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Inhibition of PLA2G15 Alleviates Palmitic Acid-Induced Lysosomal Membrane Permeabilization in Human Nucleus Pulposus Cells During Intervertebral Disc Degeneration

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ABSTRACT

Objective: Intervertebral disc degeneration (IDD) imposes substantial economic and healthcare burdens, yet its mechanisms remain incompletely understood and effective pharmacological treatments are lacking. This study aimed to elucidate key factors driving IDD progression, specifically investigating lysosomal dysfunction and lysosomal membrane permeability (LMP) in human nucleus pulposus (NP) cells, and to identify potential therapeutic targets.

Methods: We employed an integrative multiomics approach (transcriptomics, proteomics, metabolomics, and lipomics) combined with functional validation. Luciferase reporter assays investigated transcriptional regulation. The role of PLA2G15 in palmitic acid (PA)-induced lysosomal dysfunction was assessed in vitro and in vivo.

Results: Integrated analysis identified lysosomal dysfunction and increased LMP as the key molecular features in human NP cells during IDD. PLA2G15 expression was significantly upregulated under PA overload conditions. PLA2G15-mediated PA-induced alterations in lysosomal membrane lipid composition and subsequent LMP by hydrolyzing lysosomal membrane phospholipids. The transcription factor C/EBP α directly bound the PLA2G15 promoter, regulating its transcription under PA overload conditions. Crucially, in vivo inhibition of PLA2G15 mitigated PA-induced LMP by restoring normal lysosomal membrane lipid composition, thereby attenuating IDD progression.

Conclusions: This study demonstrates that PLA2G15 inhibition mitigates PA-induced IDD progression by preventing lysosomal dysfunction and LMP. Our findings reveal the significance of lysosomal membrane lipid composition alterations in human NP cells and identify PLA2G15 as a potential therapeutic target for IDD treatment.

1 | Introduction

Intervertebral disc degeneration (IDD) represents a primary contributor to low back pain, resulting in considerable disability and distress for patients. This condition significantly impacts the quality of life and imposes a substantial economic burden on healthcare systems [1]. IDD is characterized by a complex

interplay of genetic, environmental, and age-related factors, contributing to a gradual deterioration of the overall health of the discs [2]. The current therapeutic approaches for IDD are limited in their efficacy and often fail to adequately address the underlying biological mechanisms that drive the degenerative process. Although surgical interventions may be necessary in certain cases, they do not always yield satisfactory outcomes and

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may lead to complications such as adjacent segment disease and instrumentation failure [3]. As such, there is an urgent need for the development of new therapeutic options for IDD. This necessitates a thorough comprehension of the pathological mechanisms underlying IDD, as well as the formulation of strategies that can effectively address this complex condition [4].

Lysosomal dysfunction has emerged as a significant factor in the pathogenesis of IDD [5, 6]. The lysosomes play a crucial role in maintaining cellular homeostasis, particularly by degrading and recycling cellular components, including damaged organelles and misfolded proteins [6]. Impaired lysosomal function can result in the accumulation of cellular debris, contributing to inflammation and cellular stress, which are key processes involved in IDD [7]. Additionally, alterations in lysosomal membrane permeability (LMP) are also of critical significance. Disruption of lysosomal integrity and increased LMP can lead to the release of hydrolytic enzymes cathepsins, reactive oxygen and Fe^{2+} , into the cytosol [8, 9]. This cytosolic release of lysosomal contents that are typically confined within the lysosomes can have detrimental consequences for the cell and may trigger cell death pathways including apoptosis, necrosis, pyroptosis, and autophagic cell death that substantially contribute to the progression of IDD [10]. The micro-environment within the disc tissue is characterized by acidity, limited nutrient supply, hypoxia, and lipotoxicity [11, 12]. However, the mechanisms influencing lysosomal membrane integrity under these pathological conditions remain poorly understood.

The presence of an aberrant micro-environment, marked by an excessive accumulation of palmitic acid (PA), has been identified in IDD in our previous study [13]. PA is implicated in various cellular dysfunctions, particularly those associated with lysosomal integrity and dysfunction [14]. PA activates the NF- κ B signaling pathway, resulting in the upregulation of inflammatory cytokines such as IL-6, TNF- α , IL-1 β , and these cytokines can mediate cell death through the lysosomal pathways [15]. Exposure to PA in human umbilical vein endothelial cells resulted in an elevation of oxidative stress and inflammation, both of which are closely associated with lysosomal integrity [15, 16]. Similarly, PA was found to induce an inflammatory response that impairs the migration of placental trophoblasts potentially through mechanisms involving plasminogen activator inhibitor-1, which is also linked to lysosomal function [17, 18]. Our previous studies have demonstrated that PA induces lipid droplet accumulation and senescence in human nucleus pulposus (NP) cells via the endoplasmic reticulum (ER) stress pathway [12]. The induction of ER stress by PA has been associated with impaired autophagic flux and lipophagy, further complicating the relationship between lipotoxicity induced by PA, lipid metabolism, and lysosomal function [19]. Thus, the evidence presented here further emphasizes the pathogenic mechanism of PA in promoting IDD through lysosomal pathways, highlighting their significance as crucial targets for potential therapeutic interventions.

The current study utilized an integrative multiomics approach, including transcriptomics, proteomics, metabolomics, and lipidomics, to systematically elucidate the critical molecular events and perturbed pathways associated with lysosomal dysfunction and IDD. We demonstrated that PA induces LMP and further propose that this effect is mediated through the activation of

PLA2G15, which can cleave the fatty acyl linkage of glycerophospholipids, thereby hydrolyzing phospholipids in the lysosomal membrane. Hence, we further investigated the role of PLA2G15 inhibition in preventing PA-induced lysosomal membrane damage in human NP cells. Our results suggest that PLA2G15 inhibition would be a potential therapeutic target for alleviating LMP in IDD.

2 | Materials and Methods

2.1 | Patient Samples and NP Cells Culture

The institutional ethics committee approved this study, and written consent was obtained from each participant. NP samples were collected from patients undergoing discectomy. Before surgery, all patients had a standard MR lumbar spine examination, and disc degeneration was assessed using MR T2-weighted images according to the Pfirrmann classification (grades I–II for early-stage, III–V for advanced-stage). The collected NP tissues were sectioned and enzymatically digested with 0.25% Type II collagenase at 37°C for 4 h. Then, the isolated NP cells were suspended in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, and maintained in a 37°C humidified incubator with 95% air and 5% CO_2 [4]. Moreover, the cells were employed for immunofluorescence staining in order to ascertain the expression of collagen II, thereby validating the identity of the cultured cells as NP cells. The culture medium was refreshed every 3 days. NP cells from the second passage were used for subsequent experiments.

2.2 | Transcriptomics, Proteomics, Metabolomics and Lipidomics Sequencing

The transcriptomic, proteomic, metabolomic, and lipidomic sequencing were conducted by Ouyi Biotech (Shanghai, China). Detailed methodologies can be found in our previously published work [4, 12, 13]. For RNA-seq, genes were considered significantly differently expressed if they reached a difference threshold with a false discovery rate (FDR) < 0.05, p < 0.05, and a fold change > 2 or < 0.5, respectively. For protein quantization, it was required that a protein contains at least 1 unique peptide, with a FDR < 0.01. Identified proteins with a p < 0.05 and fold change > 1.2 or < 0.83 were considered to be upregulated and downregulated, respectively. The criteria for identifying significantly differentially abundant metabolites were a variable importance in the projection (VIP) value > 1 and a p < 0.05 in the orthogonal partial least squares discriminant analysis (OPLS-DA) model.

2.3 | Subcellular Fractionation

Approximately 1×10^6 NP cells were collected for subcellular fractionation and the preparation of cytosolic and lysosomal-enriched fractions, utilizing the Lysosome Enrichment Kit (Solarbio, China). Briefly, the cell pellet was re-suspended in PBS and subjected to homogenization using a dounce homogenizer on ice, with approximately 20 strokes. Then the

homogenate was subjected to differential centrifugation in accordance with the protocol of the manufacturer to spin down the crude lysosomal fractions, and the resulting supernatant fractions were collected as cytosolic fractions [8, 9]. Isolated subcellular fractionations were stored at -80°C for further analysis.

2.4 | Lysosomal Enzyme Assay and LMP Evaluation

The activities of cathepsin D (CTSD) (Proteintech, China) and alpha-N-acetylglucosaminidase (NAGLU) (Sigma-Aldrich, USA) were determined following the protocols provided by the manufacturers. Enzyme activities in the lysosomal fraction or cytosolic fractions were estimated as relative fold change in optical density.

CTSD and NAGLU are typical enzymes located within lysosomes. Under normal physiological conditions, the acidic lumen of the lysosome confines these soluble hydrolases. Upon LMP, the integrity of the lysosomal membrane is compromised, allowing the leakage of these enzymes from the lysosomal compartment into the cytosol. Therefore, the detection of CTSD and NAGLU staining in the cytosolic fraction serves as a biochemical hallmark of LMP, indicating a loss of lysosomal barrier function.

2.5 | Protein Preparation and Western Blotting

The proteins were extracted using RIPA buffer supplemented with a protease inhibitor, phenylmethylsulfonyl fluoride (Beyotime, China). Postextraction, the proteins were subjected to isolation and separation through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer onto a polyvinylidene difluoride membrane. To minimize non-specific binding, the membrane was blocked with 5% skim milk. Subsequently, the bands were incubated overnight with a primary antibody. The specific primary antibodies used are detailed below: anti-GAPDH (Servicebio, China; 1:3000 dilution), anti-LAMP1 (Zenbio, China; 1:1000 dilution), anti-CTSD (Proteintech, China; 1:5000 dilution), anti-PLA2G15 (Proteintech, China; 1:1000 dilution), anti-LC3B (Huabio, China; 1:1000 dilution), anti-P62 (Huabio, China; 1:5000 dilution), anti-Beclin-1 (Huabio, China; 1:1000 dilution), anti-C/EBP α (Proteintech, China; 1:1000 dilution). Following a series of washes, the bands were incubated with a horseradish peroxidase-conjugated secondary antibody from Proteintech in China for 1 h, and then visualized employing a chemiluminescence system.

2.6 | Transmission Electron Microscopy (TEM)

The NP cells were fixed overnight in 2.5% glutaraldehyde solution, then treated with 1% osmium tetroxide at 4°C for 1 h. Next, they were dehydrated with ethanol of gradually increasing concentrations (30%, 50%, 70%, 90%), with each step lasting 30 min, followed by three more dehydrations in pure ethanol for 30 min each. After that, the samples were treated with an embedding medium containing propylene oxide. Seventy nanometers of

ultrathin sections were cut using an LKB—V ultramicrotome and placed on copper grids. The sections were stained with lead citrate at room temperature for 10 min, rinsed three times with deionized double-distilled water, then stained with uranyl acetate at room temperature for 30 min and rinsed three more times. Finally, images were captured using a JEM-1400 transmission electron microscope system.

