Group V Secretory Phospholipase A$_2$ Regulates Endocytosis of Acetylated LDL by Transcriptional Activation of PGK1 in RAW264.7 Macrophage Cell Line

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Aims: It was suggested that group V secretory phospholipase A$_2$ (sPLA$_2$-V) existed in the nucleus. This study examined whether nuclear sPLA$_2$-V plays a role in endocytosis of acetylated low-density lipoprotein (AcLDL) in monocyte/macrophage-like cell line RAW264.7 cells.

Methods: RAW264.7 cells were transfected with shRNA vector targeting sPLA$_2$-V (sPLA$_2$-V-knockdown [KD] cells) or empty vector (sPLA$_2$-V-wild-type [WT] cells). AcLDL endocytosis was assessed by incubation with $^{125}$I-AcLDL or AcLDL conjugated with pHrodo. Actin polymerization was assessed by flow cytometry using Alexa Fluor 546-phalloidin.

Results: In immunofluorescence microscopic studies, sPLA$_2$-V was detected in the nucleus. ChIP-Seq and ChIP-qPCR analyses showed binding of sPLA$_2$-V to the promoter region of the phosphoglycerate kinase 1 (Pgk1) gene. In the promoter assay, sPLA$_2$-V-KD cells had lower promoter activity of the Pgk1 gene than sPLA$_2$-V-WT cells, and this decrease could be reversed by transfection with a vector encoding sPLA$_2$-V-H48Q that lacks enzymatic activity. Compared with sPLA$_2$-V-WT cells, sPLA$_2$-V-KD cells had decreased PGK1 protein expression, beclin 1 (Beclin1) phosphorylation at S30, and class III PI3-kinase activity that could also be restored by transfection with sPLA$_2$-V-H48Q. sPLA$_2$-V-KD cells had impaired actin polymerization and endocytosis, which was reversed by introduction of sPLA$_2$-V-H48Q or PGK1 overexpression. In sPLA$_2$-V-WT cells, siRNA-mediated depletion of PGK1 suppressed Beclin1 phosphorylation and impaired actin polymerization and intracellular trafficking of pHrodo-conjugated AcLDL.

Conclusions: Nuclear sPLA$_2$-V binds to the Pgk1 gene promoter region and increases its transcriptional activity. sPLA$_2$-V regulates AcLDL endocytosis through PGK1-Beclin1 in a manner that is independent of its enzymatic activity in RAW264.7 cells.

Key words: Group V secretory phospholipase A$_2$, Endocytosis, Phosphoglycerate kinase 1, Actin polymerization, Macrophage

Introduction
Phospholipase A$_2$s (PLA$_2$s) participate in diverse biological events through the generation of a variety of lipid mediators$^1$. Mammalian PLA$_2$ enzymes are classified into three main categories based on their biochemical features and primary structures$^1$: intracellular cytosolic PLA$_2$ (cPLA$_2$), Ca$^{2+}$...
extracellular sPLA2s promote release of arachidonic acid and other fatty acids from plasma membrane phospholipids or non-cellular lipid components. After secretion, extracellular sPLA2s promote release of arachidonic acid and other fatty acids from plasma membrane phospholipids or non-cellular lipid components1, 2. Previous reports suggested that some sPLA2s including group V sPLA2 (sPLA2-V) localized within the nucleus3-5) . However, whether their nuclear localization has pathophysiological relevance is unclear. Notably, hydrolytic enzyme activity of sPLA2s requires Ca2+ concentrations in the millimolar range11), and the Ca2+ concentration in both the nucleus and cytosol is at most a few µM10). Therefore, functional roles for sPLA2s in the nucleus are likely mediated by non-enzymatic activities.

PLA2s have been reported to participate in innate immune functions through the regulation of phagocytosis7). Macrophages contribute to the innate immune response by ingesting pathogens, internalizing them in phagosomes that later recruit lysosomal proteins to form phagolysosomes in which pathogens are killed8). sPLA2-V was previously shown to regulate phagocytosis of microorganisms independently of its effects on eicosanoid generation9, 10) . The precise mechanisms by which sPLA2-V regulate phagocytosis are unclear. Endocytosis of modified low-density lipoproteins (LDL) through clathrin or non-clathrin-mediated pathways11) is thought to be central to the formation of foam cells, which are the basis for generation of atherosclerotic lesions. Whether sPLA2-V may also play a role in endocytosis of modified LDLs such as acetylated LDL (AcLDL) also remains to be determined.

While searching for the biological role of sPLA2-V in the nucleus of macrophages, we unexpectedly found that sPLA2-V bound to the promoter region of phosphoglycerate kinase 1 (Pgk1) gene and promoted Pgk1 transcriptional activity in a non-enzymatic manner using the chromatin immunoprecipitation sequencing (ChIP-Seq) assay and the promoter assay. PGK1 is a key enzyme in the glycolytic pathway in which it catalyzes the reversible conversion of 1,3-biphosphoglycerate to 3-phosphoglycerate. PGK1 was recently shown to directly phosphorylate beclin 1 (Bclin1)12), a protein that plays a key role in autophagy. Thus, PGK1 can act as a protein kinase to enhance autophagy, a novel role that is distinct from its glycolytic function13). Beclin1 is not only a key player in autophagy, but also serves non-autophagy functions including those involved in endocytic trafficking and phagocytosis14, 15).

Using the monocyte/macrophage-like cell line RAW264.7 cells, in this study we tested the hypothesis that sPLA2-V in the nucleus can regulate PGK1 expression that leads to enhanced endocytic activity of AcLDL.

Materials and Methods

Materials

FBS, RPMI 1640 medium, penicillin/streptomycin solution, Alexa Fluor™ 488 AcLDL, Alexa Fluor™ 546 phalloidin, and pHrodo™ Indicator were purchased from Thermo Fisher Scientific (Waltham, MA, USA). AcLDL was from Alfa Diagnostic International (San Antonio, TX, USA). Sodium [125I]-iodine (carrier-free, 3.7GBq/ml) was purchased from Perkin Elmer Japan (Yokohama, Kanagawa, Japan). Paraformaldehyde solution and saponin were from Sigma (Tokyo, Japan). EcoRI and BamHI restriction enzymes were obtained from New England Biolabs Japan (Tokyo, Japan). Rabbit monoclonal antibody against c-Src (clone no. 32G6; catalog. no. 2123), rabbit monoclonal antibody against phospho-c-Src at the active site (Tyr416; clone no. D49G4; cat. no. 6943), mouse and rabbit monoclonal antibody against Myc-tag (clone no. 9B11 and 71D10, cat. no. 2276 and 2278, respectively), rabbit monoclonal antibody against Beclin1 (clone no. D40C5; cat. no. 3495), rabbit polyclonal antibody against phospho-Beclin1 at the active site (Ser30) (cat. no. 54101), rabbit monoclonal antibodies against Rab5 (clone no. C8B1; cat. no. 3547) and Rab7 (clone no. D95F2; cat. no. 9367), rabbit polyclonal antibody against Lamin (cat. no. 2032), and rabbit monoclonal antibody against β-tubulin (clone no. 9F3; cat. no. 2128) were purchased from Cell Signaling Technology Japan (Tokyo, Japan). Rabbit polyclonal antibody to sPLA2-V for immunofluorescence study was purchased from Abcam Japan, Tokyo (cat. no. ab23709). Mouse monoclonal antibody against GAPDH (clone no. 3E12; cat. no. bs-0978M) was from Bioss (Woburn, MA, USA). Rabbit polyclonal antibody against PGK1 (cat. no. LS-C48233) was obtained from LSBio (Seattle, WA, USA). Rat monoclonal antibody against Scavenger receptor class A member 1 (SR-A1) conjugated with FITC (clone no. 268318; cat. no. FAB1797F) was purchased from R and D systems (Minneapolis, MN, USA). Other chemicals were purchased from Sigma unless otherwise indicated.

Cell Culture

The RAW264.7 cell line, which is a monocyte/macrophage-like cell line derived from BALB/C mice,
was obtained from the American Type Culture Collection (Manassas, VA, USA; ATCC no. TIB-71). The cells were cultured in RPMI 1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin in an atmosphere of 5% CO₂ at 37°C. Cells were passaged at 90% confluency and used for experiments within passage number 15.

Details regarding the generation and characterization of sPLA₂-V knockout (KO) mice with a C57BL/6J background (systemically deficient in sPLA₂-V) are described in our previous report[^16]. sPLA₂-V KO male mice (20–25 wks-old) were used in the present study. Littermates of the sPLA₂-V wild-type (WT) males served as a WT group. Peritoneal macrophages were isolated from sPLA₂-V KO and WT mice by flushing the peritoneal cavities of mice with 5 mL ice-cold RPMI 1640 medium. The isolated peritoneal macrophages were washed and resuspended with RPMI 1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin, and then plated on 24- or 48-well tissue culture plates. The cells were incubated overnight at 37°C with 5% CO₂. Nonadherent cells were removed by washing three times with PBS. Peritoneal macrophages isolated as adherent Mac-2-positive cells were obtained at >95% purity. The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. A28-37), and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication, 8th Edition, 2011).

