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Impact of a Missense Mutation in *TRAPPC12* in Patients with Progressive Encephalopathy, Brain Atrophy and Spasticity Phenotype without Microcephaly and Epilepsy

Beyza Goncu [1], Gozde Yesil Sayin [2], Ayca Dilruba Aslanger [2] & Emrah Yucesan [3]

[1] Department of Medical Services and Techniques, Vocational School of Health Services, Bezmialem Vakif University, Istanbul, Turkiye
[2] Department of Medical Genetics, Faculty of Medicine, Istanbul University, Istanbul, Turkiye
[3] Department of Neurogenetics, Institute of Neurological Sciences, Istanbul University-Cerrahpasa, Istanbul, Turkiye

Abstract

Objective: Various symptoms, including microcephaly, corpus callosum agenesis, cerebellar atrophy, spasticity, and epilepsy, are associated with variations in the *TRAPPC12* gene. This diversity of features contributes to a broad range of mortality and morbidity. Identifying variations with functional consequences is crucial for accurate diagnosis and appropriate counseling for affected families. This study presented the results of a functional analysis of a previously detected mutation.

Materials and Methods: Patient-derived fibroblast cells (have a c.679T>G; p. Phe227Val variation) and the CCD1079Sk cell line (as a healthy control) were used. The relative protein expression of TRAPPC12 along with morphological changes, including Golgi integrity, endoplasmic reticulum (ER) structure, and vesicle distribution for neutral lipids, were assessed using immunofluorescent staining.

Results: Protein expression analysis revealed an absence of the mature TRAPPC12 protein and the uncharacterized protein fragment (CGI-87) via mutation compared with the wild-type. Additionally, milder outcomes were observed for Golgi integrity, slight ER structure enlargement, and further vesicle distribution changes, particularly with the truncated TRAPPC12 protein.

Conclusion: Despite the variant leading to a milder clinical phenotype without microcephaly and epilepsy, as previously reported, the study showed unstable protein expression and mild effects on Golgi and ER structures along with alterations in vesicle distribution throughout the cytoplasm. Despite the lack of mature TRAPPC12 protein expression and mild organelle impairments, the vesicle trafficking persisted. We showed that a single amino acid substitution might cause a loss of mature protein expression and also cause a milder disruption of organelles. More functional analyses are necessary to confirm these outcomes.

Keywords *TRAPPC12* · TRAMM · Neutral Lipid · Vesicle Trafficking · Progressive Encephalopathy

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Corresponding author: Beyza Goncu bgoncu@bezmialem.edu.tr

INTRODUCTION

Early-onset progressive encephalopathy with brain atrophy and spasticity (PEBAS) (MIM#614139) caused by bi-allelic variations in the *TRAPPC12* gene is an autosomal recessive condition characterized by progressive central nervous system (CNS) deterioration with seizures, hypotonia, microcephaly, dystonia, and/or global developmental delay (1).

TRAPPC12 is a constituent of the transport protein particle (TRAPP) multisubunit tethering complex and plays a role in intracellular vesicle trafficking (2). Every cell engages in intracellular trafficking, facilitating interaction for the delivery and/or recognition of its contents. The intracellular trafficking process involves multiple stages, including coating the cargo, cargo movement, tethering to the target membrane, and uncoating the cargo protein (3-5). Initially conceptualized as the tethering of two membranes, the transportation mechanism is now recognized to be much more intricate and involves a diverse array of proteins (5). The tethering function of the TRAPP complex has been extensively studied. This complex is categorized into two protein families in mammals: TRAPP II and TRAPP III (3, 5). One of the primary functions of these cells is to facilitate trafficking interaction among the endoplasmic reticulum (ER), ER-Golgi intermediate compartment (ERGIC), and Golgi apparatus (3, 5, 6). Over the course of 20 years of subsequent research, TRAPP complexes have been revealed to play a crucial role in mediating interactions between ER exit sites (Coat protein complex, COP, I and II vesicles) through the Golgi apparatus (7), exhibiting guanine nucleotide exchange factor (GEF) activity (8). A recent study highlighted that the TRAPP complex undergoes various conformational changes, leading to robust GEF activity, especially with regard to its interaction with Rab1 and Rab11; TRAPP II interacts with both, while TRAPP III exhibits distinct specificity for Rab1 (8). TRAPP III complexes are also implicated in kinetochore stability, mitotic transformation, and autophagy (9-11).