2.7 | Lyso-Tracker Staining

Lysosomes were assessed using Lyso-Tracker Red staining (Beyotime, China) following the manufacturer's instructions. The cells were incubated with Lyso-Tracker Red (50 nM) at 37°C for 30 min and subsequently imaged using confocal microscopy. The fluorescence intensity was then quantified using Image J software.

2.8 | Immunofluorescence Staining (IF)

The NP cells cultured in confocal dishes were fixed with 4% paraformaldehyde for 20 min, followed by two rinses in PBS. After permeabilization with 0.1% Triton for 15 min, the samples were blocked with 2% goat serum for 30 min. Subsequently, the samples were incubated overnight at 4°C with a primary antibody, and Alexa Fluor-594 or Alexa Fluor-488 was used as the secondary antibody at room temperature for 1 h. The primary antibodies utilized are as follows: anti-LAMP1 (Zenbio, China; 1:100 dilution), anti-CTSD (Proteintech, China, 1:200 dilution), anti-LC3B (Huabio, China, 1:100 dilution). The nucleus was stained with DAPI for 5 min. Finally, the fluorescent images were captured using a confocal microscope.

2.9 | Cell Transfection

The PLA2G15 overexpression plasmid and its control vectors, sh-PLA2G15 plasmid, sh-C/EBP α plasmid, and its control vectors were procured from Tsingke Biotechnology Co. Ltd. (Nanjing, China). The target sequence of PLA2G15 shRNA-1 plasmid was 5'-accgaaagtacttcacaatc-3', PLA2G15 shRNA-2 plasmid was 5'-ccgcgagatgatcgaggagat-3', PLA2G15 shRNA-3 plasmid was 5'-gtcatcattgactgctggatt-3'; the target sequence of C/EBP α shRNA plasmid was 5'-cgggtggacaagaacagcaa-3'. Briefly, NP cells were seeded in 35 mm dishes overnight, and then the plasmids were transfected using Lipofectamine 2000 and Opti-MEM (Invitrogen, USA) according to the manufacturer's instructions. Before further experiments, the efficacy of transfection was measured by WB.

2.10 | Animal Experiments

All animal studies were approved by the institutional animal care and use committee of the first affiliated hospital of USTC. Three-month-old male Sprague-Dawley rats were used for the animal experiments according to methods as reported previously [12, 13]. The rats were randomly divided into five groups, each consisting of five rats: Control group, PA group with PA injection, sh-Control group with PA injection + vector

adenovirus, sh-C/EBP α group with PA + sh-C/EBP α adenovirus injection, and sh-PLA2G15 group with PA + sh-PLA2G15 adenovirus injection. Following anesthesia, the rat IDD model was established by injection of 3 μ L PA to construct degenerated models. The injection procedure was performed at 7 days post-IDD induced surgery and repeated every week three times with control adenovirus, sh-C/EBP α or sh-PLA2G15 adenovirus (1.3×10^{10} vector genomes in 3 μ L) by direct injection into the rat coccygeal IVDs with a 33-gage needle (Hamilton, Switzerland). In contrast, the PA group received an intradiscal injection of saline solution into their discs. These drug injections were performed once weekly for a total duration of 3 weeks.

2.11 | Histology and Immunohistochemical Staining

For human NP tissues, the samples were initially fixed in a 4% paraformaldehyde solution. Subsequently, the NP tissues underwent dehydration and paraffin embedding before being sectioned into slices with a thickness of 5 μ m. The slides containing the samples were subjected to staining using hematoxylin and eosin (HE), Masson, and Alcian blue stains.

As for the rat tail, the Co8–Co9 level, which encompassed the entire upper endplate of the head-end vertebra and the lower endplate of the tail-end vertebra, was collected and fixed in a 4% paraformaldehyde solution at 4°C overnight. Subsequently, the samples underwent decalcification using a 10% EDTA solution for a period of 1 month. Following decalcification, the samples were dehydrated and embedded in paraffin. The paraffin blocks were then cut into 5 μ m slices in the coronal plane. The slices of the rat tail were stained using HE, safranin O/fast green (S–O), and Alcian blue staining.

Immunohistochemical (IHC) staining was conducted on both human NP tissues and rat tail samples. Prior to staining, the sections underwent deparaffinization and rehydration, followed by microwave boiling in 0.01 mol/L citrate buffer (pH 6.0) for 15 min. Subsequently, the sections were blocked with goat albumin for 30 min and incubated overnight at 4°C with the primary antibodies as follows: anti-PLA2G15 (Proteintech, China; 1:100 dilution), anti-C/EBP α (Proteintech, China; 1:200 dilution). After incubation with the primary antibody, a secondary antibody was applied to the samples for 1 h at room temperature, followed by hematoxylin staining.

2.12 | Statistical Analysis

The data were presented as the mean $\pm$ standard deviation, and a minimum of three independent experiments were conducted. Statistical differences between two groups were determined using Student's *t*-test for normally distributed data or the Mann–Whitney *U* test for nonnormally distributed data. For multigroup comparisons, one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test was used for normally distributed data, while the Kruskal–Wallis test followed by Dunn's post hoc test was applied for nonnormally

distributed data. Spearman's rank correlation test was used to calculate the correlation between two quantitative variables. Statistical significance was determined using GraphPad Prism software, with a threshold set at $p < 0.05$.

3 | Results

3.1 | Lysosomal Dysfunction Was Detected During IDD

The severity of IDD was assessed using T2-weighted MR imaging (Figure 1A). To evaluate the histological degeneration in the NP tissues, HE, Alcian blue, and Masson staining were employed (Figure 1B). In our previous works, we conducted a comprehensive transcriptomic and proteomic sequencing with the objective of elucidating the molecular characteristics of IDD (Figure 1C). The volcano plot depicts the differential expression of genes and proteins between the early-stage and advanced-stage IDD (Figure 1D,E). GSEA performed on both transcriptomic and proteomic data revealed a significant correlation between “lysosome” and the occurrence of IDD (Figure 1F–H). The KEGG analysis of proteomic data identified “lysosome” as a significantly differentially expressed item (Figure 1I). Both CTSD and NAGLU activity exhibited similar trends, with decreased levels in lysosomal fractions and increased levels in cytosolic fractions in advanced-stage IDD (Figure 1J,K). Additionally, WB analysis revealed that the levels of CTSD were decreased in lysosomal fractions and increased in cytosolic fractions in advanced-stage IDD when compared to the early-stage (Figure 1L–N). IF staining demonstrated the leakage of CTSD from lysosomes into the cytosol in advanced-stage IDD (Figure 1O). Both lyso-tracker labeling and TEM observation revealed the loss of healthy lysosomes in advanced-stage IDD (Figure 1P,Q). These findings suggest that lysosomal dysfunction and LMP occur during the progression of IDD.

3.2 | Lysosomal Membrane Lipid Composition Alterations in IDD

The composition of lysosomal membrane lipids is crucial for influencing lysosomal function; thus, we conducted a non-targeted metabolic analysis on the whole NP cells and a lipidomics analysis on the lysosomes. Figure 2A shows the workflow of analysis. Orthogonal partial least squares-discriminant analysis (OPLS-DA) was conducted to analyze the data; the volcano plot and heat-map showed a clear differentiation in metabolites between the early- and advanced-stage of whole NP cells (Figure 2B–D). KEGG pathway enrichment analysis was performed to identify significantly impacted metabolic pathways (Figure 2E). Then, we prepared lysosomal enriched fractions to analyze the lipid composition of lysosomal membranes. The OPLS-DA indicates a clear separation between the two groups (Figure 2F). Figure 2G,H shows a significant metabolic difference between early- and advanced-stage samples of lysosomes fractions. Lipidmap revealed elevated levels of lysosomal lysophosphatides and reduced levels of PC and PE in advanced stages, indicating lysosomal membrane hydrolysis (Figure 2I). KEGG pathway analysis revealed the most impacted pathway (Figure 2J). Subsequently, an integrative analysis was

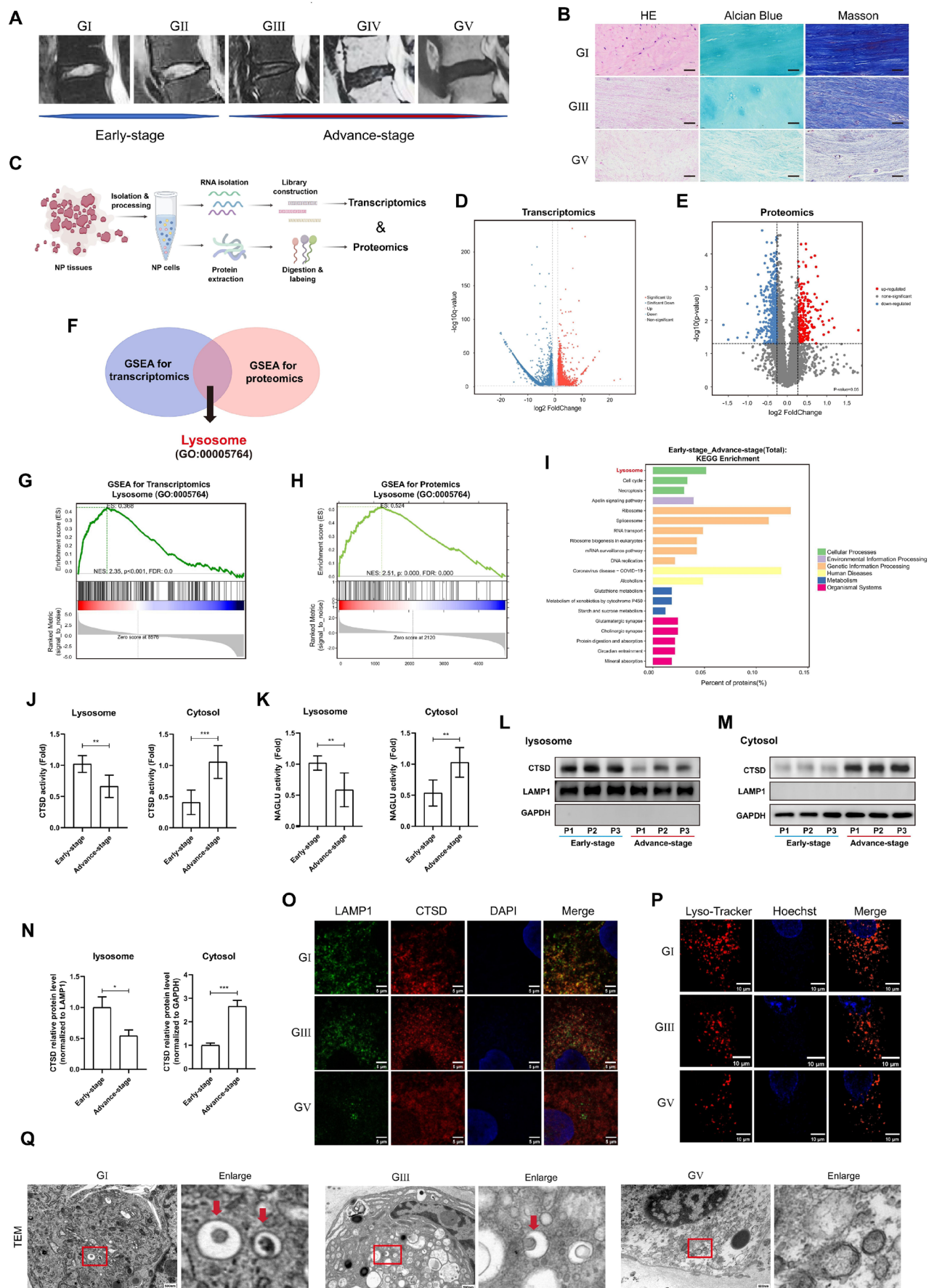


FIGURE 1 | Legend on next page.

