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Original Article

Impairment of lipophagy by PNPLA1 mutations causes lipid droplet accumulation in primary fibroblasts of Autosomal Recessive Congenital Ichthyosis patients

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ABSTRACT

Background: Autosomal Recessive Congenital Ichthyosis (ARCI) is a group of epidermal keratinization disorders. One of the disease-associated proteins, patatin-like phospholipase domain-containing protein-1 (PNPLA1), plays a key role in the epidermal omega-*O*-acylceramide synthesis and localizes on the surface of lipid droplets (LDs).
Objective: Previously, routine clinical test results showed abnormal LD accumulation in blood smear samples of our ARCI patients with *PNPLA1* mutations. To investigate the abnormal accumulation of LDs, we analyzed primary fibroblast cells of ARCI patients with PNPLA1 mutations (p.Y245del and p.D172N). We hypothesized that PNPLA1 mutations might affect lipophagy-mediated regulation of LDs and cause intracellular lipid accumulation in ARCI patients.
Methods: LD accumulation was analyzed by fluorescence staining with BODIPY®493/503 in the fibroblasts of patient cells and PNPLA1 siRNA transfected control fibroblast cells. The expression of PNPLA1 and its effects on the lipophagy-mediated degradation of LDs were analyzed by immunocytochemistry and immunoblotting.
Results: Our results showed that mutant or downregulated PNPLA1 protein causes abnormal intracellular LD accumulation. We found that PNPLA1 mutations affect neither the cellular localization nor the expression levels of the protein in fibroblast cells. When we analyzed lipophagic degradation process, LC3 expression and the number of autophagosomes were significantly decreased in fibroblast cells of the patients. In addition, co-localization of LDs with autophagosomes and lysosomes were markedly less than that of the control group.
Conclusion: PNPLA1 mutations caused disturbances in both autophagosome formation and fusion of autophagosomes with lysosomes. Our results indicate a possible role for PNPLA1 protein in LD regulation via lipophagy-mediated degradation.

1. Introduction

Autosomal Recessive Congenital Ichthyosis (ARCI), is a rare and heterogeneous group of epidermal keratinization disorders [1]. Although the severity of symptoms may vary within the subtypes, patients generally represent collodion baby phenotype accompanied with dehydration, heat loss, electrolytic imbalance, and sepsis [2]. In all ARCI patients, abnormalities are seen in the epidermal stratum corneum layer composed of corneocytes and surrounding lipid matrix layer. Impairments in

Abbreviations: ARCI, Autosomal Recessive Congenital Ichthyosis; BECN1, Beclin 1; BSA, Bovine Serum Albumin; DAPI, 4′6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LD, lipid droplet; LAMP1, lysosome-associated membrane protein type1; MAP1LC3 (herein LC3), microtubule-associated light chain 3 beta; NLSDI, neutral lipid storage disease with Ichthyosis; PFA, paraformaldehyde; PNPLA, patatin-like phospholipase domain -containing protein; PBS, phosphate buffered saline; PE, phosphatidylethanolamine; TG, triacylglyceride.

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the differentiation of keratinocytes or formation of epidermal lipid barrier lead to disease pathology. So far, many genes including ABCA12 [3], ALOX12B [4–6], ALOXE3 [4], CASP14 [7], CERS3 [8], CYP4F22 [9], LIPN [10], NIPAL4 [11], PNPLA1 [12], SDR9C7 [13], SLC27A4 [14], and TGM1 [15,16] were found to be associated with ARCI.

ARCI-associated human PNPLA1 gene (#285848) is localized on chromosome 6p21.31 and encodes the PNPLA1 protein, which is a member of patatin-like phospholipase domain-containing protein (PNPLA) family [17]. Mammalian PNPLA family members have a common patatin domain at their N-terminus that exhibits lipid hydrolase, triglyceride lipase or transacylase enzyme activity and plays critical roles in maintaining lipid homeostasis [19,20]. PNPLA1 is a transacylase enzyme, playing role in the synthesis of omega-*O*-acylceramides [21–24].

Human PNPLA1 protein localizes onto the surface of cytoplasmic lipid droplets (LDs) through its C-terminal proline-rich domain [17]. LDs are specialized organelles composed of neutral lipids such as triacylglycerides (TG) and sterol esters. LDs interact with other intracellular compartments including endoplasmic reticulum, mitochondria, and peroxisomes via proteins on their surface [25]. Through these interactions, LDs play a central role in maintaining energy balance by regulating the synthesis and degradation of lipids in the cell.

Degradation of LDs is achieved through lipolysis or macroautophagy. The lipolytic degradation of neutral lipids stored in the core of LDs is mediated by diverse lipases localized on the surface of LDs [26]. Macroautophagy is one of the major degradation pathways in which the substrates are sequestered within autophagosomes, which may further fuse with lysosomes, where the substrates are degraded [27,28]. Selective degradation of LDs by macroautophagy is called lipophagy [29].

Previously, routine clinical test results showed abnormal LD accumulation (Jordan's anomaly) in blood smear samples of our ARCI patients with *PNPLA1* mutations namely p.Y245del and p.D172N mutations). As its role in omega-*O*-acylceramides metabolism, mutations in PNPLA1 protein may lead to potent changes in intracellular lipid composition. These lipid composition changes might have an impact on the regulation of lipophagy mechanisms by affecting membrane organization, membrane trafficking, and endocytic pathways. We hypothesized that PNPLA1 mutations might affect lipophagy-mediated regulation of LDs and cause intracellular lipid accumulation. When we investigated fibroblast cells of ARCI patients with PNPLA1 mutations, we observed abnormal accumulation of intracellular LDs. When we knocked down PNPLA1 expression using specific siRNAs in control fibroblasts, we observed abnormal intracellular LD accumulation similar to that of PNPLA1 mutant cells. In addition, we noticed that PNPLA1 mutations caused disturbances in autophagosome formation and autophagosome-lysosome fusion. Our results indicate a possible role for PNPLA1 protein in the regulation of LDs via lipophagy-mediated degradation.