A wide spectrum of human diseases is linked to the alterations observed in TRAPP subunits. Changes in TRAPP subunit proteins have been correlated with diverse clinical manifestations, including global developmental delay, intellectual disability, microcephaly, spasticity, epilepsy, and encephalopathy (6, 11-14).

In this study, we evaluated the protein expression and organelle integrity, including the Golgi, ER, and vesicle distributions of the *TRAPPC12* gene variation reported by Aslanger et al. (c.679T >G; p. Phe227Val) (12).

MATERIALS AND METHODS

Approval was obtained from the Bezmialem Vakif University Ethics Committee (approval number: 2019/2659). Written informed consent was obtained from the patient and the parents of the patients were included in this study. This study evaluated a functional analysis of the previously reported (12) homozygous missense variation (NM_016030.6:c.679T>G; p. Phe227Val) of the *TRAPPC12* gene.

Sample Retrieval

Punch biopsy samples of the patient's skin were harvested after clinical determination. The derivation of fibroblast cells was performed as described previously (15). The skin biopsy in media was dissected into 10-12 pieces using sterile forceps and then cultivated. Medium was refreshed every 2-3 days. Further assays were performed once fibroblasts were confluent. CCD1079Sk (ATCC® CRL-2097™) human skin fibroblast cell line was used as the healthy control group. Cells were grown in DMEM/F12 (Panbiotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum, 0.5% non-essential amino acids (NEAA), and 1% penicillin/streptomycin. Both primary skin fibroblast and CCD1079Sk cell lines were cultivated at 37°C in 5% CO_2 and 95% relative humidity. Protein samples from cells were prepared using a 100 µL RIPA lysis buffer containing 100-mM phenylmethylsulfonyl fluoride, 100-mM sodium orthovanadate solution, and 1X protease inhibitor cocktail (Santa Cruz, Texas, USA). The lysed protein samples were frozen overnight at −80°C then centrifugation was performed at 13000 g for 10 min. The total protein concentrations were determined using the Qubit protein assay kit protocol with a Qubit Fluorometer 4.0 (Thermo Fisher Scientific MA, USA).

Western Blotting

A 75 µg protein sample with 4X Laemmli buffer (EcoTech Biotechnology, Erzurum, Turkiye) was separated on 4–12% handmade polyacrylamide gel electrophoresis**.** Proteins were transferred to 0.2 µm polyvinylidene fluoride (PVDF) membranes (EcoTech Biotechnology, Erzurum, Turkiye) via semi-dry hot transfer using 20% MeOH and a Trans-Blot Turbo Transfer System (Bio-Rad, USA). Blocking was performed for 1 h at room temperature with Tris-buffered saline containing 0.02% Tween-20 containing 5% nonfat milk. Two different primary antibodies were used for TRAPPC12. The rabbit polyclonal antibody (cat no: NBP1-84500 from Novus Biologicals, Oxfordshire, UK) recognizes 625 amino acid (a.a.) and 710 a.a. sequences of human-TRAPPC12. The second primary antibody was a mouse polyclonal antibody (cat no. H00051112-B01P from Novus Biologicals, Oxfordshire, UK),

which recognizes the full-length protein (1 a.a. - 735 a.a) of the human TRAPPC12. The secondary antibodies were anti-rabbit IgG and anti-mouse IgG (both from Cell Signaling, MA, USA), and as a reference protein, horseradish peroxidase (HRP)-conjugated rabbit monoclonal β-actin primary antibody was used (cat no. AC028, Abclonal, MA, USA). The membranes were washed five times for 5 min per wash, each between primary and secondary antibody incubations. The blots were generated using an enhanced chemiluminescence (ECL) detection kit (WesternBright ECL, Advansta, CA, USA). The ImageJ (National Institutes of Health; NIH) program (16) was used to analyze Western blot images and to measure the intensity of organelle staining.

Organelle Staining

The Golgi and ER staining were performed with BODIPY™ FL C5-Ceramide and ER-Tracker™ Red (BODIPY™ TR Glibenclamide) dyes from Thermo Fisher Scientific, respectively, according to the manufacturer's instructions. Additionally, the cytoplasmic neutral lipid droplets were stained with Nile red (Thermo Fisher Scientific) as previously described (17). Nuclei were counterstained with DAPI, and images were acquired using a laser scanning confocal microscope (Leica TCS-SPE, Leica Microsystems, Wetzlar, Germany) from the Aziz Sancar Institute of Experimental Medicine, Istanbul University, as a service purchase.