FIGURE 1 | Lysosomal dysfunction was detected during IDD. (A) Intervertebral discs on T2-weighted MR images are graded as early-stage and advanced-stage. (B) NP tissues from early- and advanced-stage IDD are examined with HE, Alcian blue, and Masson stains. Scale bar: 50 μ m. (C) Workflow of transcriptomics and proteomics integrative analysis. (D) Volcano plot show the different genes for transcriptomics. (E) Volcano plot show the different proteins for proteomics. (F–H) The integration of transcriptomics and proteomics data has identified “lysosome” as distinct GSEA items. (I) KEGG analysis revealed “lysosome” as disturbed pathways related to IDD. (J–N) CTSD and NAGLU levels and activities are reduced in lysosomes and elevated in cytosol of advanced-stage IDD compared to early stage. $n = 3$. (O) IF staining shows CTSD (red) leakage from lysosomes (green) into cytosol in advanced-stages. Scale bar: 5 μ m. (P) Lyso-tracker staining is utilized to visualize lysosomes in human NP cells. Scale bar: 10 μ m. (Q) Representative TEM images clearly illustrate lysosomal damages in advanced-stage IDD. Scale bar: 2 μ m. Compared to early-stage, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

performed, combining the sequencing data from both the whole NP cells and lysosomal fractions. This analysis revealed the “glycerophospholipid metabolism (Has00564)” pathway (Figure 2K). Together, these results demonstrated that altered membrane lipids composition and “glycerophospholipid metabolism (Has00564)” pathway may be involved in lysosomal dysfunction.

3.3 | PA Induces Lysosomal Dysfunction in Human NP Cells

IDD features a lipotoxic environment with PA accumulation, and the PA content was positively correlated with the degree of IDD (Figure 3A,B). We further examined its impact on NP cell lysosomal dysfunction and LMP. PA (50 μ M) induces the release of lysosomal contents from lysosomes into the cytosol in NP cells after 48 h of treatment (Figure 3C–H). To further assess the biological impact of PA on NP cells, we treated the NP cells with 50 μ M PA for 48 h and categorized them into Bodipy-low and high cells for proteomics analysis (Figure 3I). PCA analysis and volcano heat map showed larger variations in proteins after PA treatment (Figure 3J,K). The GSEA and KEGG analysis identified distinct components involved in the cellular processes of “lysosome” and “lysosome lumen” (Figure 3L–N). These findings suggest that PA was involved in lysosomal dysfunction and LMP in IDD.

3.4 | PLA2G15 Is Activated and Present at Lysosomes Under PA Overload Conditions

Phospholipase (PLA) is the primary enzyme responsible for the hydrolysis of membrane phospholipids. Consequently, our study focused on the differentially expressed proteins belonging to the PLA family after PA treatment. As a result, PLA2G15 and PLA2G4E were noted (Figure 4A). The “glycerophospholipid metabolism” pathway plays a role in the synthesis and maintenance of lysosomal membrane lipid composition. We further conducted an integrative analysis of the GSEA items related to “glycerophospholipid metabolism” and “lysosome” for the proteomics between early- and advanced-stage. One of the most interesting genes identified via integrative screening was also PLA2G15 (Figure 4B–D). The expression of PLA2G15 was significantly upregulated in the advanced-stage IDD compared with early-stage IDD; thus, PLA2G15 was selected for further analysis. The IF staining revealed PLA2G15 was mainly located at lysosomes (Figure 4E–J). Under the PA treatment, the expression of PLA2G15 was significantly upregulated in NP cells (Figure 4K,L), and it accumulated in the lysosomes (Figure 4M). These results suggest that PLA2G15 is activated in response to PA treatment and is localized to the lysosomes.

3.5 | PLA2G15 Inhibition Attenuates PA-Induced Lysosomal Dysfunction in NP Cells

We next aimed to explore whether inhibiting PLA2G15 could mitigate PA induced lysosomal membrane damage and. PA increased the protein levels of PLA2G15, whereas sh-PLA2G15 transfection decreased these levels. For further experiments, sh-PLA2G15-2 was selected (Figure 5A,B). We measured activity of the lysosomal enzymes in isolated lysosomal and cytosolic fraction from early-stage, PA, PA + sh-Control and PA + sh-PLA2G15 group, respectively. As expected, the PLA2G15 inhibition attenuates the leakage of lysosomal contents (Figure 5C–F), and promotes the formation of lysosomes (Figure 5G). The TEM observation also revealed that inhibition of PLA2G15 attenuates PA-induced lysosomal damage in NP cells (Figure 5H). Whereas overexpression of PLA2G15 promotes the lysosomal LMP in advanced-stage NP cells (Figure 5I–F).

3.6 | PLA2G15 Inhibition Normalizes Lysosomal Membrane Lipid Composition and Maintain the Autophagy Activity After PA Treatment

To further determine the protective mechanism of PLA2G15 inhibition, we purified lysosomes for lipids analysis. Our data revealed clear differences in the lysosomal lipid profiles between PA and PA + sh-PLA2G15 group (Figure 6A). Importantly, there were significantly higher abundances of phospholipids and lower abundances of lysophospholipids in the sh-PLA2G15 group (Figure 6B–D). KEGG pathway enrichment analysis identified that “glycerophospholipid metabolism” was the significantly impacted metabolic pathway (Figure 6E). Lysosomal damage and LMP after PA are associated with inhibition of autophagy activity; we detected that inhibition of PLA2G15 increases the expression of Beclin1 and LC3 while decreasing the expression of P62 after PA treatment (Figure 6F,G). Consistently, we observed accumulation of both LC3 and LAMP1 by IF in NP cells after PLA2G15 inhibition as compared to PA overload (Figure 6H).

3.7 | Transcription Factor C/EBP α Promotes PLA2G15 Expression

We subsequently investigated the potential molecular mechanism underlying PA-induced upregulation of PLA2G15 in NP cells. Through bioinformatics analysis, we hypothesized that C/EBP α might act as an upstream transcription factor for PLA2G15. The expression levels of C/EBP α were found to be higher in

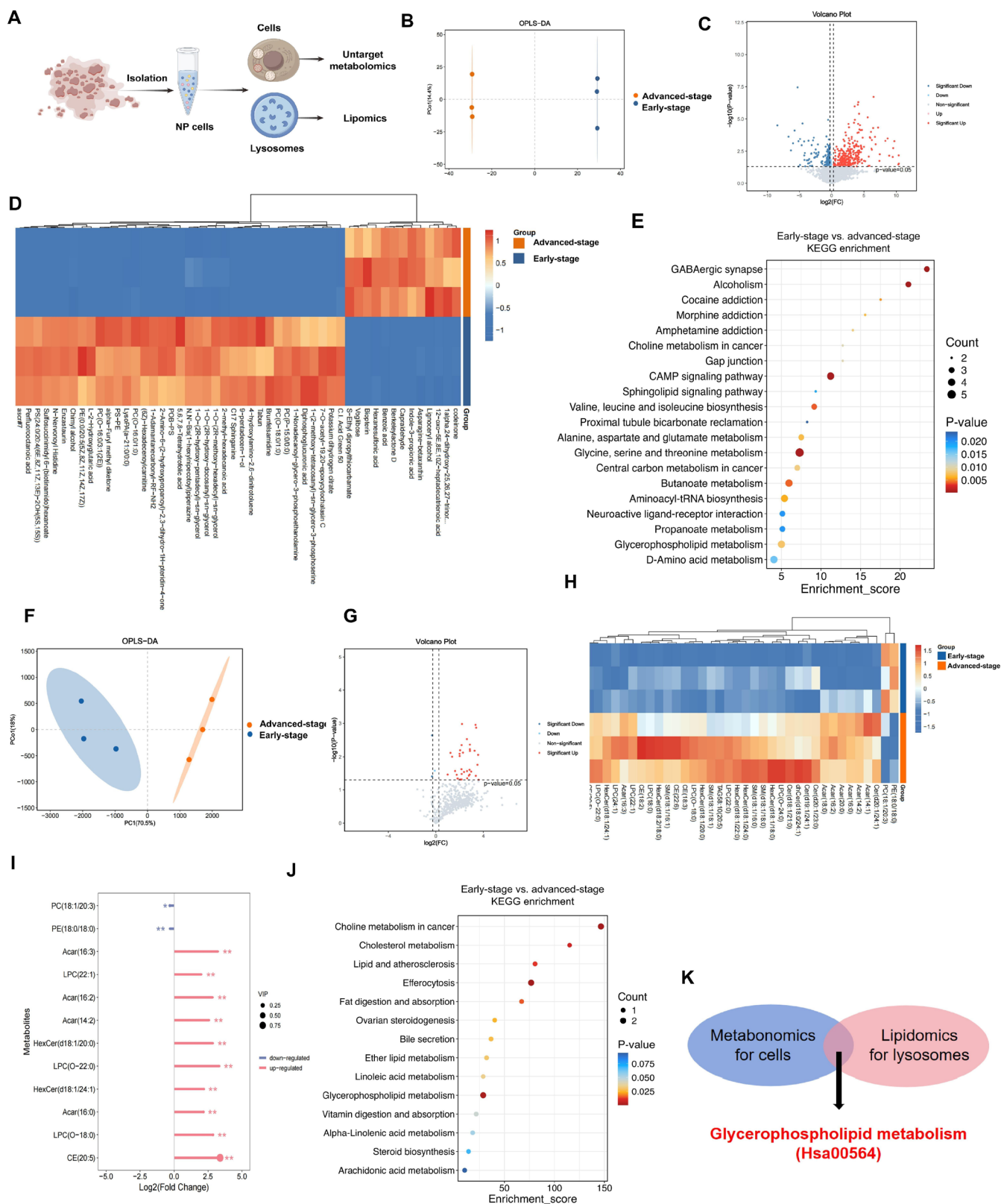


FIGURE 2 | Altered lysosomal membrane lipid composition in IDD. (A) workflow of metabolic analysis. (B) Orthogonal partial least squares-discriminant analysis (OPLS-DA) for NP cells nontargeted metabolomics. The results indicated a clear separation between the two groups. (C and D) Volcano plot and heatmaps display the significant differential metabolites. (E) KEGG analysis of the disturbed metabolic pathways related to IDD. (F) Lipidomics analysis for lysosomal fractions. The OPLS-DA for metabolic profiling indicated a clear separation between the two groups. (G and H) Volcano plot and heat-map display the significant differential metabolites. (I) Lolipommap revealed increased abundance of lysosomal lysophosphatides and decreased of PC and PE in the advanced-stage compared to the early-stage. (J) KEGG analysis of the disturbed metabolic pathways in lysosomes. (K) The integration of metabolomics and lipidomics analyses has identified “Glycerophospholipid metabolism (hsa00564)” as distinct KEGG items.