2. Materials and methods

2.1. Cell culture

The study was approved in advance by Hacettepe University the Non-Invasive Clinical Research Ethics Committee. Informed consent forms were obtained from each patient or his/her parent. Primary fibroblast cultures were established from the skin biopsy of individuals by standard protocols. Fibroblasts were maintained in fibroblast medium containing Dulbecco's modified Eagle's medium (DMEM) with 3.7 g/l NaHCO3, 4.5 g/l D-glucose, and stable L-glutamine (Biochrom), 10% fetal bovine serum (Biochrom), 100 μg/ml each penicillin/streptomycin (Biochrom) and amphotericin B (Biochrom). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

20 mM sodium oleate solution was prepared by dissolving Oleic acid (Sigma-Aldrich) in 0,025 M NaOH (Sigma-Aldrich) at 70 °C. 20 mM sodium oleate solution was mixed with 5% fatty acid-free Bovine Serum Albumin (BSA) solution (Sigma-Aldrich) to obtain oleate-BSA complex. For oleic acid treatment, the cells were grown in fibroblast medium supplemented with 400 μM oleate-BSA complex for 12 h. For lipophagy induction experiments, cells were treated with 250 nM Torin (Sigma-Aldrich) for 4 h.

2.2. Immunoblotting analyses

Cells were collected in RIPA buffer (150 mM NaCl (Sigma-Aldrich), 10 mM Tris-HCl (Sigma-Aldrich), pH 7.4, 0.1% SDS (Carlo Erba), 1% Triton X-100 (Applichem), 1% sodium deoxycholate (Sigma-Aldrich) and 5 mM EDTA (Sigma-Aldrich) with protease inhibitor cocktail (Roche) and were lysed. After centrifugation at 14,000 *g* for 15 min., the protein concentration was measured by using the bicinchoninic acid assay (Thermo Fischer Scientific). 25 μg of each protein sample was denatured with 2× Laemmli buffer, separated by 14% SDS-PAGE according to standard protocols and blotted onto nitrocellulose membrane (Thermo Fischer Scientific). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.2% Tween-20 for 1 h and hybridized with primary antibodies raised against human PNPLA1 (Sigma-Aldrich HPA037853), GAPDH (Sigma-Aldrich), LC3 (Abcam), and BECN1 (Sigma-Aldrich) for 1 h at room temperature. Specifically bound immunoglobulins were detected in a second reaction using horseradish peroxidase–conjugated antibodies (Abcam) and visualized by enhanced chemiluminescence detection (Super Signal West Femto; Thermo Fischer Scientific). PNPLA1 blocking peptide (33R-7474) was purchased from Fitzgerald Industries International. The PNPLA1 primary antibody (Sigma-Aldrich SAB2105719) was incubated overnight at 4 °C with blocking peptide in 1:5 ratio before immunoblotting.

2.3. siRNA transfection and quantitative real-time PCR (Q-RT-PCR)

10 nM PNPLA1 (Santa Cruz, sc-61373) and control siRNA (Santa Cruz, sc-37007) are combined with 100 μl Nucleofector™Solution and transfected to primary fibroblast cells by electroporation using Amaxa™Basic Nucleofector™Kit (Lonza, USA VPI-1002) according to manufacturer's instructions. Transfection was initiated by using Nucleofector™Device Program-A24 and downregulation of gene expression was assessed by immunoblotting and q-RT-PCR analysis at 48 h of post-transfection.

Total RNA was extracted using TRIzol reagent (Sigma-Aldrich) and cDNA was reverse transcribed from total RNA using M-MuLV reverse transcriptase (Fermentas) and random hexamers (Invitrogen). Q-RT-PCR for PNPLA1 mRNA quantification, SYBR Green Quantitative RT-PCR kit (Roche) and iCycler iQ thermal cycler (BioRad) were used for single step q-RT-PCR reactions. To activate the SYBR green, an initial cycle of 95 °C, 10 min was performed and followed by PCR reactions: 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Then, the dissociation curves were generated for the verification of amplification specificity (a single cycle of 95 °C for 60 s, 55 °C for 60 s and 80 cycles of 55 °C for 10 s). Changes in mRNA levels were quantified by $2^{-\Delta\Delta CT}$ method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as control. PNPLA1 primers: 5′-AACTAGGCCAAGAACAGCCC-3′; 5′-CCGAATGTCTTGGGAAGCCT-3' ; GAPDH primers 5-AGCCACATCGCTCAGACAC-3′; 5'-GCCCAATACGACCAAATCC-3'.

2.4. Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 30 min at 4 °C and rinsed with ice-cold 1X PBS (PBS;

Sigma-Aldrich). The cells were blocked with blocking solution containing 3% BSA (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich) for 45 min. at 4 °C. LDs were stained with 1 mg/ml BODIPY®493/503 (Thermo Fischer Scientific) in 150 mM NaCl solution for 10 min at room temperature. For Lysotracker®Red-DND-99 (Thermo Fischer Scientific) staining, the cells were treated with 100 nM Lysotracker®Red-DND-99 for 1 h at room temperature. For immunocytochemistry analysis, the cells were treated with primary antibody for 1 h at room temperature, rinsed with 1X PBS, and incubated with Alexa fluor® secondary antibodies (Abcam) at room temperature for 1 h. The cells were rinsed with 1X PBS and mounted with anti-fade mounting medium (Thermo-Fischer Scientific).