Statistical Analyses

Statistical analyses were performed for experimental values by t-tests using GraphPad (GraphPad Software v10, Inc., CA, USA). Data are presented as means with a significance level of $p \leq 0.05$.

RESULTS

Before further experiments, we checked the pathogenicity scores of the variation, which was previously reported by our team (*TRAPPC12* NM_016030.6:c.679T>G, rs1312522735) using prediction tools (12). The patient was shown to be homozygous, and the parents were determined to be obligate carriers. The variant was not found in public databases in the homozygous state. However, 2 of 237.118 alleles were found in the Gnomad exome database, indicating a very rare carrier rate (0.00000843) (18). The variant was classified as pathogenic based on the tools integrated into the Varsome platform (19). The region appeared to be highly conserved across species according to PyhloP5.680 PhastCons 1.00 scores (20). Additionally, to detect our variation's nature, we used the *in-silico* prediction tool Mutalyzer (Mutalyzer 2.0.35) and found a non-truncating variant. Variations in the *TRAPPC12* gene have been reported in the literature, and the results of this study are presented in Figure 1A.

TRAPPC12 Protein Expression

Patient fibroblasts and CCD1079Sk human skin fibroblasts were used as mutant and wild type, respectively. Prior to Western blotting, we replicated the cells using consecutive passages during cultivation for the wild-type and mutant types. The variation itself did not cause a stop gain mutation; therefore, we evaluated the protein level with two different primary antibodies, including one that recognizes between 625 a.a. - 710 a.a. region (tetratricopeptide region) and the other region recognizes the full-length of the human TRAPPC12 protein while β-actin was used as a reference control (Figure 1B). Our chemiluminescent detection was >70 pg sensitive. The Western blot analysis of TRAPPC12 showed that the primary antibody that identified the tetratricopeptide region showed no bands of the expected size (79 kDa). However, an absence of the intact protein according to the second primary antibody that recognizes the full-length TRAPPC12 resulted in only a cleaved-like pattern protein product compared with the wild-type (Figure 1B). Regarding the Western blot, highly reduced protein expression and possibly cleaved protein products were observed (Figure 1B right blot). The second primary antibody recognizes the tetratricopeptide region of the TRAPPC12 protein, and mutation occurred via a nonpolar phenyl (F) at position 227 to another nonpolar amino acid, Valine (V). This may result in unstable, cleaved, or limited target protein function. To confirm whether the primary antibodies recognize the amino acid sequence, we ran the standard protein BLAST. We then determined the sizes of the cleaved-like pattern proteins and CGI-87. In addition, we also identified wild-type CGI-87 expression at approximately 35 kDa, and the subcellular location of the CGI-87 was reported to be located in the Golgi apparatus, according to UniProt (UniProt ID: Q53S18) (Figure 1B). The CGI-87 protein is identical to the a.a. sequence from 1 to 349 a.a. of the TRAPPC12 a.a. sequence. Regarding the outcome of the two primary antibodies of the TRAPPC12 region, the mutant type failed to show a protein expression for TRAPPC12 and CGI-87 (TRAPPC12-related protein, an uncharacterized protein fragment) compared with the wild type. In addition, the cleaved-like pattern did not show any reference point from BLAST. Thus, the *TRAPPC12* NM_016030.6:c.679T>G variation also affected the mature TRAPPC12 protein and CGI-87 by changing the position at 227 a.a.

Intracellular Differences

To investigate variations that interfere with organelle structure via mutation, we performed ER and Golgi

