkey anti-Mouse IgG (H + L) | AB_621847      | 1:15,000 | LI-COR  |
| IRDye® 680LT Goat anti-Mouse IgG (H + L) | AB_10706161  | 1:20,000 | LI-COR  |
| IRDye® 680LT Goat anti-Rabbit IgG (H + L) | AB_10706309  | 1:20,000 | LI-COR  |

HB, Hybridoma bank; CST, Cell Signalling Technology; IRDye, Infrared Dye; [http://antibodyregistry.org/](http://antibodyregistry.org/); GAPDH was used as reference gene.
Table S6. Primers used for RT-qPCR

| Gene Name | Forward | Reverse |
|-----------|---------|---------|
| Human Primers | | |
| OPA1 | GGCGGAAGACCTCAAGAAAGT | GGCTGGCACAAAGACGTTGAT |
| MFN1 | CCAGAAAGTGGTGTTGCGACT | GTTTTGACTTGACTGCGAG |
| FIS1 | GGTGCGGAGCAAGTAGTACAATGA | CGTATTCCTTGAGGCGGTAGT |
| MAP3K9 | CTGGAAACGGGAGCTCAACAT | TGGTGTAAGACTGATGGCT |
| DCN | CCCTCCTCCTTTCCACACCT | TTTTCAAACAGGGAAACCTT |
| GAS6 | GACATAGACGAGTGCAGCAGA | ACAGGAAGATGTCCTCACAG |
| GAD1 | GGGAACTAGCGAGAACGAGG | GGTATCGTACGTTGATG |
| MT-ND2 | TCATAGCAGGCAGTTGAGGC | GTGTGGTGCTGGAGGTTTA |
| *GAPDH | CTCTCTGCTCTCCTCTGCTTCAG | TGAAGCGATGTCGCTGCTT |

| Mouse Primers | | |
| Gene Name | Forward | Reverse |
|-----------|---------|---------|
| mIl6 | GATGCTACCAAACTGGATATACTC | GGTCCTTAGCCACTCCTTCTGG |
| mc3 | ACCCCTTCATTTCCTACCCT | CCTTACTTGCTGGAATCTTGATG |
| mLcn2 | TCTGTCCCAACCAGACCAATG | GGGGAGTGCTGGGCAATAA |
| mMap3k | ATCAGGGAGATGAAGGCCTCAAG | AGGACTGTTGGGTGATAG |
| mMfn1 | CAGGGACGGAGTGAGTGCACCT | GTTTCTGCAATTAGCACCCTGGA |
| mFis1 | CTGGTGTCTGTGAGGAGATGCTGA | GAGCCCTTTACATATTCTGAGC |
| mOpa1 | CTGCAGGTCCCAAATTGGTT | CTGCAGGTCCCATAATGGTT |
| mDcn | TTCCTACTCGGCTGTAGTAC | AAGTTGAATGGCAGAAGCC |
| mGad1 | CCTTCGCCTGCAACCTTCCCTGAAC | GGGTAGTTGCTCTCCCCTTCT |
| mMt-nd2 | AGGGATCCACTGCACATAG | TGAGGGATGGGTTGTAAGGA |
| mIl17d | GGGCGTACAGGATTTTCTAC | AGAGAAGACGGGTTGTGCTG |
| mGas6 | AAAGGAGCACAGTGAAGTGA | TTTTCCCTACCTCAAGA |
|    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| *mGapd | TGCACCACCAACTGCTTAG | GGATGACCTTGCCC |    |
|       |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