**Stable Knockdown (KD) of sPLA₂-V with Short Hairpin RNA (shRNA) in RAW264.7 Cells and Transfection of Small Interfering RNAs (siRNAs)**

Pre-designed short hairpin RNA (shRNA) specifically targeting sPLA₂-V was cloned into the pLKO.1 vector (Sigma). The shRNA sequence was: 5'-CCGGGCTAGAACTCAAGTCCATGATCTCGAGATTGGACTTGAGTTCTAGCTTTTTG-3' (cat. no. TRCN0000222633). The negative control shRNA (cat. no. SHC002) sequence was: 5'-CCGGGCTAGAACTCAAGTCCATGATCTCGAGATTGGACTTGAGTTCTAGCTTTTTG-3'. Stable knockdown (KD) of sPLA₂-V in RAW264.7 cells was established as previously described[^17]. Briefly, cells cultured on a 24-well plate were transfected with 0.8 µg of the shRNA targeting sPLA₂-V using FuGENE HD transfection reagent (Promega Japan, Tokyo, Japan) according to the manufacturer’s instructions. After selection of transfected cells with puromycin (final concentration 3 µg/mL), some monoclonal cell populations were isolated from the stable cell pool by limiting dilution. Cells from each clone were collected for RT-PCR screening of sPLA₂-V mRNA knockdown. Meanwhile, the same procedure was used to isolate stable controls (RAW264.7 cells with WT sPLA₂-V) from cells transfected with the negative control shRNA vector. Efficiency of gene knockdown was confirmed by quantitative real-time (RT) PCR and immunoblotting.

Pre-designed small interfering RNAs (siRNAs) specific for mouse PGK1, Beclin1, vacuolar protein sorting 34 (VPS34), and sPLA₂-II, -IIE, and XIIA were obtained from Sigma. The sense strand sequences are listed in **Supplementary Table 1**. The sequence of negative control siRNA (cat. no. SIC002) was not provided. RAW264.7 cells were transiently transfected with 30 nM siRNAs using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific), and the cells were used for experiments within 48 hr after the transfection. The efficacy of the siRNA-mediated suppression was confirmed by RT-PCR and western blot.

Transfection of shRNA, siRNAs, or any cDNA constructs was not performed using peritoneal macrophages due to the low transfection efficiency of primary macrophages with DNA constructs[^18].

**Generation and Transfection of cDNA Constructs Encoding Mouse sPLA₂-V, sPLA₂-V-H48Q, Myc-tagged sPLA₂-V, Myc-tagged sPLA₂-V-H48Q, GFP-tagged sPLA₂-V, and Mouse PGK1 and c-Src**

pCMV6-Entry vector encoding a C-terminal Myc-tagged mouse sPLA₂-V (accession no. NM_011110.4) was purchased from OriGene Technologies (Rockville, MD, USA). At first, we generated cDNA encoding a mutant of sPLA₂-V to transfect into RAW264.7 cells having shRNA-mediated knockdown of sPLA₂-V. Site-specific mutations were introduced using mismatched primer PCR with *Pfu* DNA polymerase and *DpnI* restriction enzyme and a plasmid vector encoding a C-terminal Myc-tagged mouse sPLA₂-V as a template according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). The primers for the mutations of nucleotides without any changes of amino acids are described in **Supplementary Table 1**. Then, cDNA construct of mouse sPLA₂-V lacking the Myc-tag was generated by the similar way using the respective primers (**Supplementary Table 1**). cDNA constructs of mouse sPLA₂-V encoding the activity-ablated mutant H48Q[^19], with and without the Myc-tag, were also produced with a site-specific mutation method and a pCMV6-Entry vector carrying mouse sPLA₂-V cDNA with or without Myc-tag as a template. The primers for generation of the H48Q
ChIP-Seq and Quantitative ChIP-PCR in RAW264.7 Cells

ChIP-Seq was performed using truChIP Chromatin Shearing Kit ( Covaris, Woburn, MA, USA) and iDeal ChIP-seq kit for Transcription Factors ( Diagenode, Seraing, Belgium) according to the manufacturer’s instructions and our previous report (20). Since we previously had technical problems due to unavailability of antibodies that specifically detect sPLA2-V in tissues by immunoblotting (6), we used a monoclonal antibody against the Myc-tag and analyzed sPLA2-V KD RAW264.7 cells with re-constitutive expression of Myc-tagged sPLA2-V or Myc-tagged sPLA2-V-H48Q as well as sPLA2-V KD RAW264.7 cells transfected with empty vector. Briefly, RAW264.7 cells (4 × 10⁶ cells/10 cm dish) were crosslinked in 1% formaldehyde for 5 min at room temperature. The reaction was quenched by addition of glycine to a final concentration of 0.125 M and incubation for 5 min at room temperature. After quenching, the cells were washed and collected in PBS before incubation with lysis buffer to prepare the nuclei. The nuclei were pelleted and resuspended with shearing buffer for sonication of chromatin using a Covaris M220 Focused-ultrasonicator ( Covaris, Woburn, MA, USA) that sheared the chromatin into 200–700 bp fragments (75 W peak power, 10% duty factor, 200 cycles/burst, for 20 s). The lysate was kept cold at all times during sonication. A portion of the chromatin lysate was set aside as an input control. The remainder of the chromatin lysate was used for the ChIP experiment using mouse monoclonal antibody against Myc-tag (1:100 dilution; clone no. 9B11, catalog no. 2276, Cell Signaling Technology Japan, Tokyo, Japan). Normal mouse IgG (cat. no. 015-000-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as a negative control.

Chromatin-antibody complexes were pulled down using protein A-coated magnetic beads. The isolated DNA was purified and Illumina sequencing libraries were prepared using an Illumina TruSeq DNA Sample prep Kit ( Illumina, San Diego, CA, USA). Libraries were PCR amplified for 18 cycles. Several libraries were pooled on one NovaSeq6000 lane and sequenced. Fastq files were first trimmed using Trimmomatic to remove poor quality reads and adapters. Reads from the trimmed Fastq files were aligned to the reference mouse genome (mm10) using Bowtie to produce sam files. Duplicate reads were then removed using Picard tools. ChIP-Seq peaks were called using the MACS2 algorithm, then annotated using ChIPseeker.

Data obtained after ChIP-Seq was validated by ChIP-quantitative PCR (qPCR). Immunoprecipitated DNA samples using mouse anti-Myc-tag monoclonal antibody and control mouse IgG were amplified and quantitated using Pgk1 primers (Supplementary Table 1). Amplification reactions (20 μL total volume) contained 1 μL DNA (Input/ChIP), 500 nM of the respective primers and SYBR Green Master Mix (Toyobo, Osaka, Japan). Data were calculated by normalizing relative to input sample (percentage of input).

Assay of Pgk1 Gene Promoter Activity in RAW264.7 Cells

We used secreted luciferase from Cypridina. The Pgk1 promoter (−648 to −50) was amplified from mouse genomic DNA by PCR and cloned into the Cypridina luciferase reporter plasmid pMCS- Cypridina Luc (Thermo Fisher Scientific) at the XhoI and BamHI restriction sites. sPLA2-V-WT and sPLA2-V KD cells as well as sPLA2-V KD cells expressing sPLA2-V-H48Q were transfected with Cypridina luciferase reporter vector using FuGENE HD Transfection Reagent (Promega) according to the manufacturer’s instructions. Renilla luciferase expression vector (pTK-Green Renilla Luc, Thermo Fisher Scientific) was co-transfected to serve as an internal control. At 24 hr post transfection, the culture medium and the cells were harvested. Cypridina luciferase activity in the harvested culture medium was measured using a SpectraMax L luminometer ( Molecular Devices, San Jose, CA, USA) and a Pierce Cypridina Luciferase Glow Assay Kit (Thermo Fisher Scientific). Renilla luciferase activity in cellular lysates was measured on the luminometer using coelenterazine as a substrate. Cypridina luciferase activity was normalized to Renilla luciferase activity. Data are represented as fold-induction by normalizing the luciferase activity of the tested sample to that of the corresponding control sample.
Assay of Class III Phosphatidylinositol 3-kinase (PI3-kinase) Activity in RAW264.7 Cells