Confocal microscopy (Carl Zeiss LSM-710, Germany) images were visualized by using plan-apochromat, 63×/1.40 Oil DIC (differential interference contrast) M27 objective lens. Excitation/Emission wavelength of fluorescence signals was were as follows: Blue: 358/463 nm; Green: 493/520 nm; Red: 576/603 nm. Merged images showing the overlay of green and red signals were obtained by Carl Zeiss ZEN software. Co-localized pixels between green and red channels were acquired by the appearance of yellow signal that was confirmed by ZEN image co-localization analysis (https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zen-aim_colocalization.pdf).

2.5. Data analysis

For quantitative analysis of LDs and LC3 dots, ImageJ (http://imagej.nih.gov/ij/) 'Particle Analysis' function was used with threshold settings of size (pixel^2) from 0 to infinity and circularity from 0 to 1.

The intensity of the protein bands of the immunoblots was quantified using ImageJ.

Statistical analysis was performed by two-way ANOVA and Tukey's Honest Significant Difference tests by using GraphPad Prism 6 software (USA). Data were reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Lipid droplet accumulation in patient and control fibroblast cells

In order to investigate the effects of PNPLA1 protein on the metabolism of LDs, we used primary fibroblast cultures of ARCI patients with PNPLA1 mutations p.Y245del and p.D172N (Fig. 1A). In the fibroblast cells of patients and control subjects, neutral lipids in LDs were stained with BODIPY®493/503 (Fig. 1B and C). Under normal conditions, smear-like small LDs distributed through the whole cytoplasm were observed in control fibroblast cells. On the other hand, LD accumulation with abnormal quantity and size was clearly detected in the fibroblast cells of patients, under physiological conditions. In order to increase intracellular neutral lipid accumulation, cells were treated with the oleic acid-supplemented medium. Accumulation of LDs was prominently increased in oleic acid-treated control cells compared to counterparts that were cultured in the regular medium. Yet, there was no pronounced change in the numbers of LDs in fibroblasts of patients following oleic-acid treatment. Nevertheless, the size of LDs seemed more markedly increased in patient fibroblasts, and the signal was more pronounced around the nucleus (Fig. 1B).

We also performed quantitative measurements of the number and mean size of LDs. In fibroblasts of patients, a statistically significant increase in the number of LDs per cell was detected in both oleic acid-free and oleic acid-supplemented conditions compared to control fibroblasts ($P < 0.0001$) (Fig. 1C). In addition, the average sizes of LDs were calculated. The size of LDs in patient fibroblasts were significantly larger than that of control cells ($P < 0.01$), both in oleic acid-free and oleic acid-supplemented conditions (Fig. 1D). PNPLA1 mutations increased the number and size of LDs in patient fibroblasts under basal conditions, and the size of LDs rather than number was augmented following lipid challenge.

In order to determine the possible role of PNPLA1 in lipid mechanisms, we knocked down PNPLA1 expression using specific siRNA in control fibroblasts, and quantified the number and the size of LDs. siRNA transfection has efficiently downregulated the expression of PNPLA1 at mRNA and protein level (Fig. 1E). Strikingly, knockdown of PNPLA1 significantly increased LD accumulation under both normal and oleic acid-supplemented conditions (Fig. 1F and G). The mean size of LDs in PNPLA1 siRNA-transfected cells was also larger than that of control cells (Fig. 1H).

3.2. Determination of intracellular expression and localization of PNPLA1 protein

We wondered whether the mutations affect the cellular localization or expression of PNPLA1 protein or not. Immunoblot analysis showed that PNPLA1 protein was expressed in both control and patient fibroblast cells (Fig. 2A). Of note, this is the first study showing PNPLA1 protein expression in fibroblast cells. PNPLA1 mutations did not prominently affect the expression levels of the protein in fibroblasts.

It is known that PNPLA1 protein is localized on the surface of LDs. In order to determine the localization of PNPLA1 protein in patient-derived fibroblast cells, we stained LDs with BODIPY®493/503 and PNPLA1 protein with a specific antibody. Our confocal microscopy analyses showed that PNPLA1 protein co-localized with LDs in both control and patient-derived cells. So, p.Y245del and p.D172N mutations had no effect on the intracellular localization of the PNPLA1 protein (Fig. 2B).

For further check the expression and localization of PNPLA1, we pre-incubated PNPLA1 antibody with blocking peptide. Consistent with our previous results, PNPLA1 protein was expressed in fibroblast cells (Fig. 2C) and it localized on LDs (Fig. 2D) that is proven by attenuation of signal with blocking peptide.

3.3. Investigation of lipophagy-mediated regulation of LDs

Next, we wondered whether the abnormal accumulation of LDs was a result of an impairment in lipid degradation mechanisms, lipolysis or lipophagy. Since PNPLA1 does not have a role in TG lipolysis, we focused on the lipophagy mechanism. Beclin 1 (BECN1) protein is a key component of the lipophagy pathway playing a role in the nucleation of autophagosomes. Thus, we first analyzed cellular levels BECN1 protein under basal, exogenous oleic acid, or Torin treated conditions. We observed that in control cells, BECN1 protein levels were upregulated upon exogenous oleic acid or Torin treatment. In contrast, changes in BECN1 levels were less in treated versus non-treated patient fibroblast cells (Fig. 3A).