*was used as reference gene; m=mouse.
### Media used in this study

#### Table S7a. Neural Maturation Medium (NMM)

| Reagent                               | Company & Catalogue | Final role                                      | stock | Final Study |
|---------------------------------------|---------------------|-------------------------------------------------|-------|-------------|
| DMEM/F12 with L-Glutamine and Heps    | Thermo Fisher Scientific, 11330-057 | Basal medium                                   | 1x    | 50 %        |
| Neurobasal Medium                     | Thermo Fisher Scientific, 21103049   | Basal medium                                   | -     | 50 %        |
| B-27 without Vitamin A                | Thermo Fisher Scientific, 12587-010 | Support neuronal cell growth                    | 50x   | 2 %         |
| N-2 Supplement                        | Thermo Fisher Scientific, 17502048   | Support neuronal cell growth                    | 100x  | 1 %         |
| Glutamax (L-Glutamine)                | Thermo Fisher Scientific, 35050061   | Nutrition factor                               | 100x  | 1 %         |
| Penicillin/Streptomycin               | Sigma Aldrich, P0781                   | Antibiotics                                    | 100x  | 1 %         |
| *b-FGF-2                              | ProSpec, CYT-557                  | 25ng/µl/dPBS                                    | 10 ng/ml| Promotes proliferation |
| *EGF                                  | ProSpec, CYT-217                   | 100 ng/µl                                      | 10 ng/ml| Promotes proliferation |
| *SB4315642                            | Selleckchem, S1067                 | 10 mM/DMSO                                      | 10 µM | Inhibition of TGFβ pathway. Promotes ectodermal differentiation [1] |
| *LDN193189                            | Sigma Aldrich, SML0559             | 2 mM                                            | 0.1 µM | Inhibition of BMP pathway. Promotes neural differentiation [2] |
| Reagent                          | Company & Catalogue                          | Stock | Final | Role                                      |
|---------------------------------|---------------------------------------------|-------|-------|-------------------------------------------|
| Neurobasal Medium               | Thermo Fisher, Scientific, 21103049          | -     | 90%   | Basal medium                              |
| Non-essential Amino Acids       | Sigma Aldrich, M7145                         | 100x  | 1%    | Amino Acid supplement                      |
| N-2 supplement                  | Thermo Fisher, Scientific, 17502048          | 100x  | 1%    | Supplement to support neuronal cell growth |
| Penicillin/Streptomycin         | Sigma Aldrich, P0781                         | 100x  | 1%    | Antibiotics                               |
| *L-Ascorbic acid                | Sigma Aldrich, A4403                         | 50 mM | 200 µM| Antioxidant                               |
| *Recombinant Human IGF-1        | Peprotech, 100-11                            | 100 µg/ml | 200 ng/ml | Growth Factor                              |
| *Human Activin-A                | Thermo Fisher, PHG9014                       | 100 µg/ml | 10 ng/ml  | Growth Factor                              |
| *Recombinant Human Heregulinβ-1| Peprotech, 100-03                            | 100 µg/ml | 10 ng/ml  | Growth Factor                              |
| *b-FGF-2                        | ProSpec, CYT-557                             | 25 ng/µl | 10 ng/ml  | Growth factor Promotes proliferation      |
| Reagent                                | Company & Catalogue                  | stock | Final  | ROLE                                      |
|----------------------------------------|--------------------------------------|-------|--------|-------------------------------------------|
| Neurobasal Medium                      | Thermo Fisher Scientific, 21103049   | Fisher| 50 %   | Basal medium                              |
| DMEM/F12                               | Sigma Aldrich, D8437                 | 1:1   | 50 %   | Basal medium                              |
| Non-essential Amino Acids              | Sigma Aldrich, M7145                 | 100x  | 1 %    | Amino Acid supplement                     |
| N-2 Supplement                         | Thermo Scientific, 17502048          | 100x  | 1 %    | Supplement to support neuronal cell growth|
| L-Ascorbic Acids                       | Sigma-Aldrich, A4403                 | 50 mM | 200 µM | Antioxidant                               |
| Glutamax (L-Glutamine)                 | Thermo Scientific, 35050061          | Fisher| 1 %    | Nutrition Factor                          |
| Sodium Pyruvate                        | Thermo Fisher Scientific, 1136070    | 100 mM| 1 %    | Nutrition Factor Carbon source            |
| Fetal Bovine Serum                     | Th Geyer, BW/S181B-500               | 100x  | 2 %    | Basal media supplement                    |
| *Recombinant Human Heregulinβ-1        | Peprotech, 100-03                    | 100 µg/ml| 10 ng/ml| Growth Factor                             |
| *Human Activin A                       | Thermo Scientific, PHG9014          | Fisher| 100 µg/ml| 10 ng/ml| Growth Factor                             |
| *Recombinant Human IGF-1               | Peprotech, 100-11                    | 100 µg/ml| 200 µg/ml| Growth Factor                             |

* All growth factors were added fresh to the media every time before use.
Additional References

1. Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., & Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature Biotechnology, 27*(3), 275–280. https://doi.org/10.1038/nbt.1529

2. Vogt, J., Traynor, R., & Sapkota, G. P. (2011). The specificities of small molecule inhibitors of the TGFβ and BMP pathways. *Cellular Signalling, 23*(11), 1831–1842. https://doi.org/10.1016/j.cellsig.2011.06.019