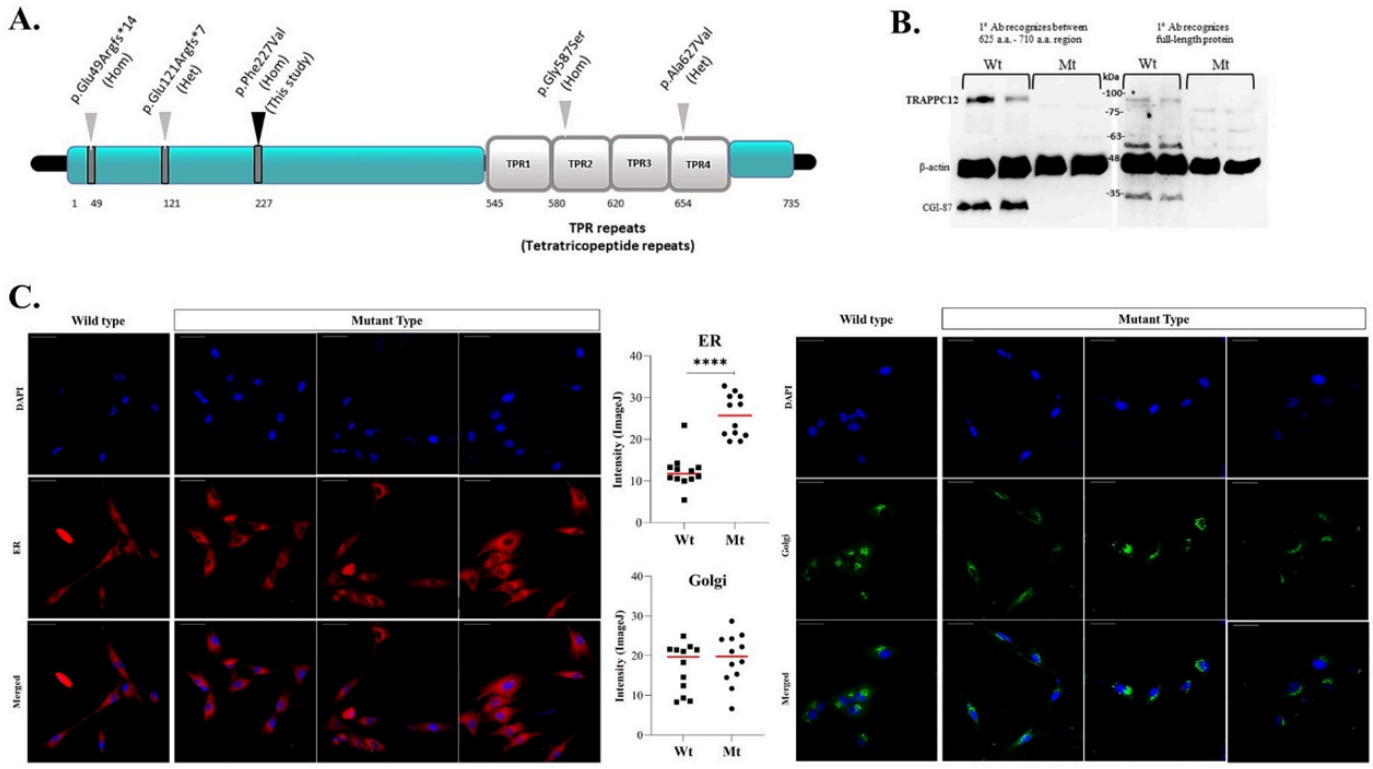

Figure 1. The variations were mapped at the protein level (NP_001308031.1). Schematic depicting aligned protein-coding sequence for the TRAPPC12. The gray arrows mark the locations of previously identified TRAPPC12 variations. A black arrow indicates the new variation. Variants are named according to GRCh37. **B.** Immunoblot images of 75µg protein samples per lane. The wild type of CCD1079Sk cells was replicated using different passages. The mutant type from patient fibroblast was replicated from passages two and three during cultivation. The left panel shows a primary antibody recognizes between 625 a.a. and 710 a.a. sequence (tetratricopeptide region) and the right panel shows a second primary antibody that recognizes a full-length protein of the human-TRAPPC12, respectively. Both primary antibodies demonstrated that the mutant type showed no visible bands or isoforms of intact TRAPPC12 which appeared to be associated with changes in the amino acid sequence. The CGI-87 bands indicate uncharacterized protein fragments and TRAPPC12-associated protein products (UniProt ID: Q53A18) from UniProt, which were detected by both primary antibodies that we used for TRAPPC12. The sequence similarity analysis of TRAPPC12 revealed 100% identity for this protein fragment. **C.** Evaluation of the Golgi and ER structures by immunostaining wild type and mutant type. The left panel shows the ER structure in red, middle panel compiles the intensity analysis between wild-type and mutant Golgi and the ER. Each dot represents a single cell. The right panel shows the Golgi structure in green. Nuclei were stained with DAPI (shown in blue) Magnification 40X. Scale bar=50µm. Intensity measurements were performed using ImageJ, and 12 cells/group were analyzed.