Next, we analyzed MAP1LC3B (microtubule-associated light chain 3 beta; LC3 herein) protein, as a marker of lipophagy. LC3 dot formation is a commonly used test for the activation of lipophagy. In this technique, induction of lipophagy leads to the association of diffuse cytoplasmic LC3 protein with autophagosomes, resulting in a punctated pattern upon fluorescent staining. BODIPY®493/503-Anti LC3 fluorescent co-staining showed that, under normal conditions, LDs were partially co-localized with LC3 dots in control fibroblast cells reflecting basal lipophagic activity (Fig. 3B). Under same conditions, co-localization of LC3 and LDs was markedly less in fibroblast cells of patients. Treatment with oleic acid stimulated co-localization of LC3 with LDs in both control and patient cells (Fig. 3B). Quantification of LC3 dot formation demonstrated that

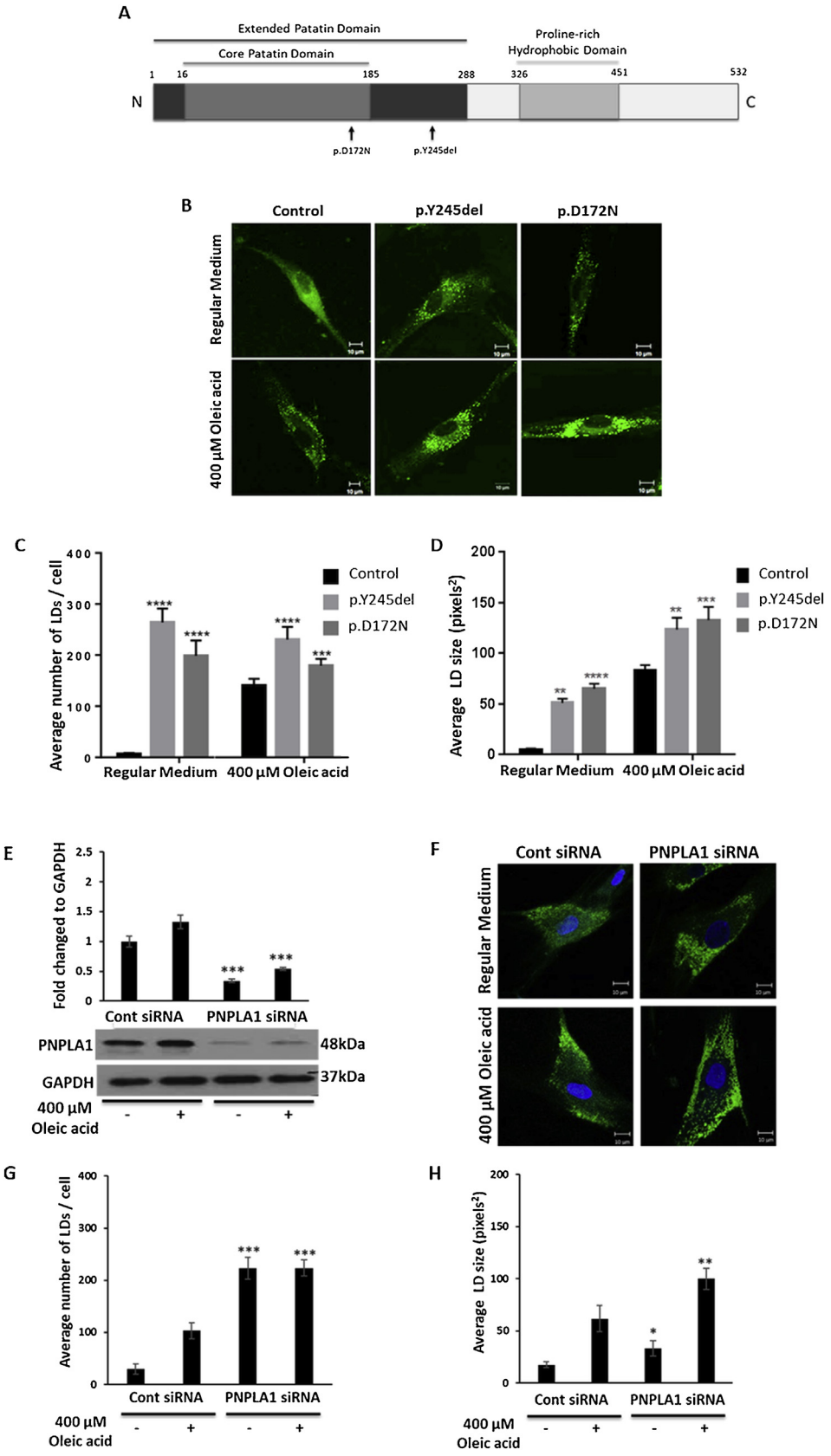

Fig. 1. PNPLA1 mutations enhance lipid droplet accumulation in fibroblasts. (A) Schematic representation of the PNPLA1 protein with conserved domains and mutations (p.D172N and p.Y245del). (B) Visualization of lipid droplet accumulation. Fibroblasts from control subjects and ARCI patients with PNPLA1 mutations (p.Y245del and p.D172N) were incubated either with regular medium or regular medium supplemented with 400 µM oleic acid for 12 h. (C) The average number of lipid droplets per cell. (D) Average lipid droplet size in the cells was quantified by Image J 'particle analysis' function. (E) Downregulation of PNPLA1 expression in control fibroblast cells upon siRNA transfection under normal and oleic acid treatment conditions. GAPDH was used as an internal control. (F) Visualization of lipid droplet accumulation in control fibroblast cells transfected with control and PNPLA1 siRNAs. (G, H) Average number and size of lipid droplets in control fibroblasts upon PNPLA1 siRNA transfection. Lipid droplets were stained with BODIPY® 493/503. Scale bar = 10 µm. Data were represented as means of ±SEM of n = 3 independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0,001$, ****$p < 0.0001$.