RAW264.7 cells were incubated with 20 µg/mL AcLDL or PBS as a vehicle for 2 hr, washed with cold PBS, and lysed with lysis buffer (Sigma). Vacuolar protein sorting 34 (VPS34) protein complexes in the cell lysates were immunoprecipitated using rabbit anti-VPS34 monoclonal antibody (clone no. D9A5; cat no. 4263, Cell Signaling Technology Japan) or IgG as a control (cat. no. 011-000-003, Jackson Immuno Research Laboratories). VPS34 protein complexes were pulled down with protein G magnetic beads and washed with PBS/0.02% Tween 20. The enzyme activity of the VPS34 protein complex was determined using Class III PI3-Kinase Kit (K-3000; Echelon BioSciences, Salt Lake City, UT, USA) according to the manufacturer’s instructions. Briefly, 12.5 µL kinase reaction buffer (20 mM Tris at pH 8.0, 200 mM NaCl, 2 mM EDTA, 20 mM MnCl₂, 100 µM ATP) was added to the immune complex bound to beads and incubated at 30°C for 3 hr. The reaction was terminated by adding 5 µL 100 mM EDTA. The quenched reaction mixture and phosphatidylinositol 3-phosphate (PI[3]P) detector protein (provided with the kit) were mixed in a PI[3]P-coated microplate for competitive binding to the PI[3]P detector protein. The amount of PI[3]P detector protein bound to the plate was determined using colorimetric detection of absorbance at 450 nm. The concentration of PI[3]P remaining in the reaction mixture was calculated based on the amount of PI[3]P detector protein bound to the plate.

Binding, Internalization, and Degradation Assays for AcLDL in RAW264.7 Cells and Mouse Peritoneal Macrophages

AcLDL was labeled with Na¹²⁵I (Parkin Elmer Japan, Yokohama, Japan) in pre-coated iodination tubes (Thermo Fisher Scientific) to yield a specific activity of 100-150 kBq/µg AcLDL protein. For the binding study, cultures of RAW264.7 cells or peritoneal macrophages on 24-well culture plates were incubated for 2 hr at 4°C with the indicated concentrations of ¹²⁵I-labeled AcLDL in RPMI 1640 medium containing 0.1% BSA in the absence or presence of 50-fold excess unlabeled AcLDL. After the reaction was stopped by rapid removal of the medium, the cells were washed three times with ice-cold PBS, and then the cell-associated radioactivity was counted after solubilization with 1 N NaOH. The specific binding is defined as the difference between binding in the presence and absence of unlabeled AcLDL.

To assess AcLDL internalization and degradation, cultures of RAW264.7 cells or peritoneal macrophages on 24-well culture plates were incubated for 2 hr at 4°C with 20 µg/mL ¹²⁵I-labeled AcLDL in RPMI 1640 medium containing 0.1% BSA in the absence or presence of 50-fold excess unlabeled AcLDL. The supernatants were removed, washed with ice-cold PBS, then further incubated in RPMI 1640 medium containing 0.1% BSA for the indicated times at 37°C. At the end of the incubation at 37°C, the culture medium was collected and kept on ice for the later measurement of degradation. The cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4°C to remove AcLDL associated with the cell surface. After washing with PBS, the internalized radioactivity was measured with a gamma counter after solubilization of the cells with 1 N NaOH. The specific internalization is defined as the difference between the internalized radioactivity in the presence and absence of the unlabeled AcLDL.

To measure AcLDL degradation, the collected culture medium as described before was precipitated with 10% trichloroacetic acid (TCA). The TCA-soluble radioactivity was measured with a gamma counter. The specific degradation is defined as the difference between the TCA-soluble radioactivity in the presence and absence of unlabeled AcLDL.

To evaluate whether the translocation of AcLDL from the early endosome compartment to the late endosome/lysosome was impaired in sPLA₂-V KD cells, a pulse-chase experiment was performed. Briefly, cells were incubated for the indicated time for up to 1 hr with ¹²⁵I-AcLDL at 18°C with or without 50-fold excess of unlabeled AcLDL. At this temperature, AcLDL degradation is inhibited by suppression of endosome-lysosome fusion and internalized AcLDL accumulates in the early-endosomal compartments. After the cells were washed with PBS to remove unbound AcLDL, a portion of the cultured cells was harvested and the cell internalized radioactivity was measured as described above. The remaining cells were further incubated for the indicated time up to 1 hr at 37°C at which time point the culture medium was collected and precipitated with 10% TCA. The TCA-soluble radioactivity as AcLDL degradation was measured as described above. The degradation efficiency was expressed as the percentage of the TCA-soluble radioactivity relative to the specific cell internalized radioactivity after 1 hr incubation at 18°C (=100%). The degradation efficiency reflects the rate of translocation of the internalized AcLDL to the late endosome/lysosome compartment.

Detection of Rab5 and Rab7 Recruitment to Endosomes Containing AcLDL and Translocation of Internalized AcLDL to Lysosomes

Fluorescein-labeled AcLDL (Thermo Fisher Scientific, Japan, Yokohama, Japan) in pre-coated iodination tubes (Thermo Fisher Scientific) to yield a specific activity of 100-150 kBq/µg AcLDL protein. For the binding study, cultures of RAW264.7 cells or peritoneal macrophages on 24-well culture plates were incubated for 2 hr at 4°C with 20 µg/mL ¹²⁵I-labeled AcLDL in RPMI 1640 medium containing 0.1% BSA in the absence or presence of 50-fold excess unlabeled AcLDL. The supernatants were removed, washed with ice-cold PBS, then further incubated in RPMI 1640 medium containing 0.1% BSA for the indicated times at 37°C. At the end of the incubation at 37°C, the culture medium was collected and kept on ice for the later measurement of degradation. The cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4°C to remove AcLDL associated with the cell surface. After washing with PBS, the internalized radioactivity was measured with a gamma counter after solubilization of the cells with 1 N NaOH. The specific internalization is defined as the difference between the internalized radioactivity in the presence and absence of the unlabeled AcLDL.

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using L each sample, 50,000 cells were analyzed by flow cytometry. translocation of internalized AcLDL to lysosomes. For each histogram were used to assess the degree of fluorescence intensities, and geometrical means of histograms representing the distribution of cell out. For flow cytometry, data were collected as confocal microscopy and flow cytometry were carried in cells by flow cytometry 22). At the indicated time assessed by measuring the relative content of F-actin such as those found in lysosomes. RAW264.7 cells were incubated with 20 µg/mL AcLDL conjugated with pHrodo at 37°C for the indicated time, and confocal microscopy and flow cytometry were carried out. For flow cytometry, data were collected as histograms representing the distribution of cell fluorescence intensities, and geometrical means of each histogram were used to assess the degree of translocation of internalized AcLDL to lysosomes. For each sample, 50,000 cells were analyzed by flow cytometry. The level of lysosomal acidification was examined using LysoTracker Red (Thermo Fisher Scientific) that emits green fluorescence under acidic conditions such as those found in lysosomes. RAW264.7 cells were incubated with 20 µg/mL AcLDL conjugated with pHrodo at 37°C for the indicated time, and confocal microscopy and flow cytometry were carried out. For flow cytometry, data were collected as histograms representing the distribution of cell fluorescence intensities, and geometrical means of each histogram were used to assess the degree of translocation of internalized AcLDL to lysosomes. For each sample, 50,000 cells were analyzed by flow cytometry. The level of lysosomal acidification was examined using LysoTracker Red (Thermo Fisher Scientific) that emits red fluorescence after accumulation in acidic compartments such as lysosomes. RAW264.7 cells were incubated with 20 µg/mL AcLDL conjugated with pHrodo at 37°C for the indicated time, and confocal microscopy and flow cytometry were carried out. For flow cytometry, data were collected as histograms representing the distribution of cell fluorescence intensities, and geometrical means of each histogram were used to assess the degree of translocation of internalized AcLDL to lysosomes. For each sample, 50,000 cells were analyzed by flow cytometry.

Flow Cytometric Analysis

The magnitude of actin polymerization was assessed by measuring the relative content of F-actin in cells by flow cytometry22). At the indicated time after addition of AcLDL, cells were fixed with 4% paraformaldehyde and permeabilized with 0.025% saponin. Then, the fixed and permeabilized cells were incubated with Alexa Fluor 546-conjugated anti-rabbit IgG (cat. no. A11010, Thermo Fisher Scientific) for confocal microscopy.

Translocation of internalized AcLDL to lysosomes was detected using AcLDL conjugated with pHrodo (Thermo Fisher Scientific), a pH indicator that emits green fluorescence under acidic conditions such as those found in lysosomes. RAW264.7 cells were incubated with 20 µg/mL AcLDL conjugated with pHrodo at 37°C for the indicated time, and confocal microscopy and flow cytometry were carried out. For flow cytometry, data were collected as histograms representing the distribution of cell fluorescence intensities, and geometrical means of each histogram were used to assess the degree of translocation of internalized AcLDL to lysosomes. For each sample, 50,000 cells were analyzed by flow cytometry.