visualization using confocal laser scanning microscopy (Figure 1C). The Golgi staining showed corrupted integrity, and Golgi clusters expanded around the juxta nuclear region compared with the wild type. In addition, a comparison of the intensity between the wild-type and mutant types revealed no significant changes. However, the ER staining intensity analysis showed an increase in the mutant type ($p<0.0001$) (Figure 1C). Moreover, we visualized vesicles with neutral lipids to assess the vesicle trafficking process. We used Nile red staining to understand the intracellular distribution of lipid droplets. The cellular distribution of the lipid droplets did not show significant changes and only slight disruption was observed. Compared with the wild type, a wild type, heterogeneous distribution of cells their stage was observed (Supplementary Figure S1).

DISCUSSION

PEBAS is a severe disorder, and its etiopathogenesis has not been fully understood. To identify the pathogenesis of the disease, two independent groups noticed that Golgi apparatus is associated with neuronal loss, leading to the PEBAS phenotype (12, 21). According to the literature, many pathways are associated with progressive encephalopathy (22). Among these pathways, the TRAPP complex is particularly remarkable. The complex is a highly conserved modular multi-subunit protein complex that plays a role in endoplasmic reticulum-to-Golgi transport (11).

A significant number of proteins, including TRAPP complexes, regulate intracellular trafficking. Since the discovery of specific subunits of the TRAPP complexes contributed to the molecular processes between the ER-ERGIC-Golgi apparatus (23). Particularly, two different complexes exist, TRAPP II and TRAPP III, in metazoans and humans (8). One study on the

cryo-EM structure of metazoan TRAPP III demonstrated that there are two arms and one core of the complex. The one arm includes TRAPPC8 and TRAPPC11, and the other arm includes TRAPPC12 and TRAPPC13 together (24). The Ras-related protein Rab-1 (Rab1) contacts half of TRAPPC8 which is a GEF (8, 25), and provides a starting point for the completion of the TRAPP III structure. According to Galindo et al., the vertexes of the arms between subunits of the TRAPP III complex and Rab1 mediate vesicle membrane attachment (24). Additionally, among subunits of the TRAPP III complex, particularly TRAPPC12, also known as TTC15, has been reported for various cellular processes (8-10, 23). In 2015, Milev et al. reported that depletion of TRAPPC12 prohibited the migration of chromosomes to the metaphase, resulting in failed chromosome congression. In addition, TRAPPC12-depleted HeLa cells exhibited a disrupted kinetochore structure (10). Subsequent research by Milev et al. also identified a novel function of TRAPPC12 phosphorylation during mitosis, which acts as a mitotic regulator and provides Centromere-associated protein E (CENP-E) recruitment to the kinetochores (10). The distinctive features of TRAPPC12 differ from those of other TRAPP complexes; therefore, Milev et al. renamed the TRAPPC12 as TRAMM (trafficking of membranes and mitosis) (9).

The TRAPPC12 structure and its functions are being studied at the molecular level, as well as considering its relationship to the TRAPP complex, as mentioned above. Genetic studies identified a possible effect of TRAPPC12 variations, on etiopathogenesis. For instance, in 2017, Milev et al. showed that three individuals from two unrelated families (one consanguineous and one nonconsanguineous) had either a homozygous deleterious variant (c.145delG [p. Glu49Argfs*14]) or compound-heterozygous variants (c.360dupC [p. Glu121Argfs*7] and c.1880C>T [p. Ala627Val]) (1). According to their study, the clinical characteristics of the three patients were highly similar, i.e., severe disability, microcephaly, spasticity, hearing loss, visual impairment, and specific brain imaging findings such as; severe cortical atrophy, simplified gyri, and hypogenesis of the corpus callosum. In our case, the patient did not have epilepsy but presented with EEG abnormalities without seizures. In addition, Milev et al. reported that their patients had agenesis of the corpus callosum and severe cerebral atrophy, although our patient had no agenesis of the corpus callosum and only mild cerebral atrophy (1). Based on this difference, we report our case as "mild".