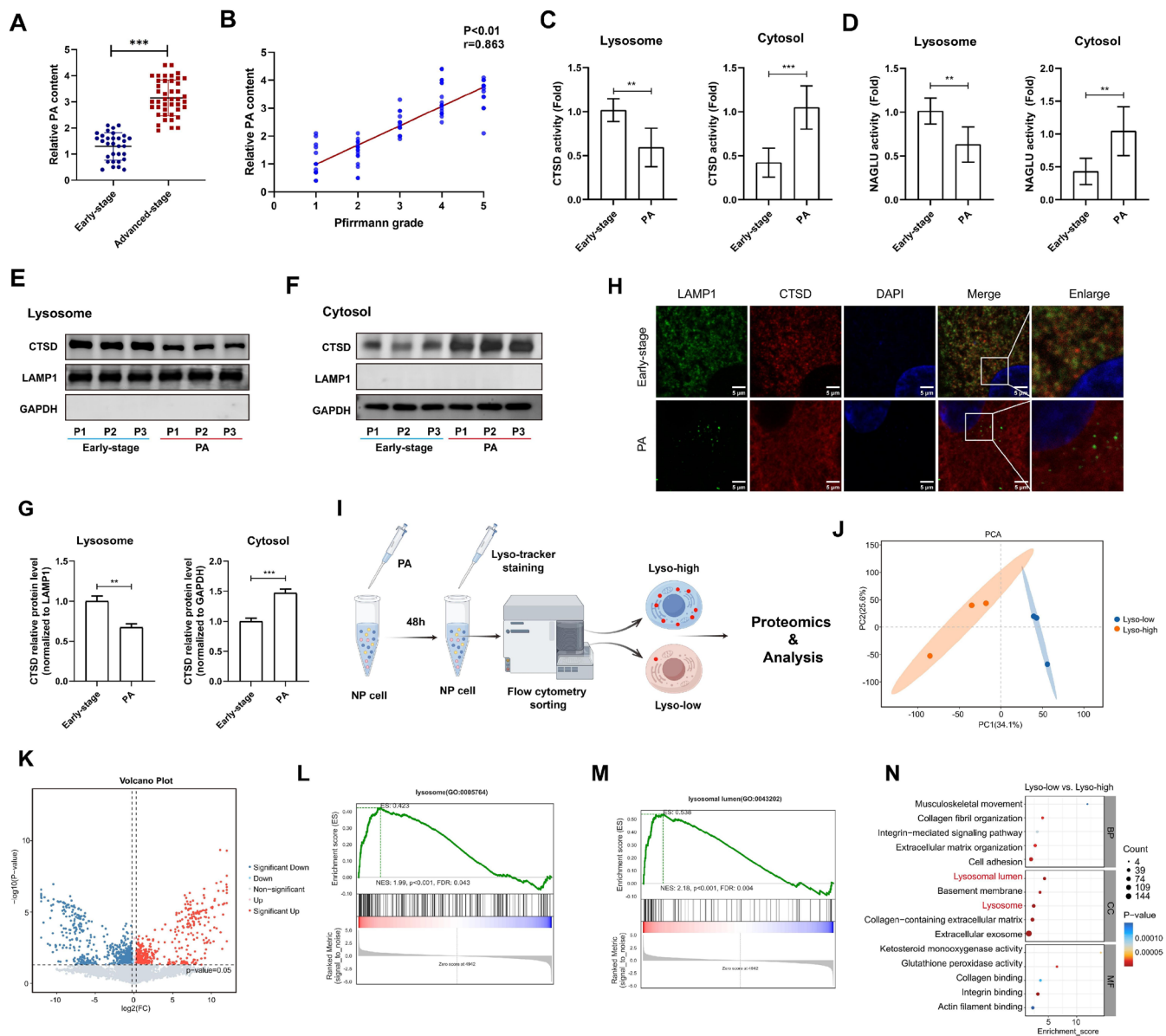


FIGURE 3 | PA induces lysosomal dysfunction. (A) The relative PA levels of NP cells determined in 31 early-stage and 43 advanced-stage IDD patients. (B) Single-factor linear regression analysis of the relationship between the relative PA contents and Pfirrmann grade. (C and D) Activity of lysosomal CTSD and NAGLU is reduced in lysosomal fractions and elevated in cytosolic fractions in human NP cells treated with PA. $n = 3$. (E–G) WB analysis and quantification of CTSD in lysosomal fractions and cytosolic following PA treatment. $n = 3$. (H) IF staining reveals leakage of CTSD from lysosomes into the cytosol following PA treatment. Scale bar: 5 μ m. (I) Workflow of PA treatment and flow cytometry sorting for Bodipy-low and -high NP cells. (J) Proteomics analysis for Bodipy-low and -high NP cells. PCA analysis indicates a clear separation between the two groups. (K) Volcano map of different proteins. (L–N) GSEA and KEGG analysis of the different proteins were related to “lysosome” and “lysosomal lumen.” Compared to early-stage or control, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

advanced-stage compared to the early-stage (Figure 7A–D). Furthermore, treatment with PA resulted in increased expression of C/EBP α in NP cells (Figure 7E,F). Consequently, a thorough analysis of the PLA2G15 promoter revealed putative C/EBP α -binding sites within its proximal promoter region (Figure 7G). Luciferase reporter analysis showed that C/EBP α interacted with WT-PLA2G15 and promoted its transcription (Figure 7H). To further elucidate the impact of C/EBP α induction on PA-mediated upregulation of PLA2G15 expression, we assessed the protein levels of C/EBP α in NP cells treated with PA and subjected to shRNA-C/EBP α (Figure 7I,J). As expected, the PLA2G15 expression

levels were significantly decreased after sh-C/EBP α transfection (Figure 7K–M). Therefore, we conclude that PA promotes PLA2G15 expression in a C/EBP α -dependent manner. The proposed regulatory mechanism is shown in Figure 7N.

3.8 | PA Induces LMP in a C/EBP α -Dependent Manner

The present study further investigated the potential effects of C/EBP α inhibition on NP cells against LMP induced by PA.

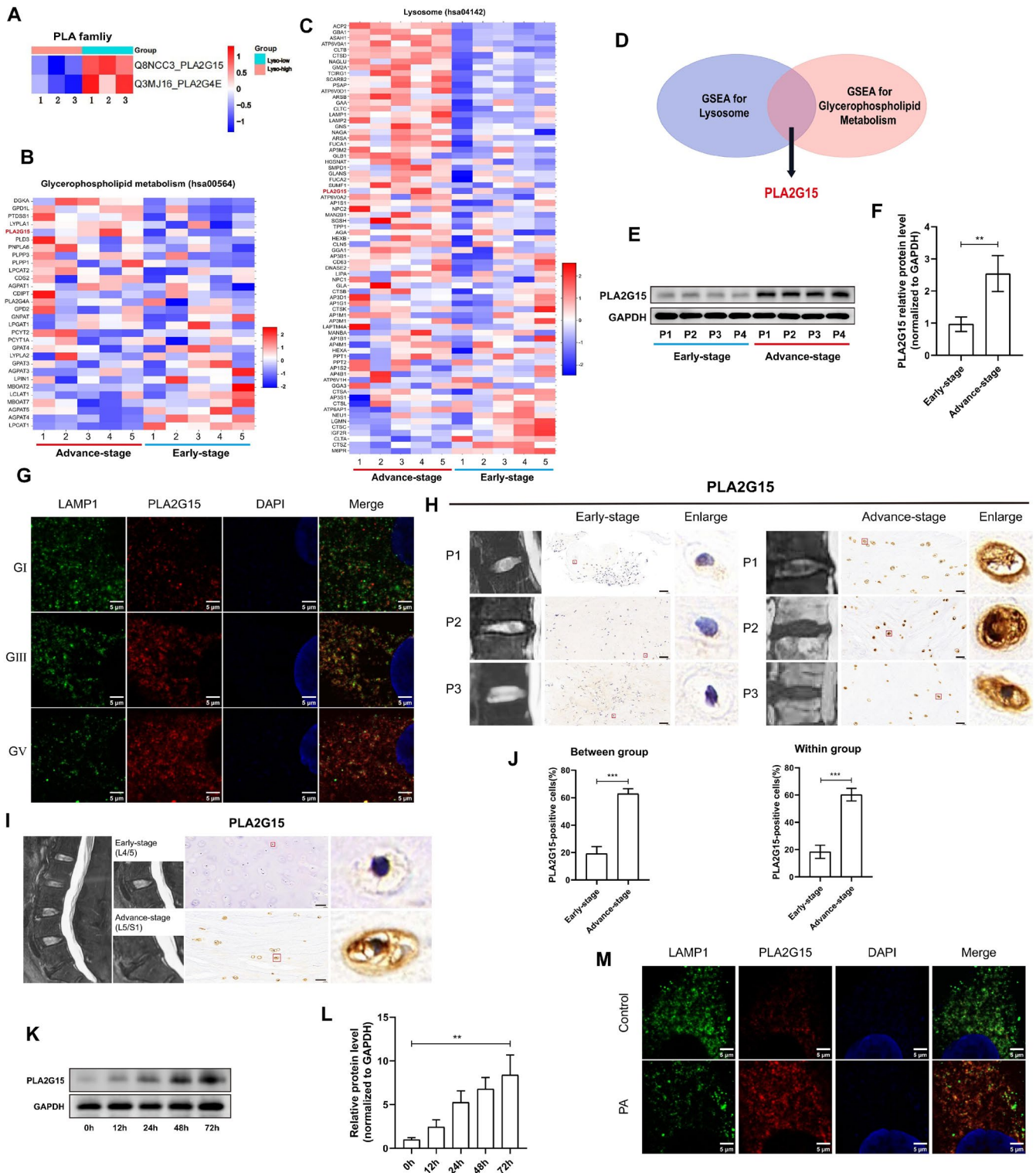


FIGURE 4 | PLA2G15 is activated and present at lysosomes after PA treatment. (A) Different expressed proteins belong to PLA family between Bodipy-low and -high NP cells. (B) GSEA enrich proteins for “glycerophospholipid metabolism pathway.” (C) GSEA enrich proteins for “lysosome.” (D) Integrative analysis revealed an upregulation of PLA2G15 protein in IDD. (E and F) WB analysis and quantification of PLA2G15 protein levels in human NP tissues. (G) The IF staining of PLA2G15 revealed its localization within lysosomes and upregulation in advanced-stage. Scale bar = 5 μ m. (H) IHC staining and quantification of PLA2G15 expression in human NP tissues. Scale bar = 50 μ m. $n = 8$. (I) PLA2G15 IHC staining was performed on pairs of human NP tissues with varying degrees of IDD severity from each patient. Scale bar = 50 μ m. $n = 8$. (J) The positivity rate is depicted in the statistical chart. (K and L) The expression of PLA2G15 was upregulated under PA treatment. (M) The IF staining demonstrated that PA induces unregulated localization of PLA2G15 in lysosomes. Scale bar = 5 μ m. Compared to early-stage or untreated group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