Immunofluorescence Study

When nuclear localization of sPLA2-V and Myc-sPLA2-V was examined, cells were treated for 10 min with 20 µg/mL of AcLDL or PBS as a vehicle in 24-well plates with cover slips, washed with PBS, fixed with 2% paraformaldehyde, and permeabilized with 0.1% Triton X-100. The fixed cells were incubated with anti-sPLA2-V polyclonal antibody (1:100 dilution, cat. no. ab23709, Abcam) or anti-Myc-tag monoclonal antibody (1:500 dilution, clone no. 71D10, cat. no. 2278, Cell Signaling Technology) followed by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG (cat. no. A11008, Thermo Fisher Scientific). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Immunofluorescence study for the intracellular presence of Rab5 and Rab7 was described before.

Confocal Microscopy in Cells

Images were acquired with an Olympus FluoView 1000 confocal microscope system (Olympus, Tokyo, Japan) and processed using FV10-ASW software version 1.0 (Olympus) using appropriate wavelength of excitation laser and band-pass filter for emitted light according to the manufacture’s instruction. Images of the cells were taken consecutively with 1 µm intervals from the bottom edge to the top edge of the nucleus. The number of images captured was approximately 5–7 per cell. Among them, the single image that represents the most center of the nucleus was selected. Thus, the images of the nucleus do not include cytosolic staining above and below the nucleus. Images of sPLA2-V KD RAW264.7 cells with expression of GFP alone or GFP-sPLA2-V were taken after fixation with 4% paraformaldehyde. When pHrodo indicator or LysoTracker Red was used, cells were incubated with pHrodo-conjugated AcLDL or Lysotracker Red, and unfixed cells containing pHrodo or LysoTracker Red were viewed using the same confocal microscopy system.

Immunostaining of Isolated Nuclei

Nuclei were isolated by cell lysis buffer using

Scientific) was incubated with RAW264.7 cells at 4°C for 2 hr in 24-well plates with cover slips, then removed, and the cells were washed with ice-cold PBS twice and further incubated at 37°C for the indicated times in RPMI 1640 medium containing 0.1% BSA. Then, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.025% saponin and incubated with a primary antibody against Rab5 or Rab7 followed by Alexa Fluor 546-conjugated anti-rabbit IgG (cat. no. A11010, Thermo Fisher Scientific) for confocal microscopy.

Data are presented as histograms showing the distribution of cell fluorescence intensities, and geometrical means of each histogram were used to assess actin polymerization or SR-A1 expression. For each sample, 50,000 cells were analyzed by flow cytometry.
Nuclei EZ Prep Nuclei Isolation Kit (Nuc-101, Sigma) according to the manufacturer’s instruction. Cells were lysed by ice-cold Nuclei EZ Lysis Buffer. After centrifugation at 500×g for 5 min at 4°C, the nuclei pellet was washed with the same lysis buffer, followed by the centrifugation for 5 min at 4°C to remove remaining cytoplasmic contents. The final nuclear pellet was resuspended in Nuclei EZ Storage Buffer, being seeded on the silane-coated slides by cytospin centrifugation, fixed with 2% paraformaldehyde, and permeabilized with 0.1% Triton X-100. The fixed nuclei were served to immunofluorescence and confocal microscopic studies with similar procedure performed with whole cells, as described before.

**Western Blotting Analysis**

Protein (15 µg) from extracts of cultured cells was applied to a 4–20% SDS-PAGE gel (Bio-Rad Laboratories, Tokyo, Japan) under reducing conditions and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was treated with blocking buffer at room temperature for 1 hr and then incubated at 4°C overnight with primary antibody. After washing, the membrane was incubated at room temperature for 1 hr with HRP-conjugated goat anti-mouse or anti-rabbit IgG as the secondary antibody (dilution 1:10,000; cat. no. 115-035-146 and 111-035-003, respectively, Jackson ImmunoResearch Laboratories). Protein bands were visualized with Amersham ECL prime western blotting detection reagents (Cytiva, Tokyo, Japan). Samples were normalized relative to the intensity of the GAPDH or β-tubulin bands.

**Real-time Quantitative PCR (RT-PCR)**

The efficiency of the transfection of vectors encoding sPLA2-V and sPLA2-V-H48Q with or without Myc-tag, and GFP-tagged sPLA2-V as well as the efficiency of knockdown of sPLA2-V were evaluated by RT-PCR. Total RNA was isolated from cell homogenates with TRI Reagent (Sigma), reverse-transcribed into cDNA (ReverTra Ace qPCR RT Kit; Toyobo, Osaka, Japan), and assayed by qPCR using a 7500 Real Time PCR System (Thermo Fisher Scientific) and SYBR Green/ROX Master Mix (Toyobo). The ratio of each mRNA relative to the GAPDH mRNA was calculated using the ΔΔCt method. Primer pairs for respective genes are listed in Supplementary Table 1.

**Statistics**

All data are expressed as means ± SE. Statistical analysis was performed using GraphPad Prism software (version 5.01, GraphPad, La Jolla, CA). Differences between two groups were assessed by using an unpaired t-test. Analysis of more than two groups was carried out either by one-way ANOVA or two-way ANOVA followed by a Scheffe test for post hoc comparison of group means. P<0.05 was considered significant.

**Results**

**Nuclear Localization of sPLA2-V and Myc-sPLA2-V in RAW264.7 Cells and Peritoneal Macrophages**

Fluorescence microscopy using an antibody against sPLA2-V showed that the immunoreactivity of sPLA2-V was detected in the nucleus as well as cytoplasmic area of sPLA2-V knockdown (KD) RAW264.7 cells with re-constitutive expression of sPLA2-V (Fig. 1A). The immunoreactivity of Myc-sPLA2-V was also detected in the nucleus as well as cytoplasmic area of sPLA2-V KD RAW264.7 cells with re-constitutive expression of Myc-sPLA2-V using an anti-Myc-tag antibody (Fig. 1B). The immunoreactivity was not detected in sPLA2-V KD RAW264.7 cells transfected with empty vector (lower panels in Figs. 1A and 1B). In addition, endogenous sPLA2-V was detected using an antibody against sPLA2-V in the nucleus as well as cytoplasmic area of the peritoneal macrophages from sPLA2-V WT mice, but the immunoreactivity was not detected in cells from sPLA2-V KO mice (Fig. 1C). In these immunofluorescence microscopies, extent of the immunoreactivity of sPLA2-V or Myc-sPLA2-V in the nucleus appeared to be lower than that in the cytoplasmic area. In Fig. 1D, immunofluorescence study using anti-sPLA2-V antibody shows cell images taken consecutively with 1 µm intervals from the bottom edge to the top edge of the nucleus in peritoneal macrophages from sPLA2-V WT mice. The images in Fig. 1D show that the immunoreactivity of endogenous sPLA2-V existed within the nucleus as well as in the cytoplasmic area of the peritoneal macrophages. GFP-tagged sPLA2-V protein was detectable in the nuclear region as well as in the cytoplasmic region (Fig. 2A). When the cells were stimulated with AcLDL, the extent of expression of sPLA2-V, Myc-sPLA2-V, and GFP- sPLA2-V in the nucleus showed no remarkable changes (Figs. 1A, 1B, 1C, and 2A). In agreement with these immunofluorescence studies using whole cells, immunoreactivity of sPLA2-V or Myc-sPLA2-V was detected in the isolated nuclei from sPLA2-V KD RAW264.7 cells with re-constitutive expression of each of them and from peritoneal macrophages of sPLA2-V WT mice (Supplementary Fig. 1). Since we
Fig. 1. Immunofluorescence microscopic studies using anti-sPLA₂-V antibody or anti-Myc-tag antibody for nuclear localization of sPLA₂-V
Scale bars were 10 µm. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI) (blue). A and B, Immunoreactivity (green) of sPLA₂-V and Myc-sPLA₂-V was detected in the nucleus as well as in the cytoplasmic area in sPLA₂-V KD RAW264.7 cells with re-constitutive expression of sPLA₂-V or Myc-sPLA₂-V, respectively, 10 min after addition of 20 µg/mL of AcLDL or PBS as a vehicle. Their immunoreactivities in the nucleus appeared to be lower than those in the cytoplasmic area. The immunoreactivity was not detected in sPLA₂-V KD RAW264.7 cells transfected with empty vector (lower panels). C, Immunoreactivity of endogenous sPLA₂-V was detected in the nucleus as well as in the cytoplasmic area of primary peritoneal macrophages from sPLA₂-V wild-type (WT) mice but not from sPLA₂-V knockout (KO) mice. D, In peritoneal macrophages from sPLA₂-V WT mice, confocal microscopic images of the cells were taken consecutively at 1 µm intervals from the bottom edge to the top edge of the nucleus. Immunoreactivity of endogenous sPLA₂-V was detected within nucleus as well as in the cytoplasmic area.