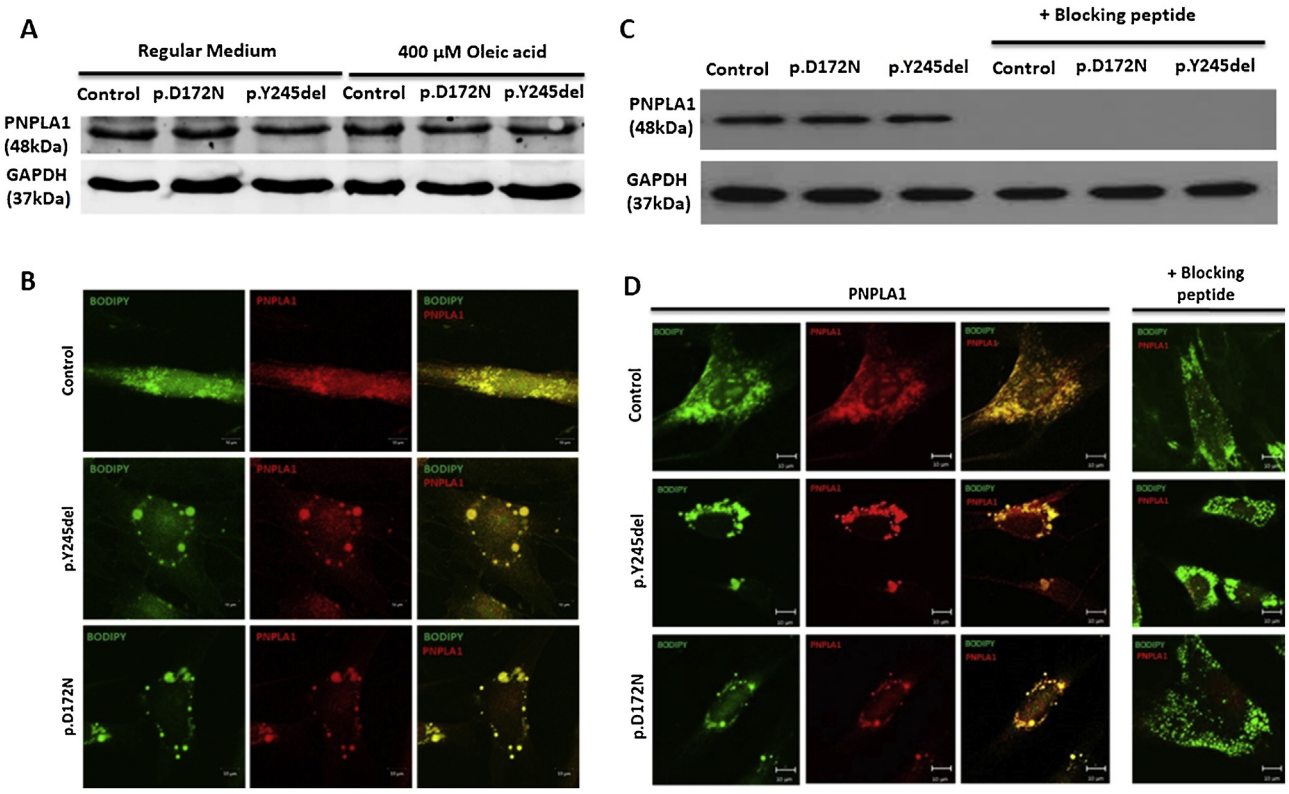

Fig. 2. Expression and subcellular localization of wild type and mutant PNPLA1 in the fibroblasts. (A) Fibroblasts from control subjects and ARCI patients with PNPLA1 mutations (p.Y245del and p.D172N) were incubated with regular medium or regular medium supplemented with 400 μM oleic acid for 12 h. Expression levels of the PNPLA1 protein was measured by immunoblotting. (B) Fluorescence images of control and patient cells were obtained by indirect immunostaining with PNPLA1 antibody and fluorescent staining of lipid droplets with BODIPY®493/503. (C) Fluorescence and (D) Immunoblotting images showing PNPLA1 protein expression in the presence and absence of PNPLA1 blocking peptide. GAPDH was used as a loading control. Data were represented as means of ±SEM of n = 3 independent experiments. Scale bar = 10 μm.

the number of LC3 dots increased in both control and patient cells; however, basal number of LC3 dots per cell in patient fibroblast cells was significantly lower than that of control cells (Fig. 3C). Following oleic acid treatment, LC3 dot numbers increased in both cases (and even to a higher extent in patient fibroblasts) and the level of lipophagy in the latter was still less compared to control (Fig. 3C). LC3 protein is lipidated through conjugation to a phosphatidylethanolamine during lipophagy activation, leading to the conversion of 18 kDa cytosolic LC3-I form to 16 kDa lipid-conjugated LC3-II form. Analysis of LC3 lipidation in control cells showed that, exogenous oleic acid or Torin treatment led to a prominent increase in LC3-II/LC3-I ratios reflecting lipophagy activation in these cells. However, we observed a lower level of increase LC3-II/LC3-I ratio in patient cells (Fig. 3D). In summary, basal lipophagy levels were lower in patient fibroblasts compared to controls and upon stimulation, patient cells failed to activate lipophagy to comparable levels that were observed in control cells.