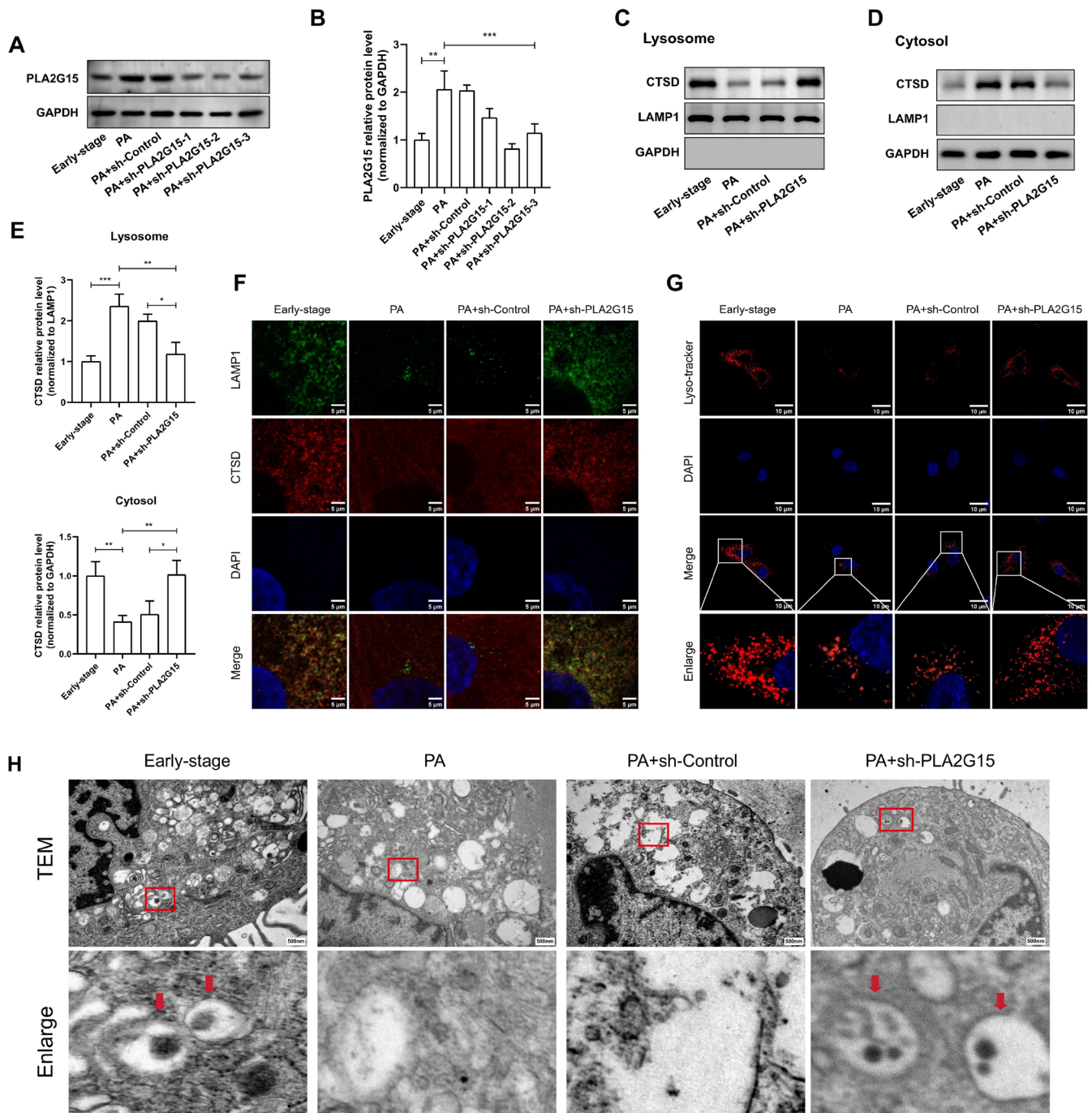


FIGURE 5 | PLA2G15 inhibition attenuates PA-induced lysosomal dysfunction and LMP. (A and B) The NP cells in each group were transfected and showed high transfection efficiency. $n=3$. (C–E) WB analysis revealed that the levels of CTSD were increased in lysosomal fractions and decreased in cytosolic fractions after PLA2G15 inhibition. (F) IF staining demonstrated that the leakage of CTSD from lysosomes into the cytosol was alleviated after transfection with sh-PLA2G15. Scale bar = 5 μm. (G) The lyso-tracker staining for lysosomes revealed that inhibition of PLA2G15 attenuates PA-induced lysosomal damage in NP cells. Scale bar = 10 μm. (H) TEM observation for lysosomes after transfection. Scale bar = 0.5 μm. Compared to early-stage or indicated group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The leakage of lysosomal contents was significantly reduced, and CTSD showed less diffusion after sh-C/EBPα treatment (Figure 8A–D). The lyso-tracker staining and TEM observation also revealed that inhibition of C/EBPα attenuates PA-induced lysosomal damage in NP cells (Figure 8E,F). In summary, the findings suggested that suppressing C/EBPα led to the inhibition of PLA2G15 expression, safeguarded lysosomes against PA overload conditions, and prevented LMP.

3.9 | Inhibition of PLA2G15 Stabilized the Lysosomal Membrane In Vivo

To determine this target for translational potential, we further evaluated whether intervention with PLA2G15 is a potential therapeutic strategy for IDD in vivo. We established an IDD model in SD rats through PA injection; MR images demonstrated that PA injection effectively induced IDD. Furthermore,

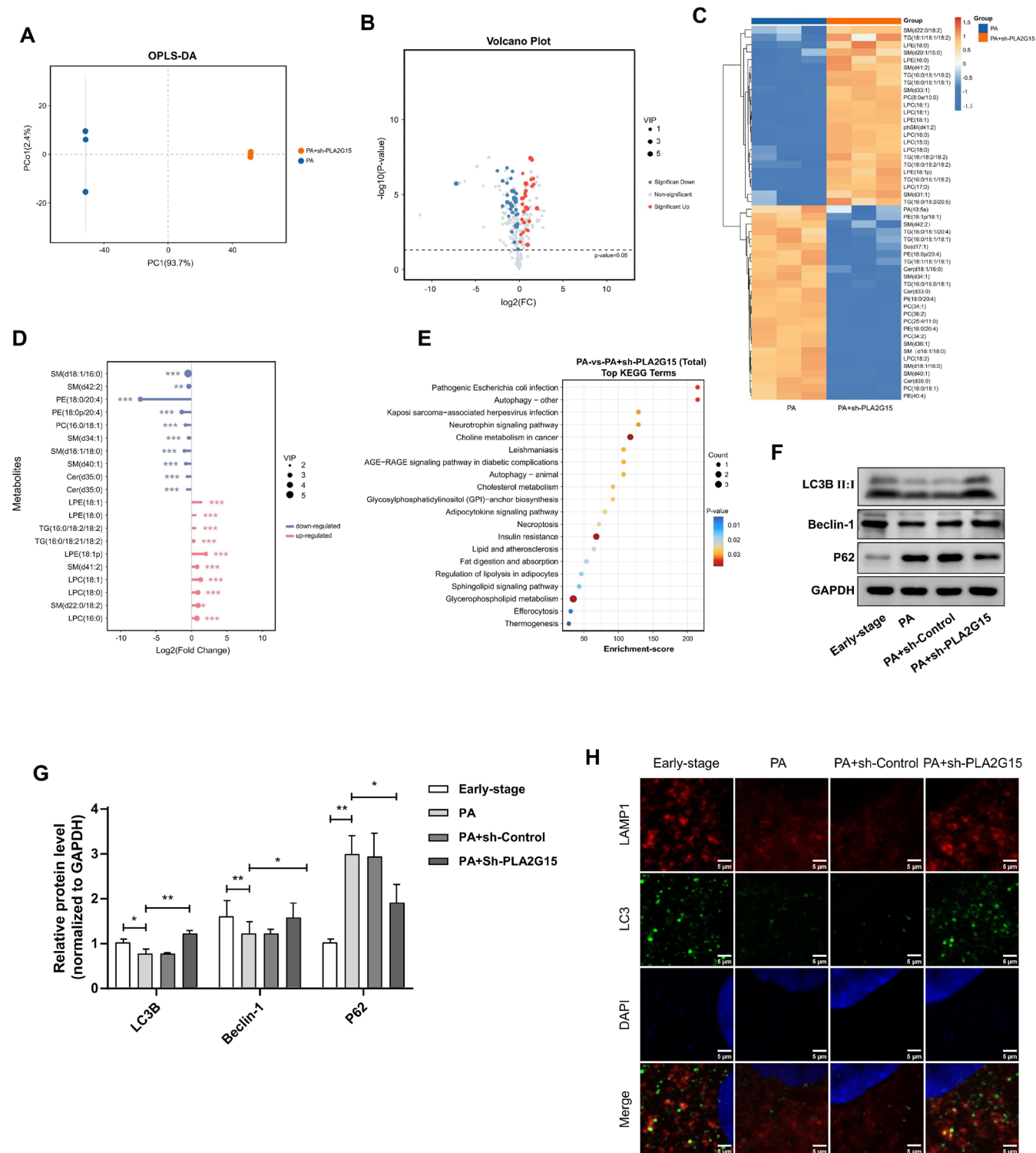


FIGURE 6 | PLA2G15 inhibition normalizes lysosomal membrane lipid composition and maintains the autophagy activity after PA treatment. (A) Lipidomics analysis for the lysosomal fractions treated with sh-PLA2G15 after PA overload, OPLS-DA analysis indicates a clear separation between the two groups. (B and C) Volcano map and heat-map of different metabolites. (D) KEGG analysis of the disturbed metabolic pathways. (E) Lipidomics analysis shows the difference between the two groups. (F and G) WB analysis and quantification of LC3B, Beclin-1 and P62 proteins after transfection. $n = 3$. (H) IF staining of LC3 and LAMP1 in NP cells after PLA2G15 inhibition. Scale bar = 5 μm . Compared to early-stage or indicated group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

the Pfirrmann MRI grade was observed to be lower in both the sh-C/EBP α and sh-PLA2G15 treatment groups compared to the PA group (Figure 9A,B). The histological features were analyzed using HE, S-O, and Alcian blue staining. The intervertebral

disc spaces in PA groups were markedly narrowed, whereas the structure and morphology of the intervertebral disc in both the sh-C/EBP α and sh-PLA2G15 groups exhibited significant improvement (Figure 9C). After transfection, the lysosomal