Fig. 2. Nuclear localization of GFP-tagged sPLA₂-V and Myc-sPLA₂-V and efficacy of knockdown of sPLA₂-V by shRNA and transfection of various types of sPLA₂-V in sPLA₂-V KD RAW264.7 cells
A, sPLA₂-V KD RAW264.7 cells were transfected with plasmid vectors encoding GFP alone or GFP-tagged sPLA₂-V. GFP-tagged sPLA₂-V was detected in the nucleus as well as in the cytoplasmic area. Scale bars were 10 µm. B, Immunoblotting analysis of nuclear (NE) and cytoplasmic (CE) fractions from sPLA₂-V KD cells transfected with empty vector and sPLA₂-V KD cells expressing Myc-tagged sPLA₂-V and Myc-tagged sPLA₂-V H48Q using anti-Myc-tag antibody, anti-Lamin antibody (nucleus marker), and anti-β tubulin (cytoplasmic marker). Duplicate experiments were performed (exp. 1, exp. 2) in cells at baseline. C, RT-PCR shows successful knockdown of sPLA₂-V after transfection with shRNA for sPLA₂-V and re-constitutive expression of sPLA₂-V, sPLA₂-V-H48Q, Myc-tagged sPLA₂-V, Myc-tagged sPLA₂-V-H48Q, or GFP- sPLA₂-V in sPLA₂-V KD RAW264.7 cells. D, Evaluation of efficacy of shRNA targeting sPLA₂-V. Immunoblotting study using anti-Myc-tag antibody showed that shRNA targeting sPLA₂-V effectively abolished expression of the transfected Myc-tagged non-mutant original sPLA₂-V in sPLA₂-V KD RAW264.7 cells.

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Myc-tagged sPLA2-V-H48Q, and GFP-sPLA2-V in sPLA2-V KD RAW264.7 cells after transfection with the respective plasmid vectors (Fig. 2C). Immunoblotting study using anti-Myc-tag antibody showed that shRNA targeting sPLA2-V effectively abolished expression of the transfected Myc-tagged non-mutant original sPLA2-V in sPLA2-V KD RAW264.7 cells (Fig. 2D).

**Table 1. ChIP-Seq peak annotations**

| Genes         | Binding          | Chromosome | Distance to TSS (kb) |
|---------------|------------------|------------|---------------------|
| Pgk1          | WT, H48Q         | X          | 0                   |
| 4930467E23Rik | WT               | 8          | 33                  |
| 6550403H02Rik | KD, WT, H48Q    | 3          | 169                 |
| Atp8b5        | KD, WT, H48Q    | 4          | 34                  |
| Dlgap1        | H48Q             | 17         | 179                 |
| Elast2        | H48Q             | 4          | -236                |
| F9            | H48Q             | X          | -69                 |
| Frem3         | KD, WT, H48Q    | 8          | 30                  |
| Gm5458        | KD, WT, H48Q    | 14         | 185                 |
| Inpp4b        | KD               | 8          | -19                 |
| Lrrc4c        | WT, H48Q         | 2          | 1199                |
| Mapk10        | KD               | 5          | -29                 |
| Mdnc          | H48Q             | 6          | 482                 |
| Mir101c       | H48Q             | 9          | 38                  |
| Mir101c       | H48Q             | 9          | 35                  |
| Mir101c       | H48Q             | 9          | 18                  |
| Poc5          | KD, WT, H48Q    | 13         | -28                 |
| Sel1l         | KD, WT           | 12         | -322                |
| Slc22a22      | WT, H48Q         | 15         | 57                  |
| Slc22a26      | H48Q             | 19         | -17                 |
| Zic3          | H48Q             | X          | 308                 |

ChIP-Seq peaks were called using the MACS2 algorithm with a $P$-value of $<0.05$ and then annotated using ChIPseeker. The input sample was used by MACS and PeakSeq as a negative control when scoring significant peaks. WT, nucleus of sPLA2-V knockdown (KD) RAW264.7 cells with re-constitutive expression of Myc-tagged sPLA2-V; H48Q, nucleus of sPLA2-V KD RAW264.7 cells with expression of Myc-tagged sPLA2-V H48Q; KD, nucleus of sPLA2-V KD RAW264.7 cells transfected with empty vector.

previously experienced technical problems partly due to unavailability of antibodies that specifically detect sPLA2-V in tissues by immunoblotting,$^{16}$ we performed immunoblotting analyses using an anti-Myc-tag antibody in sPLA2-V KD RAW264.7 cells that had re-constitutive expression of C-terminally Myc-tagged sPLA2-V (KD + Myc-sPLA2-V) or Myc-tagged sPLA2-V-H48Q (KD + Myc-sPLA2-V-H48Q). In agreement with the results obtained by immunofluorescence and fluorescence microscopic studies, immunoblotting of subcellular fractions revealed that Myc-tagged sPLA2-V and Myc-tagged sPLA2-V-H48Q protein was detected in the nuclear fraction as well as the cytoplasmic fraction, although their expression levels in the nuclear fraction appeared to be much lower than those in the cytoplasmic fraction (Fig. 2B). The successful purification of nuclear extracts was confirmed by the absence of $\beta$-tubulin, a cytoplasmic marker, in the nuclear extract (Fig. 2B). RT-PCR showed successful knockdown of sPLA2-V after transfection of RAW264.7 cells with shRNA specific for sPLA2-V and successful expression of sPLA2-V, sPLA2-V-H48Q, Myc-tagged sPLA2-V, Myc-tagged sPLA2-V-H48Q, and GFP-sPLA2-V in sPLA2-V KD RAW264.7 cells after transfection with the respective plasmid vectors (Fig. 2C). Immunoblotting study using anti-Myc-tag antibody showed that shRNA targeting sPLA2-V effectively abolished expression of the transfected Myc-tagged non-mutant original sPLA2-V in sPLA2-V KD RAW264.7 cells (Fig. 2D).

**ChIP-Seq and ChIP-qPCR in RAW264.7 Cells**

The results showing nuclear localization of sPLA2-V prompted us to examine a role for sPLA2-V in the nucleus by testing whether sPLA2-V binds to DNA in the nucleus using ChIP-sequence (seq) and ChIP-quantitative PCR (qPCR). ChIP-Seq peaks were called using the MACS2 algorithm with a $P$-value of $<0.05$, and then annotated using ChIPseeker (Table 1 and Supplementary Table 2; data are available at the NCBI Gene Expression Omnibus under accession number GSE156528). Upon selecting peaks with a $P$-value cutoff of 0.05 and an enrichment >2-fold over input, significant enrichment of 8, 8, and 16 peaks in the nucleus of sPLA2-V KD RAW264.7 cells transfected with empty vector.

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lysosomal degradation of the internalized AcLDL. The assay of internalization was affected by simultaneous gene promoter activity in RAW264.7 cells.

Pgk1 gene in the nucleus.

The Pgk1 gene was not identified as a significant peak in the nucleus of sPLA2-V KD cells. Meanwhile, the Pgk1 gene was the strongest binding start site of the Pgk1 gene transcriptional activity showed that sPLA2-V KD RAW264.7 cells expressing Myc-tagged sPLA2-V was used as a ChIP-seq control. ChIP-seq analysis showed a Myc-tagged sPLA2-V-binding peak at the upstream region of the Pgk1 gene locus. B, ChIP-qPCR validation of Myc-tagged sPLA2-V-binding to the Pgk1 gene. Data are shown as a percentage expression of input control. The amplification sites (binding site and unrelated site) for PCR are indicated in panel A (ChIP-seq). Each bar represents the mean ± SEM of 2–3 independent experiments. C, Promoter assay for Pgk1 gene transcriptional activity. sPLA2-V WT cells, sPLA2-V KD cells, and sPLA2-V KD cells with re-constitutive expression of sPLA2-V or sPLA2-V-H48Q were transfected with Cypridina and Renilla luciferase expression vectors. The promoter activity is expressed as the relative luciferase activity normalized to Renilla activity. Values in each bar were normalized to that of WT (1). Each bar represents the mean ± SEM of 6–9 independent experiments. **, P < 0.01 vs. WT. ††, P < 0.01 vs KD. Upper panel shows a schematic illustration of the promoter construct used in this Cypridina luciferase reporter assay.