Supplemental Figures Legends

Figure S1. (Related to Figure 3, 4, and 5). Transcriptome-based PCA and volcano plot of the differentially expressed genes

a) Expression-based Principal Component (PC) analysis shows consistent separation between FTD3 patient astrocytes and isogenic controls. (b) Volcano plot of genes with a mean of DESeq2-normalized counts $\geq 10$ differentially expressed between FTD3 patient astrocytes and isogenic controls. Genes with absolute log2FoldChange $\geq 1$ and adjusted p-value $\leq 0.05$ are selected as significantly differentially expressed and colored in blue or red if down- or upregulated, respectively. (c) Left panel: top 15 significantly enriched KEGG and Reactome pathways identified by functional enrichment analysis of all 1133 significantly differentially expressed genes between FTD3 patient astrocytes and isogenic controls. The gene ratio corresponds to the ratio between the differentially expressed and all expressed genes annotated with a term. For each enriched term, the size of the dot indicates the number of differentially expressed genes annotated with this term, while the color shows the FDR of the enrichment test. Right panel: The same representation as in (c) is used for the top 15 significantly enriched Gene Ontology (GO) functions.

Figure S2. (Related to Figure 2). Characterization of autophagy changes at different rapamycin treatment concentrations. a, d, g) Representative ICC images of LC3B and S100β co-labelling of astrocytes at 0nM, 200nM and 500nM rapamycin
treatment. LC3B is a marker of autophagosomes, and FTD3 astrocytes show an increase in LC3 labelling. S100B is utilized as a cytoplasmic marker to visualize LC3B intracellular distribution. Rapamycin treatment does not decrease LC3 labelling. Scale bar: 25 µm. b-c, e-f, h-i) LC3B puncta number quantification of FTD3 astrocytes and controls at different rapamycin concentrations. The rapamycin treatments do not rescue LC3B increased markers in FTD3 lines to control line levels. Unpaired student’s t-test was performed, standard error of the mean (SEM) is presented and significant differences are indicated by *p < 0.05 and **p <0.01. N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line.

Figure S3. (Related to Figure 2). Characterization of autophagy changes at 500 nM rapamycin treatment. a) Representative ICC images of P62, AQP4 and RAB7 co-labelling of astrocytes with 500 nM rapamycin treatment. Scale bar: 25 µm. b) Representative ICC images of P62, AQP4 and LAMP1 co-labelling of astrocytes 500 nM rapamycin treatment. Scale bar: 25 µm. c-j) ICC quantifications of P62, RAB7, and P62 mean area and colocalization puncta number of FTD3 astrocytes and controls. Quantifications demonstrate a FTD3 dependent increase in P62 labeling, P62 mean area of distribution, RAB7 markers and P62-RAB7 co-labelling, which cannot be rescued with rapamycin treatment. k-n) LAMP1 and colocalization puncta number quantification of FTD3 astrocytes and controls with 500 nM rapamycin treatment. Quantifications demonstrate an up-regulation in LAMP1 and LAMP1-P62 colocalization in heterozygous FTD3 patient lines compared to controls indicating an increase in autophagic vesicle fusion. In contrast, a significant down-regulation in LAMP1 puncta numbers and LAMP1-P62 colocalization is shown in the homozygous induced CHMP2B astrocyte line compared to the wildtype control demonstrating a decrease in autophagosome-lysosome fusion. Unpaired student’s t-test was performed, standard
error of the mean (SEM) is presented and significant differences are indicated by *p < 0.05 and **p < 0.01. N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line.

**Figure S4. (Related to Figure 2).** Ultrastructural analysis of autophagosome accumulations visualized with transmission electron microscopy (TEM). Representative TEM images of 10 weeks mature FTD3 heterozygous lines compared to respective controls. FTD3 astrocyte lines demonstrate an increase in electron-dense cargo-filled vesicles.  

a) Control no rapamycin treatment  
b) 500nM rapamycin treatment.

Examples of autophagosomes and autolysosomes are indicated with black arrows. (A= autophagosomes and L= autolysosomes. N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line.

**Figure S5. Off-Target effect on CRISPR-Cas9 mediated editing.** Representative sequencing chromatographs for wild-type, heterozygous CHMP2B mutation and homozygous CHMP2B mutation are shown with the potential off-target region of the Cas9-gRNA complex underlined in black (bottom).

**Figure S6. (Related to Figure 5).** Additional Cytokine Profile.  
a-c) Cytokine Profiling of IL-2, TNF α and IL-1β) of heterozygous FTD3 related astrocyte lines compared to controls. An increase in cytokine secretion is found in astrocyte media from FTD3 patients and heterozygous CHMP2B induced lines compared to controls. Standard error of the mean (SEM) is presented and significant differences are indicated by *p<0.05 and **p<0.01. N=3, n=3, N indicates the number of experimental repetitions; n indicates the number of technical replicates per cell line.  
d-e) Karyogram of heterozygous induced
CHMP2B mutation (left) and homozygous induced CHMP2B mutation (right). Both cell lines expressed normal karyotype profile.