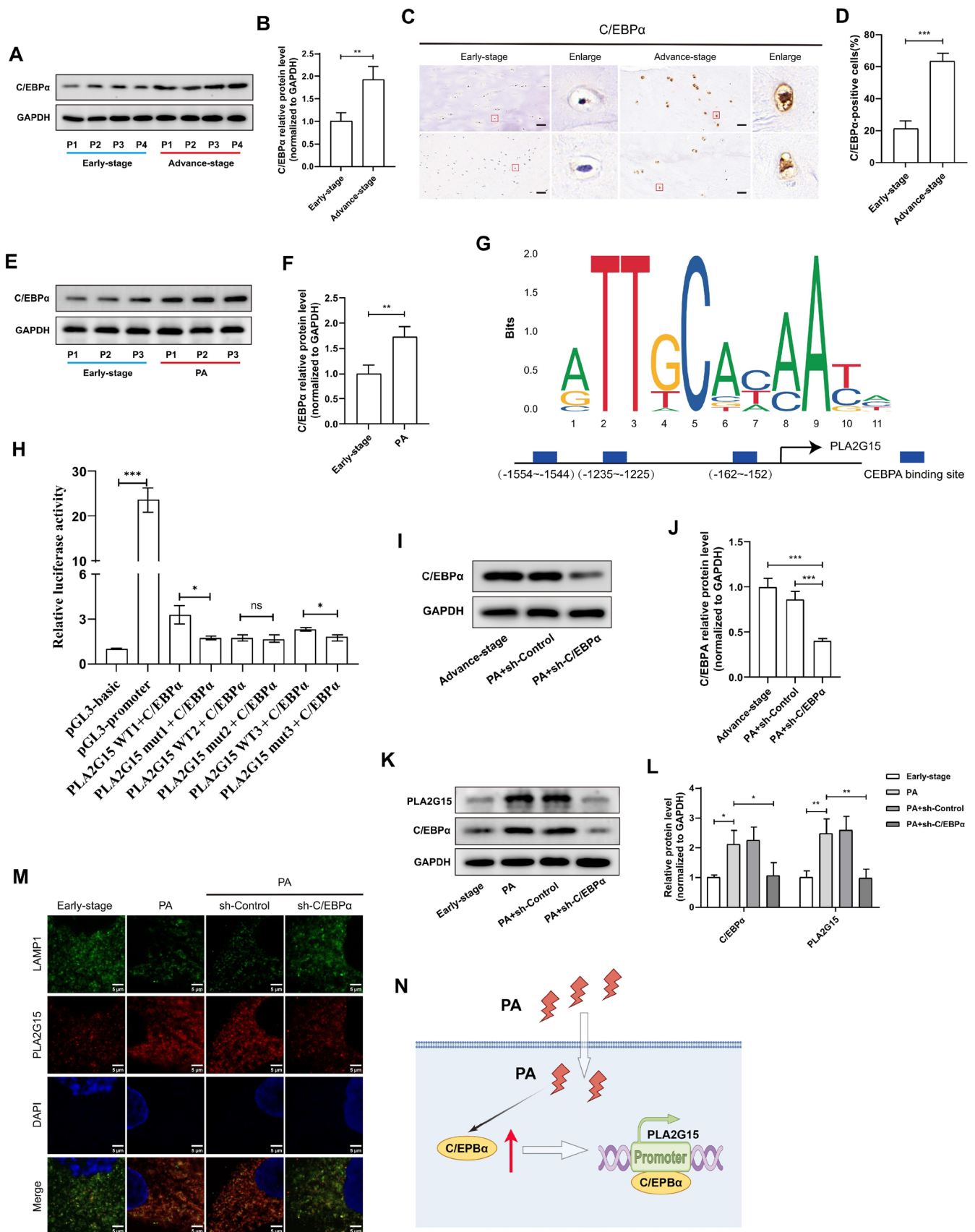


FIGURE 7 | Legend on next page.

FIGURE 7 | PA promotes PLA2G15 expression in a manner dependent on the transcription factor C/EBP α . (A and B) WB analysis and quantification of C/EBP α expression in early- and advanced stage. (C and D) IHC evaluation of C/EBP α in human NP tissues, the statistical results of the positive cell rate are presented in the chart. Scale bar = 50 μ m. n = 8. (E and F) Expression of C/EBP α was increased upon PA treatment. (G) Bioinformatics identification of the putative C/EBP α binding sites identified in the PLA2G15 promoter regions. (H) Luciferase reporter analysis showed that C/EBP α interacted with WT-PLA2G15 and promoted its transcription. (I and J) WB analysis and quantification of C/EBP α expression following plasmid transfection. (K and L) PLA2G15 expression levels were decreased after sh-C/EBP α transfection. (M) The IF staining for PLA2G15 exhibited a significant reduction following sh-C/EBP α treatment in human NP cells. Scale bar = 5 μ m. (N) The proposed regulatory mechanism in this section. Compared to early-stage or indicated group, * p < 0.05, ** p < 0.01, and *** p < 0.001.

contents leakage and damage of lysosomes in rat NP cells were significantly relieved (Figure 9D–I). These results collectively demonstrate the effects of PLA2G15 inhibition in stabilizing the lysosomal membrane in vivo.

Taken together, these results indicate that inhibition of PLA2G15 alleviates PA-induced human NP cell LMP by inhibiting the hydrolysis of lysosomal membrane phospholipids (Figure 10, Created By Figdraw).

4 | Discussion

Lysosomes function as central hubs for cellular degradation, where both autophagosomal and endosomal pathways converge to direct intracellular and extracellular materials into the highly acidic lysosomal compartment [5, 10]. This environment, characterized by its digestive properties, is enclosed by a mono-layer membrane. This membrane not only sustains the unique conditions essential for lysosomal activity but also safeguards the cell from potentially harmful substances contained within the lysosomes [9, 20]. Lysosomal dysfunction is a prominent pathological feature in IDD [11, 21]. As demonstrated in our results, evidence of lysosomal content leakage into the cytosol in IDD suggests an increase in LMP and a decrease in the barrier function of the lysosomal membrane. Furthermore, this disruption of lysosomal membrane integrity can trigger a cascade of cellular dysfunction, including activation of lytic enzymes within the cytosol that may lead to damage to cellular organelles and ultimately result in lysosome-dependent cell death [22]. Additionally, influx of cytosolic fluid into the lysosomal lumen can alter the acidic pH of the lysosome, further disrupting its normal physiological functions and may contribute to IDD progression [23]. In the current study, we demonstrate that activated PLA2G15 can directly hydrolyze the fatty acyl bond of lysosomal membrane phospholipids, thereby destabilizing the lysosomal membrane barrier and playing a role in PA-induced LMP.

The intervertebral disc, an avascular structure, experiences a relative state of closure in vivo, with its nutrient supply and metabolic waste removal entirely reliant on the slow diffusion process through the cartilage endplates [24]. As IDD progresses in severity, the vertebral endplates undergo calcification, which diminishes the disc diffusion capabilities. This reduction in diffusion capacity makes the intervertebral disc micro-environment increasingly vulnerable to the deleterious effects of toxic metabolites [25]. Notably, PA levels were observed to be significantly upregulated in samples from advanced-stage IDD. PA, one of the prevalent free fatty acids

in the human body, has been implicated in inducing ER stress, lipid accumulation, and cellular senescence in the context of IDD [12]. Moreover, the accumulation of harmful PA can trigger a series of cellular responses, such as the activation of inflammatory pathways and the induction of apoptosis, pyroptosis, or ferroptosis, thereby further aggravating the degenerative process [26–28]. A comprehensive understanding of the molecular mechanisms driving these changes is essential for the development of therapeutic strategies aimed at halting or reversing the progression of IDD.

The results of our research indicate that PA induces dysfunction within the lysosomes, compromising their structural integrity and leading to an elevation in LMP among human NP cells. PA damages lysosomes through a variety of mechanisms. In renal cells, Cai et al. [29] observed that an overload of PA can lead to apoptosis, which is associated with impaired autophagic flux and reduced lysosomal biogenesis. This impairment is often characterized by the phosphorylation of transcription factor EB (TFEB), a key regulator of lysosomal function, resulting in decreased nuclear translocation of TFEB and subsequent lysosomal dysfunction [19, 29]. Similarly, in macrophages, Liu et al. [30] noted that PA has been shown to exacerbate inflammation and contribute to nonalcoholic steatohepatitis by inducing lysosomal dysfunction. Besides, PA has been shown to significantly alter cellular membrane fluidity. The incorporation of PA into membrane phospholipids can lead to a decrease in membrane fluidity, which is crucial for lysosomal biogenesis and formation, signaling, and lipids transport processes [31, 32]. Understanding these mechanisms is crucial for developing therapeutic strategies to mitigate the adverse effects of PA on lysosomes.