 Binding, Internalization, and Degradation Assay of AcLDL in RAW264.7 Cells

Next, we determined whether sPLA2-V may have a role in endocytosis of AcLDL in RAW264.7 cells. The specific binding activity of 125I-labeled AcLDL was similar in sPLA2-V WT and sPLA2-V KD cells (Figs. 4A and 4B). The amount of specific internalization of 125I-labeled AcLDL after 1 hr incubation at 37°C was lower in sPLA2-V KD cells compared with sPLA2-V WT cells (Fig. 4C). The assay of internalization was affected by simultaneous lysosomal degradation of the internalized AcLDL. The magnitude of the specific internalization of 125I-labeled AcLDL continued to be lower in sPLA2-V KD cells compared to sPLA2-V WT cells, even when bafilomycin A1 (Baf A), a lysosomal inhibitor, was co-incubated to block simultaneous degradation of the internalized 125I-labeled AcLDL (Fig. 4D). The specific degradation of AcLDL was significantly lower in sPLA2-V KD cells compared to sPLA2-V WT cells.
Fig. 4. Impairment of AcLDL internalization and degradation in sPLA2-V KD RAW264.7 cells

Each bar represents the mean ± SEM of 5–8 independent experiments. Details of the methods are described in the text. A, Dose-response binding of $^{125}$I-labeled AcLDL to the cell surface of sPLA2-V WT cells (left panel) and sPLA2-V KD cells (right panel) after incubation for 2 hr. B, sPLA2-V WT and -KD cells show similar degrees of cell surface-specific binding of $^{125}$I-labeled AcLDL after incubation with 20 µg $^{125}$I-labeled AcLDL for 2 hr. n.s. indicates statistically not significant. C, Comparison of specific internalization of $^{125}$I AcLDL in sPLA2-V WT cells, sPLA2-V KD cells, and sPLA2-V KD cells with re-constitutive expression of sPLA2-V WT or sPLA2-V-H48Q that lacks enzymatic activity, * $P < 0.05$, ** $P < 0.01$ vs. WT. †† $P < 0.01$ vs. KD. D, Effect of the lysosomal inhibitor bafilomycin A1 (Baf A) on specific internalization of $^{125}$I AcLDL. Cells were similarly incubated with $^{125}$I AcLDL for 2 hr at 4°C, washed, and then further incubated for the indicated time at 37°C in the presence or absence of 1 µM Baf A. The specific internalization was compared, ** $P < 0.01$ vs. WT. †† $P < 0.01$ vs KD. E, Comparison of the specific degradation of AcLDL at the indicated time after incubation with AcLDL. ** $P < 0.01$ vs. WT. † † $P < 0.01$ vs KD. F, Comparison of magnitude of cell surface expression of SR-A1 between sPLA2-V WT and KD cells. MFI indicates mean fluorescence intensity. G, Pulse chase experiment in RAW264.7 cells. Intracellular processing of internalized $^{125}$I AcLDL for lysosomal degradation. Cells were incubated for the indicated time up to 1 hr with $^{125}$I-AcLDL at 18°C. The cells were then washed and a portion of the cells was taken to measure specific internalization of AcLDL. The remaining cells were further incubated for the indicated time up to 1 hr at 37°C, and TCA-soluble radioactivity was measured at each time point. The amount of the internalized and degraded AcLDL (per cell protein) at each time point was normalized to that of internalized AcLDL after incubation for 1 hr at 18°C (= 100%). The mean amount of internalized AcLDL after the incubation for 1 hr at 18°C was 481 ng/mg protein and 378 ng/mg protein for sPLA2-V WT cells and sPLA2-V KD cells, respectively. The data are representative of three experiments. H, Comparison of degradation relative to internalized $^{125}$I-AcLDL after 1 hr incubation at 18°C on the pulse chase experiment (panel G) between sPLA2-V WT cells and sPLA2-V KD cells. sPLA2-V KD cells had lower rate (percentage) of degraded AcLDL after 1 hr incubation at 37°C relative to the amount of the specific internalized AcLDL after 1 hr incubation at 18°C. The rate reflects translocation of internalized AcLDL to the lysosome compartment. n=5 in each experiment. ** $P < 0.01$ vs. WT.

(Fig. 4E). Impairment of internalization and degradation of AcLDL in sPLA2-V KD cells was reversed by re-constitutive expression of sPLA2-V WT or sPLA2-V-H48Q (Figs. 4C and 4E). Magnitude of cell-surface expression of SR-A1, a main AcLDL receptor, was comparable between sPLA2-V WT and
sPLA₂-V KD cells (Fig. 4F), a result that was comparable with that regarding the binding activity of 

\[ ^{125}\text{I} \text{AcLDL} \]. Together, sPLA₂-V in RAW264.7 cells has a crucial role in the internalization and degradation of AcLDL that may be mediated via a mechanism that is independent of its enzymatic activity.

**Pulse-chase Experiment in RAW264.7 Cells**

Next, we examined a role for sPLA₂-V in intracellular processing of internalized AcLDL. RAW264.7 cells were incubated with 

\[ ^{125}\text{I} \text{AcLDL} \] at 18°C for the indicated time up to 1 hr. At this temperature, 

\[ ^{125}\text{I} \text{AcLDL} \] bound to cell-surface receptors will be internalized and accumulate in early endosomes without further processing or fusion of early endosomes to late endosome/lysosomes\(^{21} \) (Fig. 4G). After incubation at 18°C, the treated cells were washed to remove unbound 

\[ ^{125}\text{I} \text{AcLDL} \] and chased at 37°C for the indicated time up to 1 hr. The amount of the internalized and degraded AcLDL (per cell protein) at each time point was normalized to that of the internalized AcLDL after incubation for 1 hr at 18°C (=100%). Intracellular processing of internalized AcLDL for lysosomal degradation was defined as percentage of the amount of degraded 

\[ ^{125}\text{I} \text{AcLDL} \] after incubation for 1 hr at 37°C relative to that of the internalized 

\[ ^{125}\text{I} \text{AcLDL} \] after incubation for 1 hr at 18°C. After shifting the temperature to 37°C, sPLA₂-V KD cells had a lower rate of AcLDL degradation compared with sPLA₂-V WT cells (Fig. 4H). These results indicated an impairment of intracellular processing of internalized AcLDL for lysosomal degradation in sPLA₂-V KD RAW264.7 cells.

**Maturation of Endosomes Containing AcLDL and Its Translocation to Lysosomes in RAW264.7 Cells**

To evaluate maturation of endosomes containing AcLDL, we examined whether the intracellularly internalized AcLDL co-localized with endosomal proteins that are recruited to maturing endosomes in RAW264.7 cells. In the immunofluorescence study, intracellular AcLDL co-localized with Rab5, an early endosome marker, 5 min after incubation with AcLDL (Fig. 5A). Sequentially, AcLDL co-localized with Rab7, a late endosomal marker, 5–30 min after incubation with AcLDL. Next, to evaluate translocation of the intracellularly internalized AcLDL to lysosomes, we incubated RAW264.7 cells with AcLDL conjugated with pHrodo, a pH indicator that emits green fluorescence under acidic conditions. Green fluorescence from pHrodo conjugated to AcLDL appeared in the RAW264.7 cells 30 and 60 min after co-incubation (Fig. 5B). These results indicated that endosomes containing AcLDL had matured and were translocated to lysosomes. Compared with sPLA₂-V WT cells, sPLA₂-V KD cells had lower amounts of fluorescence emission from pHrodo conjugated to AcLDL, and this decrease was restored by re-constitutive expression of sPLA₂-V or sPLA₂-V-H48Q that lacks catalytic enzymatic activity (Fig. 5C). We further examined lysosomal acidification using LysoTracker Red that is membrane permeable and accumulates in acidic organelles including lysosomes (Figs. 5D and 5E). After incubation for 30 min with 50 nM LysoTracker Red, the extent of red fluorescence from LysoTracker was similar between sPLA₂-V WT and KD cells (Fig. 5D), suggesting that both cell types had similar lysosomal acidification. Thus, the low fluorescence intensity of pHrodo conjugated to AcLDL in sPLA₂-V KD cells could be attributed to impaired translocation of AcLDL conjugated with pHrodo to the lysosome but not dysfunctional lysosomal acidification. Taken together, sPLA₂-V knockdown impaired translocation of the intracellularly internalized AcLDL to the lysosome, and sPLA₂-V played a role in the translocation of internalized AcLDL to the lysosome independently of its enzymatic activity. These results were also consistent with those obtained for the pulse-chase experiment using 

\[ ^{125}\text{I} \text{AcLDL} \].