The importance of their study from our point of view is that they provide functional data that exhibits changes in Golgi morphology, membrane trafficking dysfunction, and mitotic delay in fibroblasts (1). In 2020, two case reports by Gass et al. and Aslanger et al. were published, respectively (12, 26). Gass et al. reported compound heterozygous variations in *TRAPPC12* in two fetuses with ventriculomegaly. The family had a previously deceased fetus with hydrocephaly, polydactyly, and interhemispheric cysts, an effect on cilia (26). Aslanger et al. reported two patients from unrelated families with homozygous *TRAPPC12* variants. The first patient was a 2-year-old boy with severe progressive cortical atrophy, moderate cerebellar atrophy, epilepsy, and microcephaly who had previously reported variations (c.1880C > T (p. Ala627Val)) in TRAPPC12. The second patient, a 9-year-old boy, was carrying novel variation (c.679T > G (p. Phe227Val) and manifested mild cortical atrophy, severe cerebellar atrophy without epilepsy, and microcephaly. The absence of cardinal manifestations like epilepsy and microcephaly, led us to investigate the pathogenic mechanism of this variation (12). The variation evaluated in this study is an extension of our previous report.

Herein, we found mature TRAPPC12 protein loss or high protein-level decrease, but contrary to the previous report by Milev et al., our variation did not have stop-gain variation (1). It is also noteworthy that CGI-87 (UniProt ID: Q53S18), a protein with 100% similarity to the TRAPPC12 a.a. sequence, was absent in the wild type. Immunostaining provided a more comprehensive outcome. Our results showed that the variation caused mild structural changes in the Golgi and made in a slight enlargement of the cell size, which was validated by the ER staining. The lack/loss of TRAPPC12 mature protein raises the possibility of COPII-associated membrane clustering to the juxta nuclear region of Golgi. Instead, our findings demonstrate that the TRAPPC12 protein is unstable and may be degraded by proteasome and has a marked effect on the architecture of the Golgi complex.

To understand whether the differences at the organelle level affected vesicle trafficking, we visualized the vesicles that carried neutral lipids (by Nile red staining). When examined using Nile red staining, we observed heterogeneous vesicle distribution depending on cell stage. In wild-type comparisons, we assumed that morphological differences were heterogeneous but homogeneous within the cell groups. However, Nile Red is a dye that dissolves in acetone, and when it is applied to cells, the cells become round, regardless of the concentration. Afterward, we became aware that it would be inappropriate to make a definitive interpretation of the possible changes in the vesicle distribution. Another limitation of the study was that the other molecules found in the core structure of TRAPPC12 could not be examined in terms of their relevance to the mutation. In addition, the vesicle traffic neither showed accumulation nor stopped

via variation (c.679T>G (p. Phe227Val). Consequently, the mild disarrangement of the Golgi and ER structure showed that the neutral lipid-carrying vesicle distribution was altered throughout the cytoplasm. Further studies are required to examine the relationship between the mutagenicity of the changes in cell morphologies caused by this mutation.

CONCLUSION

In conclusion, genetic diagnosis confirms the specific cause of the related diseases however the exact diagnosis requires more functional consequences at the protein and cellular levels. The precise pathways associated with the determined genes and their protein products play an important role in elucidating intracellular processes. Herein, the cellular impact of homozygous c.679T>G (p. Phe227Val) variation provides an understanding of the disruption of variation in cellular behavior. To prove this point, further experiments with proteasome inhibitors could help detect possible protein degradation. Further functional analysis, including high-throughput structural and functional relationships with other TRAPP molecules will underline the exact diagnosis of patients with PEBAS.

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Ethics Committee Approval: Approval was obtained from the Bezmialem Vakif University Ethics Committee (approval number: 2019/2659). Written informed consent was obtained from the patient and the parents of the patients were included in this study.

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Author Contributions: Conception/Design of Study – B.G., G.Y.S., A.D.A., E.Y.; Data Acquisition – B.G.; Data Analysis/Interpretation – B.G., E.Y.; Drafting Manuscript – B.G., G.Y.S.; Critical Revision of Manuscript – E.Y.; Final Approval and Accountability – B.G., G.Y.S., A.D.A., E.Y.

Conflict of Interest: The authors declare no conflict of interest.