In order to investigate lipophagic delivery of LDs to lysosomes, we checked co-localization of LDs with lysosomes immunostained with lysosome-associated membrane protein type1 (LAMP1) (Fig. 4A). Under basal conditions, LDs in control fibroblasts clearly showed co-localization with lysosomes, while there was a partial co-localization of LDs with lysosomes in fibroblast cells from patients. When the cells were treated with exogenous oleic acid, still a limited co-localization of lysosomes and LDs was observed in patient fibroblast cells. For further analysis of the lysosomal association of LDs, BODIPY®493/503 and Lysotracker®Red-DND-99 co-immunofluorescence staining method was used. Lysotracker®Red-DND-99 is a special fluorescent dye that allows specific staining of acidic lysosomes in cells. In the absence of oleic acid, LDs in the control fibroblast cells showed partial co-localization with acidic lysosomes (Fig. 4B). Under similar conditions, there was no or minimal co-localization of acidic lysosomes and LDs in the fibroblast from patients. Following oleic acid treatment, LDs in patient fibroblasts showed a minimal level of co-localization with acidic lysosomes in the cells. To conclude, both lipophagy and lipophagic vesicle maturation (i.e. fusion with lysosomes) were defective in fibroblasts from ARCI patients with PNPLA1 mutations.

4. Discussion

This work shows that PNPLA1 gene mutations lead to abnormal LD accumulation in fibroblast cells of ARCI patients. Based on our results, the accumulation of LDs prominently arises from the defects in lipophagic degradation of LDs. Ordinarily, in fibroblast cells, LDs are degraded through lipophagy mechanisms at basal levels. However, in the presence of PNPLA1 mutations in fibroblasts of ARCI patients, lipophagy mechanism was failed to be activated even upon exogenous lipid stimulation.

ARCI pathology arises from defects in the formation of the epidermal cornified envelope and permeability barrier. Recently, ARCI-causing PNPLA1 protein was shown to play role in omega-*O*-acylceramide synthesis by catalyzing omega-*O*-esterification of ceramides with linoleic acid [21–23]. Acylceramides are epidermis-specific sphingolipids playing a role in skin permeability barrier formation [30]. Lack of acylceramides in epidermis due to mutations in genes playing a role in acylceramide synthesis was shown to cause ARCI by causing dry, thickened and scaly skin [12,24].

In this study, we aimed to investigate the cellular functional effects of the PNPLA1 protein in fibroblast cells of ARCI patients