**Reduced Actin Polymerization in sPLA₂-V KD RAW264.7 Cells**

We assessed the degree of actin polymerization in response to AcLDL by flow cytometry using Alexa Fluor 546-phalloidin\(^{22} \) in RAW264.7 cells because actin polymerization has an important role in the early stage of endocytic vesicle internalization and translocation of cargo from early endosomes to late endosomes/lysosomes\(^{33} \), \(^{24} \). The degree of actin polymerization in response to AcLDL was lower in sPLA₂-V KD cells compared to sPLA₂-V WT cells (Figs. 6A and 6B). The impairment of actin polymerization in response to AcLDL in sPLA₂-V KD cells was reversed by re-constitutive expression of sPLA₂-V or sPLA₂-V-H48Q after transfection with the respective expression vectors (Fig. 6B). Thus, sPLA₂-V appears to have a crucial role in actin polymerization via mechanisms that are independent of its enzymatic activity in RAW264.7 cells.

**PGK1 Expression, Beclin1 Phosphorylation and class III PI3-kinase Activity and Their Relation with Actin Polymerization and Translocation of Internalized AcLDL to Lysosomes in RAW264.7 Cells**

It was shown that PGK1 phosphorylates Beclin1.
**Fig. 5.** Maturation of endosomes containing AcLDL and fusion of endosomes and lysosomes in RAW264.7 cells

A, Immunofluorescence images showing colocalization of Alexa Fluor™ 488 AcLDL (green) with Rab5 and Rab7 (red) after incubation with AcLDL for the indicated times in sPLA₂-V WT and KD cells. Scale bars were 10 µm. The data are representative of three experiments. B, Fluorescence images of AcLDL conjugated with pHrodo (green) to visualize acidic cell compartments after incubation for the indicated times are also shown. Scale bars were 10 µm. Right panels are phase contrast images corresponding to the respective fluorescence images of AcLDL conjugated with pHrodo. The data are representative of three experiments. C, Comparison of the mean fluorescence intensity (MFI) of pHrodo at the indicated time after incubation with AcLDL conjugated with pHrodo for sPLA₂-V WT cells, sPLA₂-V KD cells, and sPLA₂-V KD cells with re-constitutive expression with sPLA₂-V or sPLA₂-V-H48Q that lacks enzymatic activity. \( n = 5 \) –7 in each experiment. **, \( P < 0.01 \) vs. WT. ††, \( P < 0.05 \), ††, \( P < 0.01 \) vs. KD. D, Comparison of the area exhibiting Lysotracker Red fluorescence that represents acidic cell compartments between sPLA₂-V WT and -KD cells. Cells were incubated with 50 nM Lysotracker Red for 30 min in RPMI medium. The area of red fluorescence in each cell was measured using ImageJ and expressed as the percentage of red fluorescence area relative to the cell surface area, and averaged over at least 20 cells. \( n = 5 \) in each experiment. n.s. indicates statistically not significant. E, Panel shows representative confocal microscopic images. Scale bars are 10 µm.

**Fig. 6.** Impairment of actin polymerization in sPLA₂-V KD RAW264.7 cells

A, Confocal microscopy of F-actin polymerization detected by Alexa Fluor 546-phalloidin (red) at baseline (0 min) and 5 min after incubation with Alexa Fluor 488-labelled AcLDL (green) in sPLA₂-V WT and -KD cells. Cell protrusions probably due to actin polymerization are seen 5 min after incubation with AcLDL. Scale bars were 10 µm. B, Comparison of mean fluorescence intensity (MFI) of F-actin polymerization using flow cytometry after incubation with AcLDL for the indicated time for sPLA₂-V WT, sPLA₂-V KD, and sPLA₂-V KD cells with re-constitutive expression of sPLA₂-V or sPLA₂-V-H48Q. \( n = 5 \)–7 in each experiment. **, \( P < 0.01 \) vs. WT. ††, \( P < 0.01 \) vs. KD.
Beclin1-class III PI3-kinase pathway in a manner that is independent of its enzymatic activity in RAW264.7 cells.

At the active site (S30), leading to enhancement of Beclin1-VPS34 complex (class III PI3-kinase) activity toward phosphatidylinositol 3-phosphate (PI[3]P) production. In agreement with these previous reports, reduction of PGK1 expression by siRNA suppressed Beclin1 S30 phosphorylation in sPLA2-V WT cells (Figs. 7A and 7B). Successful suppression of PGK1 expression by siRNA was confirmed by immunoblotting (Fig. 7C). Compared with sPLA2-V WT cells, sPLA2-V KD cells had lower levels of PGK1 expression (Fig. 7D), and class III PI3-kinase activity (Fig. 7E), and these levels were restored by re-constitutive expression of sPLA2-V or sPLA2-V-H48Q. Representative immunoblots to assess PGK1 expression and Beclin1 phosphorylation are shown in Fig. 7G. Taken together, sPLA2-V regulates the PGK1-Beclin1-class III PI3-kinase pathway in a manner that is independent of its enzymatic activity in RAW264.7 cells.

In sPLA2-V WT cells, suppression of PGK1 or Beclin1 expression by respective siRNAs impaired actin polymerization in response to AcLDL (Fig. 8A) and translocation of internalized AcLDL to lysosomes (Fig. 8B) as estimated by flow cytometry using Alexa Fluor 546-phalloidin and pHrodo-conjugated AcLDL, respectively. Successful suppression of Beclin1 expression by siRNA was confirmed by immunoblotting (Fig. 8C). The impairment of actin polymerization and translocation of AcLDL to lysosomes in sPLA2-V KD RAW264.7 cells was reversed by overexpression of PGK1 (Figs. 8D and 8E). An immunoblotting analysis confirmed successful expression of PGK1 after transfection of a plasmid

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**Fig. 7.** PGK1 expression, Beclin1 S30 phosphorylation, and PI3-kinase activity in RAW264.7 cells

A, Suppression of Beclin1 phosphorylation at S30 (p-Beclin1/Beclin1) by siRNA (#1 and #2)-mediated reduction of PGK1 expression. Values were normalized to that of control siRNA after incubation with PBS as a vehicle (1). n = 5 in each experiment. ***, P < 0.01 vs. control siRNA. B, Representative immunoblots for panel A. C, Successful suppression of PGK1 expression by siRNA confirmed by immunoblotting. D, E, F, Comparison of PGK1 expression (D), Beclin1 phosphorylation at S30 (E), and PI3-kinase activity (F) after incubation for 2 hr with 20 µg/mL AcLDL or PBS as a vehicle in sPLA2-V-WT and KD cells, and sPLA2-V KD cells with re-constitutive expression of sPLA2-V or sPLA2-V-H48Q. G, Representative immunoblots showing PGK1 expression and Beclin1 phosphorylation in various types of RAW264.7 cells. Values in panels D and E were normalized to that of WT after incubation with PBS as a vehicle (= 1). Each bar represents the mean ± SEM of 5–6 independent experiments. *, P < 0.05, **, P < 0.01 vs. WT; †, P < 0.05, ††, P < 0.01 vs. KD.
searched for intracellular signals that could contribute to reducing actin polymerization in sPLA2-V KD RAW264.7 cells, we observed reduced levels of c-Src phosphorylation at the active site (Y416) in sPLA2-V KD cells compared with that seen in sPLA2-V WT cells (Figs. 9A and 9B). Overexpression of c-Src relieved impairment of actin polymerization in response to AcLDL and translocation of intracellularly internalized pHrodo-conjugated AcLDL to lysosomes in sPLA2-V KD cells (Figs. 9C and 9D, respectively).

The immunoblotting analysis confirmed successful expression of c-Src after transfection of a plasmid vector encoding c-Src in sPLA2-V KD cells (Fig. 9E).

Suppression of PGK1, Beclin1, or VPS34 by the vector encoding the \textit{Pgk1} gene in sPLA2-V KD cells (Fig. 8F). Thus, the PGK1-Beclin1 pathway appears to regulate actin polymerization in response to AcLDL and translocation of internalized AcLDL to lysosomes in RAW264.7 cells. Moreover, sPLA2-V contributes to actin polymerization and translocation of internalized AcLDL to lysosomes through the PGK1-Beclin1 pathway in RAW264.7 cells (Fig. 8G).

**Role of c-Src in Actin Polymerization and Translocation of AcLDL to Lysosome**

Endocytosis is an actin-dependent process\textsuperscript{23, 24}, and a variety of intracellular signals can converge to modulate actin reassembly at endocytosis. When we searched for intracellular signals that could contribute to reducing actin polymerization in sPLA2-V KD RAW264.7 cells, we observed reduced levels of c-Src phosphorylation at the active site (Y416) in sPLA2-V KD cells compared with that seen in sPLA2-V WT cells (Figs. 9A and 9B). Overexpression of c-Src relieved impairment of actin polymerization in response to AcLDL and translocation of intracellularly internalized pHrodo-conjugated AcLDL to lysosomes in sPLA2-V KD cells (Figs. 9C and 9D, respectively). The immunoblotting analysis confirmed successful expression of c-Src after transfection of a plasmid vector encoding c-Src in sPLA2-V KD cells (Fig. 9E). Suppression of PGK1, Beclin1, or VPS34 by the
may regulate c-Src phosphorylation at Y416 in RAW264.7 cells.