Herein, we performed a comprehensive lipidomic analysis on the lysosomal fractions derived from individuals with IDD. Our investigation revealed a significant increase in the abundance of ceramide and lysophospholipids within the lysosomal membranes, alongside a notable decrease in the levels of phospholipids. These findings suggest a possible breakdown of the membrane lipids and a compromise in the structural integrity of the lysosomal membranes. The stability and fluidity of lysosomal membranes are dependent on the specific types of lipids that constitute them [31, 33]. Changes in the composition of these lipids can bring about alterations in the properties of the membrane. Such modifications might have profound implications, potentially leading to lysosomal malfunction and the induction of LMP [9]. Furthermore, phospholipids are recognized for their free radical scavenging properties. Diminished levels of phospholipids would probably render the lysosomal membrane more susceptible to further damage, such as that inflicted

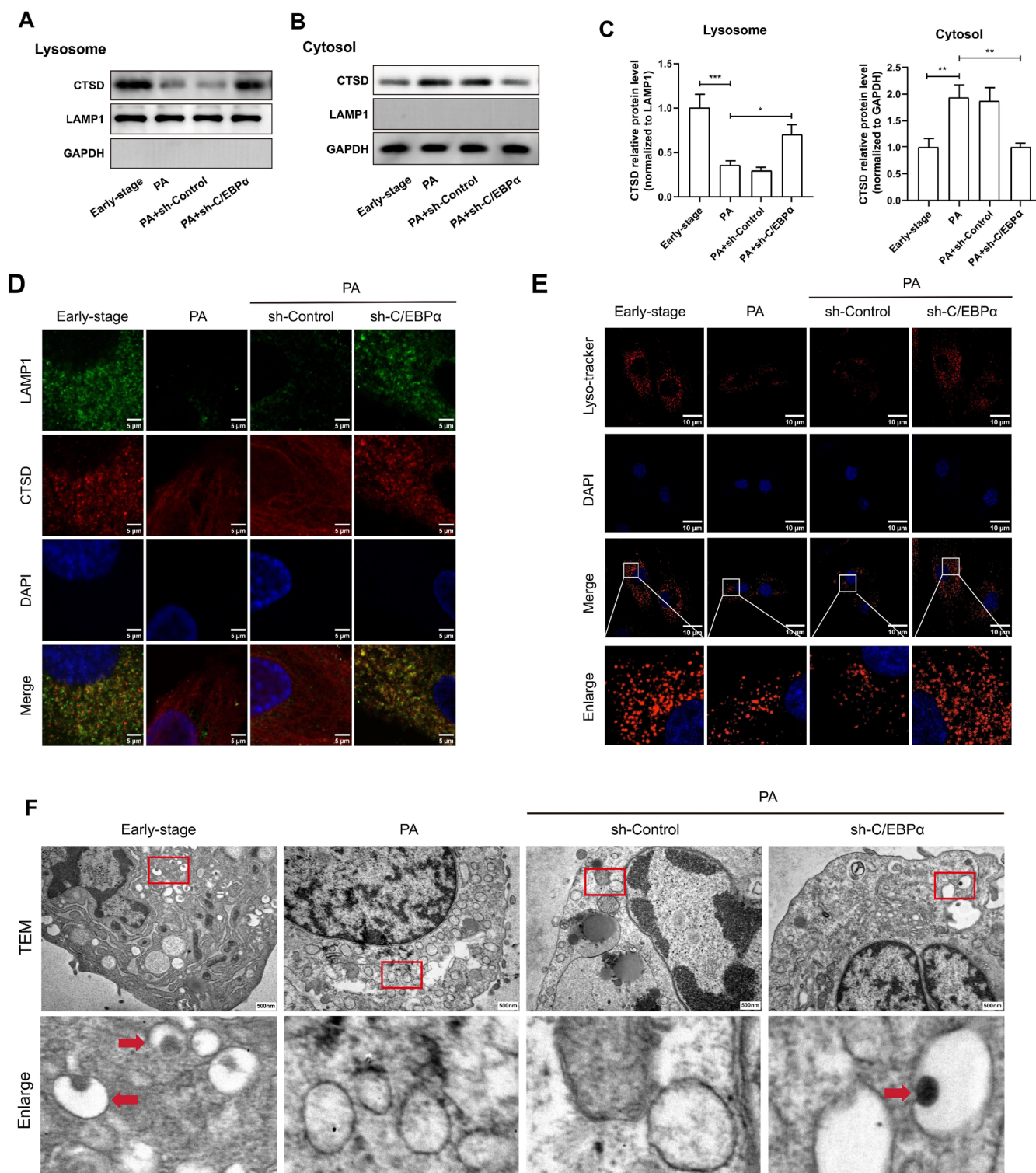


FIGURE 8 | PA induces LMP in a C/EBPα dependent manner. (A–C) WB analysis revealed that the levels of CTSD were increased in lysosomal fractions and decreased in cytosolic fractions after C/EBPα inhibition. $n = 3$. (D) IF staining demonstrated that the leakage of CTSD from lysosomes into the cytosol was alleviated after transfection. Scale bar = 5 μm. (E) The lyso-tracker staining for lysosomes revealed that inhibition of C/EBPα attenuates PA-induced lysosomal damage in NP cells. Scale bar = 10 μm. (F) TEM observation for lysosomes after transfection. Scale bar = 0.5 μm. Compared to early-stage or indicated group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

by reactive oxygen species and other oxidative stressors produced at the injury site [8, 34]. Thus, the molecular mechanisms through which PA contributes to the degradation of lysosomal membrane phospholipids within NP cells remain to be elucidated and need further investigation.

Through an integrative multiomics analysis, we have identified many differentially expressed genes involved in both membrane lipid synthesis and degradation pathways. Of particular interest is the lysosomal-localized PLA2G15, which has been identified as a key player in processes related to lysosomal function

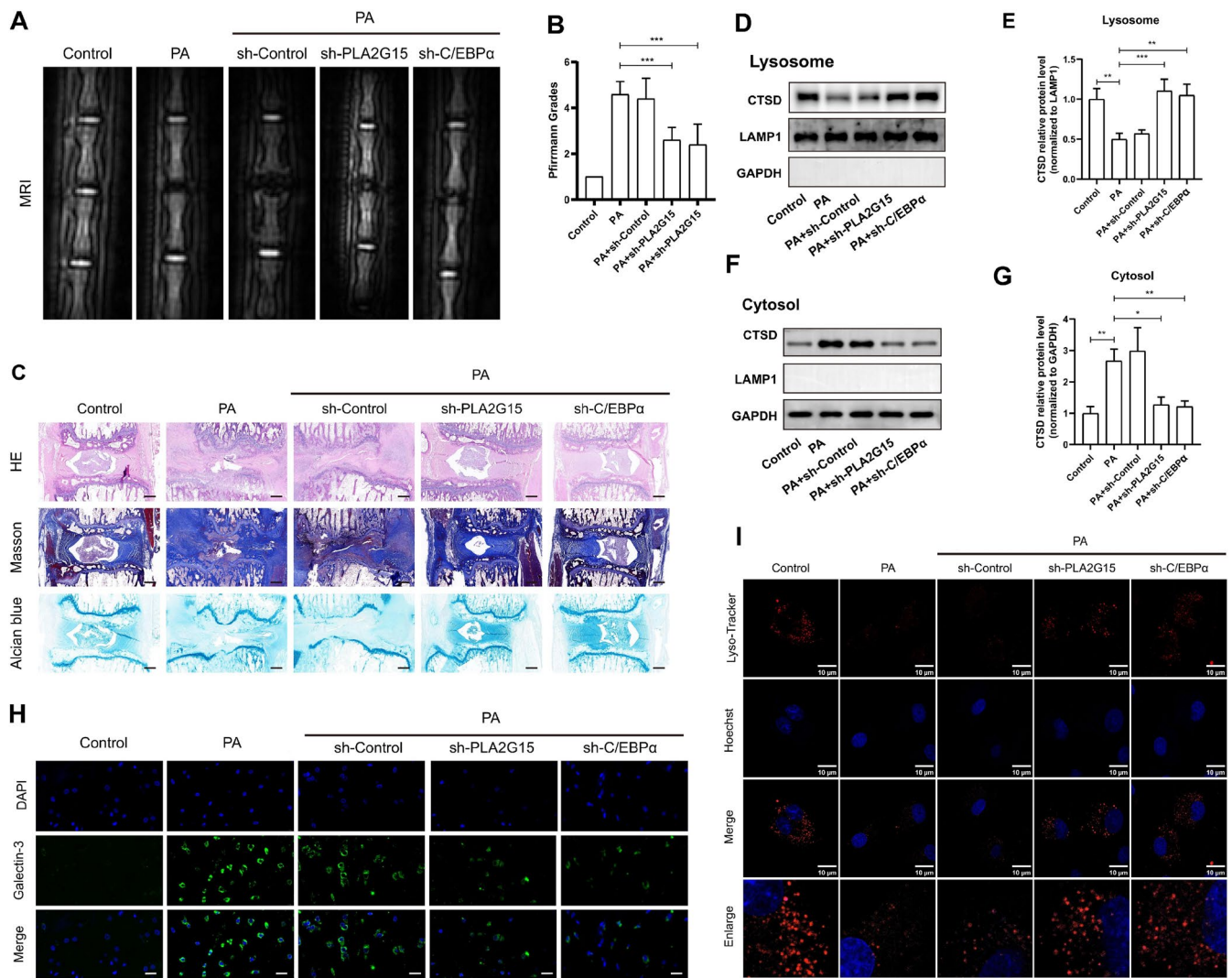


FIGURE 9 | Inhibition of PLA2G15 stabilized the lysosomes and LMP in vivo. (A and B) Representative MR T2-weighted images of rat tail, acquired 8 weeks PA injection, were assessed to evaluate the extent of degeneration based on the Pfirrmann system. $n = 5$. (C) Histological analysis was performed using HE, Alcian blue, and S-O staining on discs collected 8 weeks after surgery. Scale bar = 1 mm. (D–G) WB analysis revealed that the levels of CTSD were increased in lysosomal fractions and decreased in cytosolic fractions after transfection in vivo. $n = 3$. (H) IF staining for lysosomes injury marker galectin-3 proteins. Scale bar = 20 μm . (I) The lyso-tracker staining for lysosomes in rat NP cells. Scale bar = 10 μm . Compared to indicated group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

and glycerophospholipid metabolism. As an enzyme, PLA2G15 plays a pivotal role in the hydrolysis of membrane phospholipids, thereby significantly altering the lipid composition of lysosomal membranes [35]. The activity of PLA2 can result in the liberation of free fatty acids and lysolipids, thereby modulating the physical properties of the lysosomal membrane, including its fluidity and permeability [36]. This enzymatic action holds particular relevance in various physiological and pathological contexts such as inflammation, neurodegeneration, and metabolic disorders. In brain trauma, Sarkar et al. [9] demonstrated that activation of PLA2G4A impairs lysosomal integrity and function, consequently impairing autophagy and causing neuronal cell death. Similarly, activation of PLA2G4E hinders random skin flap survival by promoting LMP-induced necrosis [8]. We discovered that the expression of PLA2G15 in advanced-stage IDD was significantly elevated compared to that in early-stage. Moreover, elevated PLA2G15 expression was observed following PA treatment, and PLA2G15 colocalized with

lysosomes. Additionally, our experiments demonstrated that reduced PLA2G15 expression maintained lysosomal phospholipid abundance, decreased LMP, and promoted the biosynthesis and maintenance of lysosomes under conditions of PA overload. These findings indicate that PLA2G15 plays a major role in LMP in human IDD and is involved in mediating PA-induced LMP.