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Author Details

Beyza Goncu
[1] Department of Medical Services and Techniques, Vocational School of Health Services, Bezmialem Vakif University, Istanbul, Turkiye
0000-0001-6026-8218 bgoncu@bezmialem.edu.tr

Gozde Yesil Sayin
[2] Department of Medical Genetics, Faculty of Medicine, Istanbul University, Istanbul, Turkiye
0000-0003-1964-6306

Ayca Dilruba Aslanger
[2] Department of Medical Genetics, Faculty of Medicine, Istanbul University, Istanbul, Turkiye
0000-0003-1770-1762

Emrah Yucesan
[3] Department of Neurogenetics, Institute of Neurological Sciences, Istanbul University-Cerrahpasa, Istanbul, Turkiye
0000-0003-4512-8764

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APPENDIX

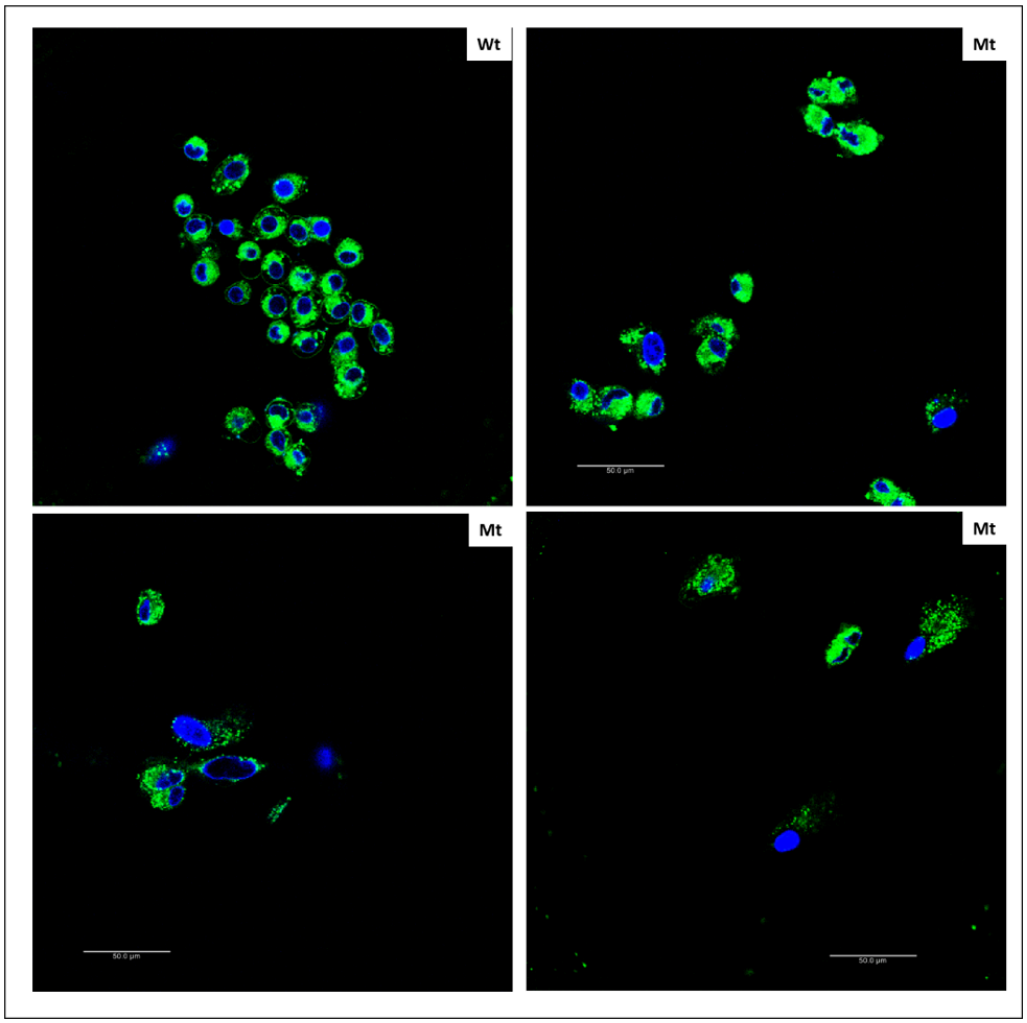

Figure S 1. Detection of neutral lipids in human skin fibroblasts from the patient (Mt) and CCD1079Sk cells (Wt) using the Nile red staining. The green fluorescence observed in the presence of Nile red indicates the presence of neutral lipid-containing vesicles. Nuclei was stained with DAPI (shown in blue). Scale bar=50µm.