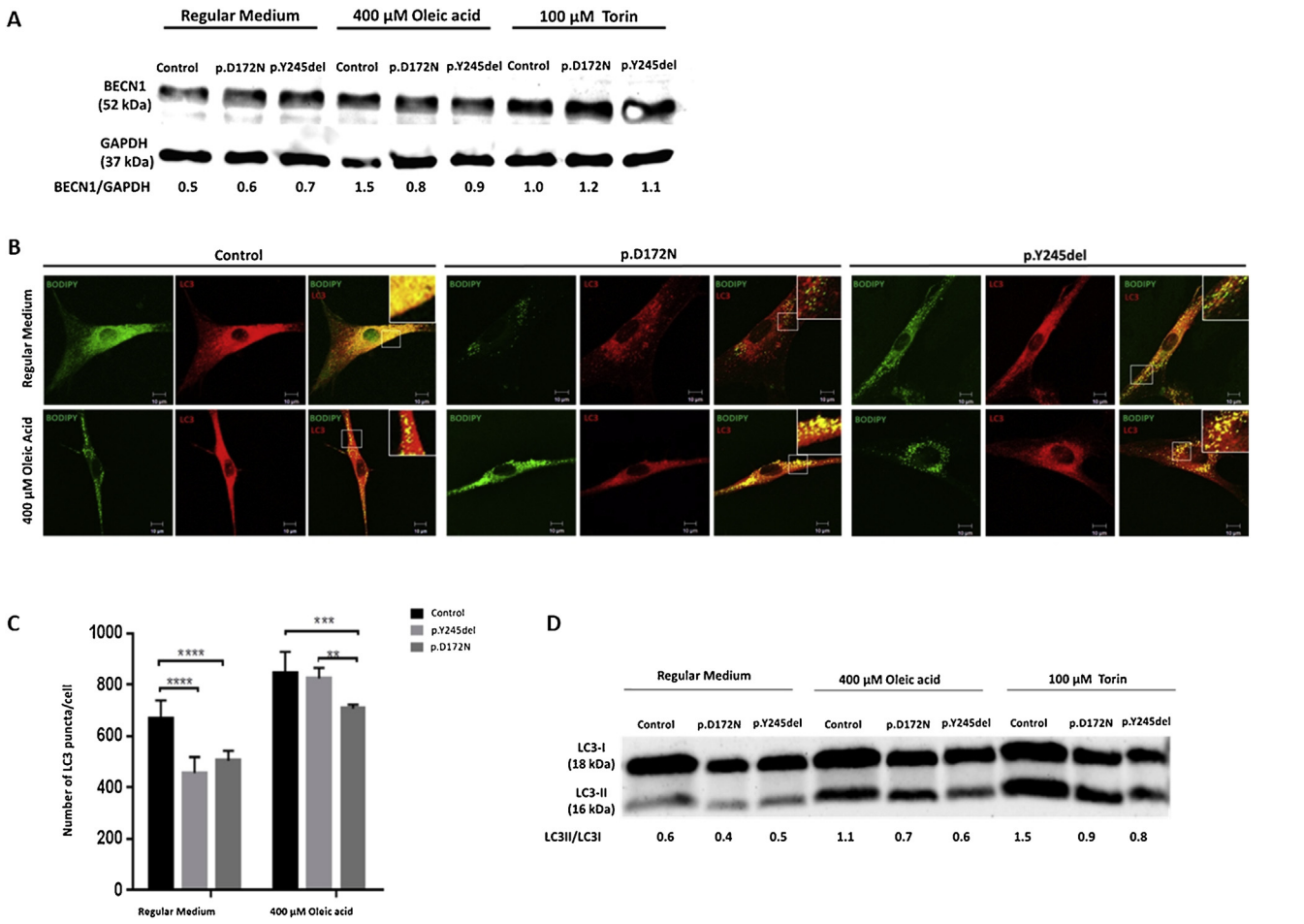

Fig. 3. Effects of the PNPLA1 mutations on autophagosome formation. The effect of the PNPLA1 mutations on autophagy initiation was determined by immunoblotting assays for (A) BECN1 and (D) LC3 protein. Cells were treated with regular medium, 400 µM oleic acid for 12 h, or 100 µM Torin for 4 h. BECN1/GAPDH and LC3II/LC3I densitometric ratios in immunoblots were analyzed by Image J software. (B) Confocal microscopy images showing the interaction between lipid droplets and LC3 protein. The cells were incubated with regular medium or regular medium supplemented with 400 µM oleic acid for 12 h. Lipid droplets were stained with BODIPY® 493/503 (green) and endogenous LC3 (red) was detected by indirect immunostaining with LC3 antibody. (C) Quantification of LC3 dot formation. LC3 dots were quantified within the minimum of 20 cells by Image J 'particle analysis'. Scale bar = 10 µm. Data were shown as mean ± SEM of n = 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0,001$, $^{****}p < 0.0001$. Scale bar = 10 µm.

with mutations (p.D172N and p.Y245del). These mutations may potentially disrupt the function of the protein according to in-silico protein function prediction tools [31]. We have found that these mutations do not affect expression levels of the PNPLA1 protein in fibroblast cells. Previously, it was suggested that PNPLA1 mRNA is not expressed in fibroblasts but rather expressed in keratinocytes of the epidermal layer [12]. However, we could observe expression of the PNPLA1 protein in fibroblast cells. Expression of PNPLA1 protein but the failure of detection of the mRNA might be due to the lack of mRNA-protein correlation in these cells. In the case of skin disorders such as psoriasis, the expression levels of mRNA and protein were shown to be modestly associated [32]. Therefore, mRNA expression levels may not be sufficient to clearly reflect the cellular aspects related to skin diseases.

We observed that PNPLA1 protein co-localizes with LDs in both patient and control fibroblast cells. PNPLA1 protein was also shown to be located on the surface of LDs in HeLa cells through the C-terminal domain [17]. In addition, recently, Kien et. al. (2018) showed that α/β hydrolase domain-containing 5 (ABHD5) protein, also known as comparative gene identification-58 (CGI-58), acts as a coactivator of PNPLA1 and recruits PNPLA1 protein onto the surface of LDs [33]. On the other hand, it was reported that GFP-tagged mouse PNPLA1 is indeed a membrane-associated protein but it does not co-localize with ER or LDs [18]. Due to the low similarity in the C-terminal sequences of human and mouse PNPLA1, the subcellular localization of PNPLA1 may differ among these species. According to our results, as p.D172 N and p.Y245del mutations did not affect C-terminal proline-rich domain, they did not change intracellular localization of the PNPLA1 protein.

Recently, it has been speculated that sphingolipids have a regulatory impact on the biogenesis and growth of LDs [34]. We wondered whether changes in cellular omega-*O*-acylceramide metabolism due to PNPLA1 mutations affect intracellular LD accumulation. Here, we report that PNPLA1 mutations cause abnormal intracellular LD accumulation in fibroblasts of ARCI patients. Studies on ARCI did not specify such intracellular LD accumulation, so far. However, some ichthyoses types including neutral lipid storage disease with ichthyosis (known as Chanarin-Dorfman syndrome) and nonbullous congenital ichthyosiform erythroderma are characterized by the accumulation of cytosolic LDs in various tissues including epidermal basal layer and leukocytes [35]. This study is the first one reporting abnormal LD accumulation in ARCI patients with PNPLA1 mutations.