The recent publications have revealed that PLA2G15 is a Bis(monoacylglycerol)phosphate (BMP) hydrolase, responsible for degrading the lipid constituent, BMP, of intralysosomal vesicles [37–39]. PLA2G15-deficient cells and tissues accumulate multiple BMP species in comparison to their PLA2G15 wild-type controls [37]. The accumulation of BMP is involved in many disorders including neurodegenerative diseases, Gaucher disease, acid sphingomyelinase and metachromatic leukodystrophy [40]. However, in the context of IDD, our metabolomic analysis of whole NP cells and lipidomic analysis of lysosomes did not detect differences in BMP content between advanced- and

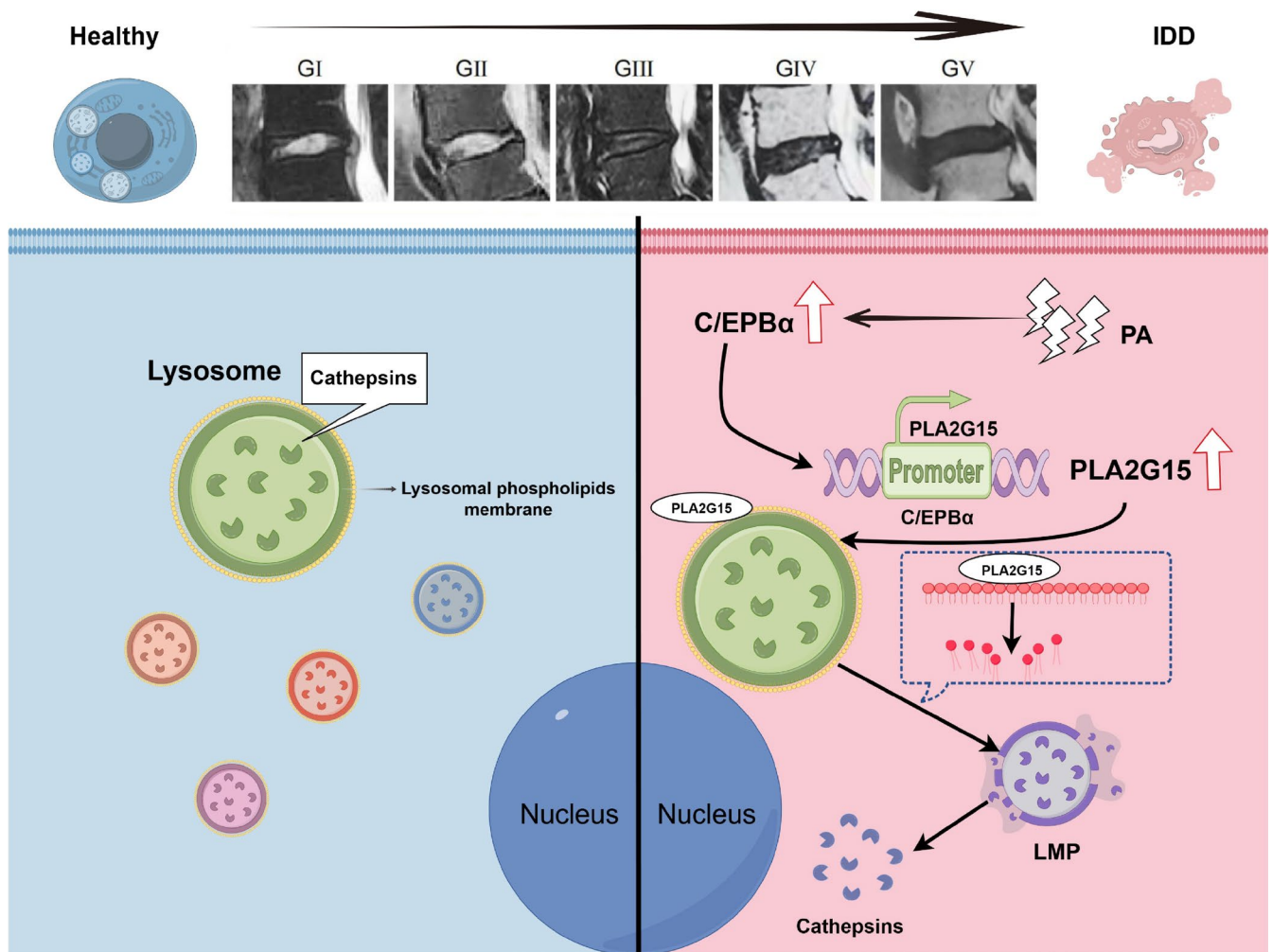


FIGURE 10 | An illustration summarizing the pathological mechanisms of PA-induced LMP in NP cells. PA promotes the expression of PLA2G15 in a C/EBPα dependent manner. The upregulation of PLA2G15 subsequently triggers the hydrolysis of lysosomal membrane lipids, ultimately leading to LMP in NP cells.

early-stage IDD. The expression and fatty acid composition of BMP are highly tissue-specific. For instance, BMP constitutes up to 18% of phospholipids in alveolar macrophages to support their high phagocytic activity; the pathophysiological context of IDD may not trigger the same BMP accumulation phenotype seen in lysosomal storage disorders or alveolar macrophages [40, 41].

PLA2G15 exhibits broad substrate specificity beyond BMP. However, PLA2G15 is not exclusively a BMP hydrolase; it is a multifunctional lysosomal phospholipase with broad substrate recognition. Previous studies have definitively shown that PLA2G15 can hydrolyze a range of glycerophospholipids, including PC, PE, phosphatidylglycerol (PG), and phosphatidylserine (PS), with a preference for the sn-2 position of unsaturated fatty acyl chains [42, 43]. Furthermore, in PLA2G15 knockout mouse models, significant accumulation of PC and PE has been observed [44]. Our finding that PC/PE levels are altered is consistent with this known broader activity.

PLA2G15-mediated hydrolysis of membrane phospholipids can modify the composition of membrane lipids, compromising the structural integrity of the membrane and leading to LMP

through various mechanisms in IDD. The lysophospholipids generated from this enzymatic action possess detergent-like properties and have the ability to directly induce pore formation [45, 46]. Moreover, activation of PLA2G15 subsequent to PA overload is associated with decreased levels of glycerophospholipids, such as PC and PE, within the lysosomal membrane. Disruption of these glycerophospholipids can impact membrane structure and fluidity. Reduced levels of glycerophospholipids may render the lysosomal membrane more vulnerable to further damage, including that caused by PA overload, ROS, and other stress conditions produced at the injury lysosomal membrane [10]. This cascade of events highlights the critical role of PLA2G15 in the regulation of lysosomal membrane stability and function. Thus, further studies should also explore the potential mechanisms by which PLA2G15 activation under PA overload.

In current study, we have demonstrated that C/EBPα playing a role in the expression of PLA2G15. As a transcription factor, C/EBPα is pivotal in various biological processes, regulating metabolic pathways and cellular stress responses [47]. PA is known to induce ER stress by disrupting ER calcium homeostasis and activating the unfolded protein response (UPR). The PERK/eIF2α arm of the UPR, in particular, can selectively enhance

translation of transcription factors including C/EBP homologous protein (CHOP) and potentially C/EBP α . Furthermore, C/EBP α itself plays a critical role in lipid metabolism and is required for postnatal maintenance of systemic energy homeostasis and lipid storage [48–50]. Our results align with these previous findings, suggesting that C/EBP α is upregulated in response to PA stimulation in human NP cells. Functionally, we have found that C/EBP α acts as a transcription factor for PLA2G15 and can promote the expression of PLA2G15 under conditions of PA overload. Since the functional role of the C/EBP α has not been studied in the pathogenesis of IDD, our study was also demonstrated that the inhibition of C/EBP α alleviates PA-induced human NP cells LMP and IDD.

The molecular mechanisms underlying LMP remain incompletely elucidated. Previous studies have suggested that LMP can be triggered by a variety of factors, including proapoptotic Bcl-2 family proteins, p53, caspases, cathepsins, ROS, and kinases [10, 34]. It is worth noting that different forms of stress can induce LMP via distinct pathways. Although our investigation primarily focuses on the impact of lysosomal impairment resulting from PA on the hydrolysis of membrane lipid composition, the cellular consequences of LMP extend far beyond this scope. In addition to disrupting lysosome-dependent cellular processes such as autophagy and endocytosis, the release of lysosomal contents can inherently exhibit cytotoxicity [11, 51]. This can exacerbate cellular dysfunction and accelerate lysosome-dependent cell death. Therefore, maintaining lysosomal integrity and function is crucial for cellular homeostasis. In this study, we report that within the PA over-load microenvironment, C/EBP α modulates the expression of PLA2G15, which in turn leads to the hydrolysis of lysosomal membrane lipids and ultimately contributes to the development of IDD, providing a therapeutic target to inhibit LMP.

There are some limitations to the present study. First, we primarily focused on the NP cell LMP, neglecting the downstream signaling pathways that could arise from LMP and lysosomal dysfunctions. We will conduct further research on these issues in the future. Second, the samples included for metabolomics and lipidomics analysis were relatively little, which suggests that the resulting profile might only partially represent the diverse metabolic changes present in NP cells associated with IDD. Nonetheless, our study offers mechanistic insights into the observed lysosomal dysfunction and LMP under PA overload conditions by integrating omics data with validation from large cohorts and functional assessment data. Third, the PA-induced IDD models used in the present study do not accurately replicate the progression of human disc degeneration. But the selection of this IDD model was based on the lysosomal dysfunction in vivo induced by PA, which may potentially provide a viable method for investigating the efficacy of potential therapeutic targets.

5 | Conclusion

Our study found that lysosomal dysfunction was the most prominent feature in IDD. We further demonstrated that LMP is responsible for lysosomal dysfunction in IDD. The lipid composition changes in the lysosomal membrane, mediated by

PLA2G15, are the cause of LMP. Additionally, our findings indicated that C/EBP α significantly promotes PLA2G15 expression under PA overload stress in human NP cells. Consequently, we propose that suppressing PLA2G15 to reduce LMP could be a promising therapeutic approach for IDD.

Author Contributions

Conceptualization: Xi Chen. Methodology: Liqun Duan, Shuangshuang Tu, Chuanfu Li, Yijun Dong, and Yongjin Li. Software: Shuangshuang Tu and Yongjin Li. Data curation: Yijun Dong. Investigation: Liqun Duan, Jiang Jiang, and Chuanfu Li. Validation: Jiang Jiang, Shuangshuang Tu, Chuanfu Li, and Yijun Dong. Formal analysis: Jiang Jiang, Shuangshuang Tu, Yijun Dong, and Yongjin Li. Supervision: Wenzhi Zhang. Funding acquisition: Wenzhi Zhang. Visualization: Yongjin Li. Project administration: Wenzhi Zhang and Xi Chen. Resources: Xi Chen. Writing – original draft: Liqun Duan. Writing – review and editing: Xi Chen.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Upregulation of PLA2G15 results in LMP. (A–C) WB analysis revealed that the levels of CTSD were decreased in lysosomal fractions and increased in cytosolic fractions after PLA2G15 upregulation, $n = 3$. (D) IF staining demonstrated the leakage of CTSD from lysosomes into the cytosol after PLA2G15 transfection. Scale bar = 5 μ m. (E) The lyso-tracker staining for lysosomes revealed that PLA2G15 transfection aggravates the lysosomal damage in NP cells. Scale bar = 10 μ m. (F) TEM observation for lysosomes after PLA2G15 transfection. Scale bar = 0.5 μ m. Compared to early-stage, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.