Abnormal accumulation of LDs in fibroblast cells of ARCI patients with PNPLA1 mutations may be related to the failure in degradation mechanisms of LDs, lipolysis or lipophagy. It is known

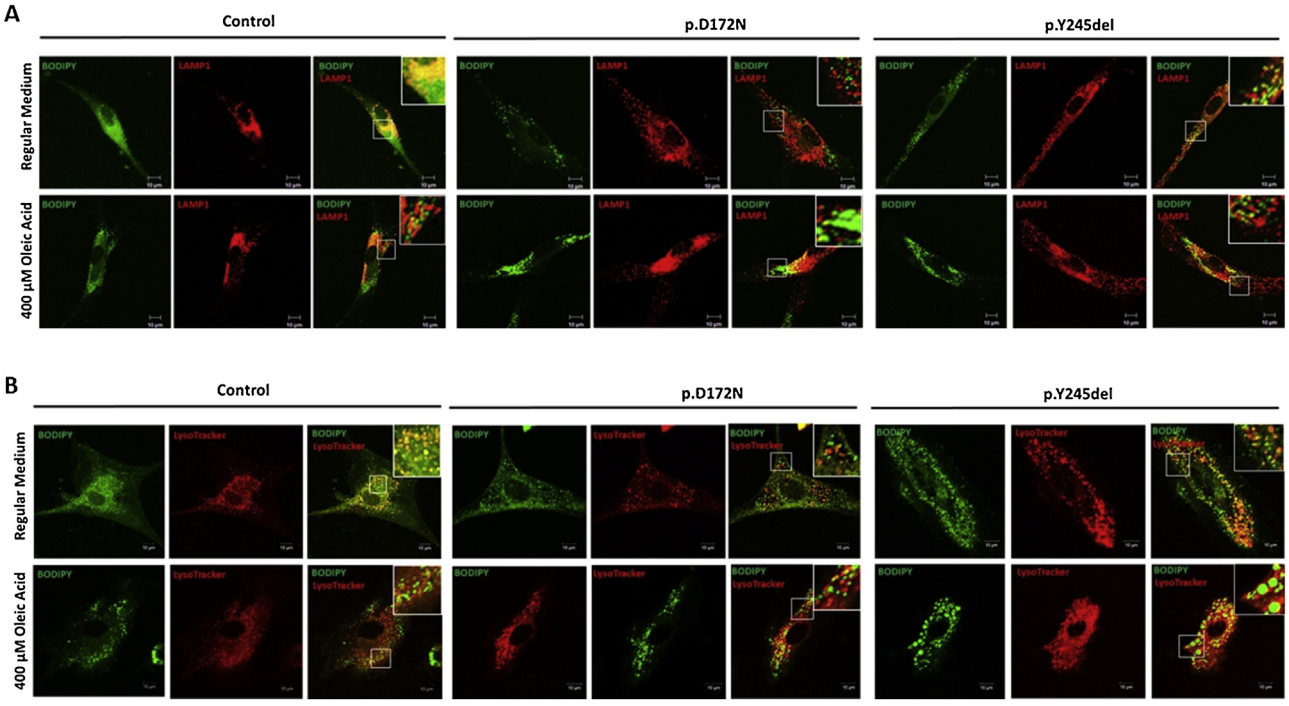

Fig. 4. Interaction of lysosomes with lipid droplets. (A) Co-localization of lipid droplets and lysosomes. Lipid droplets were stained with BODIPY® 493/503 (green) and LAMP1 (red) was detected by indirect immunostaining with LAMP1 antibody. (B) Lipid droplets were stained with BODIPY® 493/503 (green) and intracellular acidic lysosomes were stained with Lysotracker® Red-DND-99. The cells were treated with regular medium or regular medium supplemented with 400 μM oleic acid for 12 h. Scale bar = 10 μm.

that PNPLA1 protein lacks triglyceride lipase activity and is not involved in the lipolytic degradation process [12]. Therefore, alternatively, we checked if the mutations in the PNPLA1 protein might have an effect on the lipophagic degradation of LDs. Our results showed that both autophagosome formation and autophagosome/lysosomal fusion of lipophagy mechanism was defective in PNPLA1 mutant cells. Therefore, mutant PNPLA1 resulted in the abnormal accumulation of LDs in the cytosol due to impaired lipophagy in fibroblasts of ARCI patients, contributing to the pathology.

Cellular lipophagic degradation of LDs may be controlled by several direct or indirect regulators. In hepatocytes, it was shown that sirtuin-1 (SIRT1) protein directly regulates the activity of ATGL-mediated induction of lipophagy and control hepatic LD metabolism [36]. In addition, one of the members of PNPLA protein family, PNPLA5, was shown to be required for autophagosome formation [37]. Similarly, another member of PNPLA protein family, PNPLA3, was shown to play a role in lipophagic flux [38]. It was reported that PNPLA3 variants associated with increased risk of development of hepatic steatosis lead to reduced autophagic flux and lipophagy in human hepatocyte cell lines [38]. Up to now, no direct role of PNPLA1 in autophagy/lipophagy mechanism was described in the literature and this is the first study reporting its role in the lipophagy mechanism.

It is clear that lipids play major roles in the control of lipophagy by regulating signaling mechanisms for initiation, elongation, autophagosome formation or autophagosome/lysosome fusion. Hence, the role of PNPLA1 in the lipophagy may be related to its role in LD biology or its role in ceramide metabolism, since sphingolipids also contribute to the regulation of autophagy [39]. In addition, mutations in PNPLA1 protein may also lead changes in phospholipid metabolism, which may indirectly regulate degradation of LDs via lipophagy. Changes in lipid metabolism due to PNPLA1 mutations might potentially affect lipophagic degradation of LDs and lead to accumulation of cytoplasmic LDs in fibroblasts cells of ARCI patients.

To sum up, our results implicated the involvement of PNPLA1 protein in lipophagy as a pioneer study. Disturbances in lipophagy mechanism due to ARCI related mutations of PNPLA1 gene may be contributing or aggravating the defects, leading to the disease pathology. Detailed studies of these defects will further identify underlying molecular mechanisms related to ARCI pathology.

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Conflict of interest

The authors have no conflict of interest to declare.

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