Role of Other sPLA2 Isoforms in Intracellular Translocation of AcLDL and Pagk1 Gene Expression in RAW264.7 Cells

We examined whether other sPLA2 isoforms than sPLA2-V may have a role in intracellular translocation of AcLDL to lysosomes and PGK1 expression in RAW264.7 cells. First, we measured expression levels respective siRNAs was associated with a reduction in c-Src phosphorylation at Y416 in sPLA2-V WT cells (Figs. 9F, 9G, 9H, 9I, 10A, and 10B). Successful suppression of VPS34 expression by siRNA was confirmed by immunoblotting (Fig. 10C). Taken together, reduction of c-Src phosphorylation at Y416 was an additional mechanism that mediated decreases in actin polymerization and translocation of internalized AcLDL to lysosomes in sPLA2-V KD RAW264.7 cells (Fig. 8G). PGK1-Beclin1-VPS34
of all sPLA2s in RAW264.7 cells by quantitative RT-PCR using 0.5 µg of total RNA. In this study, we arbitrarily determined the significant expression of mRNA as cycle threshold (Ct) value of each sPLA2 < Ct value of negative control. The Ct values of negative control were > 31. Among sPLA2s, sPLA2-IID (Ct = 24.7 ± 0.8), -IIE (Ct = 23.5 ± 0.4), -V (Ct = 22.7 ± 0.1), and -XIIA (Ct = 22.1 ± 0.1) were significantly expressed in RAW264.7 cells on the basis of the Ct value. Next, we examined whether siRNA-mediated knockdown of sPLA2-IID, IIE, or -XIIA may reduce PGK1 expression and intracellular translocation of pHrodo-conjugated AcLDL to lysosomes in sPLA2-V WT Raw264.7 cells. As shown in Supplementary Fig. 2, knockdown of these sPLA2s had no effects on intracellular translocation of pHrodo-conjugated AcLDL and Pagk1 gene expression in RAW264.7 cells. Thus, other sPLA2s did not seem to have similar activities as sPLA2-V.

Primary Cultures of Peritoneal Macrophages from sPLA2-V KO Mouse Had An Impairment of Endocytosis of AcLDL and Actin Polymerization and Reduction of PGK1 Expression, Beclin1 Phosphorylation, and c-Src Phosphorylation

Primary cultures of peritoneal macrophages from sPLA2-V KO mice had impairments in internalization (Fig. 11A) and degradation of 125I AcLDL (Fig. 11B), yet had similar levels of cell-surface-specific binding as compared with peritoneal macrophages from sPLA2-V WT mice (Fig. 11C). Flow cytometry using Alexa Fluor 546-phalloidin showed a lower degree of actin polymerization in response to AcLDL in sPLA2-V KO peritoneal macrophages compared with sPLA2-V WT cells (Fig. 11D). sPLA2-V KO peritoneal macrophages showed a lower magnitude of mean fluorescence intensity (MFI) of pHrodo after incubation with pHrodo-conjugated AcLDL (Fig. 11E). Furthermore, levels of PGK1 expression and Beclin1 phosphorylation at S30 were reduced in peritoneal macrophages from sPLA2-V KO mice compared with those from sPLA2-V WT mice (Figs. 11F and 11G). c-Src phosphorylation at Y416 was also decreased in peritoneal macrophages isolated from sPLA2-V KO mice compared to those from sPLA2-V WT mice (Fig. 11H). Representative immunoblots for panels G and H are shown in Fig. 11I.

Discussion

The present study is the first to demonstrate that sPLA2-V in the nucleus binds to the promoter region of the Pagk1 gene and increases its transcriptional activity in RAW264.7 cells. Similarly, sPLA2-V-
H48Q, which lacks enzymatic activity\(^{19}\), also bound and promoted transcriptional activity of the \(Pgk1\) gene. The hydrolytic activity of sPLA\(_2\)s requires mM concentrations of \(Ca^{2+}\)\(^{1}\), whereas the calcium concentration in both the nucleus and the cytosol is at most a few \(\mu\)M\(^{6}\). In the present study, the suppressed expression of \(PGK1\) in sPLA\(_2\)-V KD RAW264.7 cells could be restored by transfection with sPLA\(_2\)-V-H48Q. Taken together, sPLA\(_2\)-V could promote \(Pgk1\) gene transcription via a mechanism that does not involve enzymatic activity.

Immunofluorescence microscopic studies showed that immunoreactivity of sPLA\(_2\)-V and Myc-sPLA\(_2\)-V, that were re-constitutively expressed, was detected in the nucleus as well as cytoplasmic area of sPLA\(_2\)-V KD RAW264.7 cells. Also, immunoreactivity of endogenous sPLA\(_2\)-V was detected in the nucleus as well as cytoplasmic area of primary peritoneal macrophages. In addition, fluorescence microscopy showed nuclear localization of GFP-tagged sPLA\(_2\)-V in sPLA\(_2\)-V KD RAW264.7 cells. Furthermore, immunoreactivity of sPLA\(_2\)-V or Myc-sPLA\(_2\)-V was detected in the isolated nuclei from sPLA\(_2\)-V KD RAW264.7 cells with re-constitutive expression of each of them and from sPLA\(_2\)-V WT peritoneal macrophages, which may support the finding obtained with whole cells. In the immunoblotting, Myc-sPLA\(_2\)-V was shown in the nuclear subcellular...
fractions of sPLA₂-V KD RAW264.7 cells with expression of Myc-sPLA₂-V. These studies showed that extent of expression of sPLA₂-V and Myc- or GFP-tagged sPLA₂-V localized in the nucleus appeared to be very lower than that in the cytoplasmic area. Thus, it may require further evaluation of nuclear localization of sPLA₂-V using other methods. According to computational approaches, sPLA₂-V has neither DNA binding motifs nor sequence motifs corresponding to either nuclear localization signals or nuclear export signals. The mechanism underlying the nuclear localization of sPLA₂-V is entirely unclear at the present time. sPLA₂-V has neither the group I and II sPLA₂-specific the disulfide bridge between cysteines 11 and 77, nor does it contain the 6-amino acid C-terminal extension characteristic of group II sPLA₂s. It is speculated that these structural characteristics of sPLA₂-V that are different from those of other sPLA₂s may be involved in the nuclear localization of sPLA₂-V. In addition, since molecular weights of sPLA₂-V and GFP-tagged sPLA₂-V were approximately 14 kDa and 41 kDa, respectively, they are small enough to enter and exist in the nucleus by passive diffusion through nuclear pores. The Pgk1 gene is constitutively expressed and is ubiquitously present in all somatic cells. The promoter region of Pgk1 gene has multiple binding sites for transcriptional factors and possess constitutive enhance activity. It is fully unclear how the sPLA₂-V protein interacts with Pgk1 promoter region to enhance the promoter activity. There is a possibility that sPLA₂-V may presumably bind to unknown cytosolic proteins which could import to the nucleus, and that the protein complex with sPLA₂-V may interact with the promoter region of Pgk1 gene so that the transcription of Pgk1 gene could proceed.

PGK1 is known to play a key role in the glycolysis pathway. Recently, PGK1 was shown to phosphorylate Beclin1 at the active site (S30) in a manner that is independent of its role in the glycolysis. This phosphorylation promotes activation of the Beclin1-VPS34 (class III PI3-kinase) complex. Beclin1 is a core component of this class PI3-kinase complex, and Beclin1 phosphorylated at S30 activates class III PI3-kinase to generate PI(3)P, which in turn interacts with the early endosome antigen 1, Rab5, and Rab7 on the endosomal membrane and contributes to endosomal maturation. PI(3)P is also required for actin polymerization during phagosome formation. On the basis of the present studies using approach of PGK1 overexpression and siRNA-mediated reduction of PGK1 and Beclin1 expression, PGK1-Beclin1 pathway regulates AcLDL endocytosis in RAW264.7 cells, and that sPLA₂-V regulates AcLDL endocytosis through the PGK1-Beclin1 pathway (Fig. 8G). Since actin polymerization has an important role in the early stages of endocytic vesicle internalization and translocation of cargo from early endosomes to late endosome/lysosomes, impairment of actin polymerization might contribute to the reduction of both internalization and intracellular trafficking of AcLDL to lysosomes in sPLA₂-V deficient cells. Previous clinical report described that PGK1 deficiency was associated with hemolytic anemia, rhabdomyolysis, and/or mental retardation. It remains unclear whether the present findings may have a role in the pathogenesis of these disorders in humans.

The results of this study suggested that c-Src plays a regulatory role in sPLA₂-V-mediated internalization and intracellular trafficking of AcLDL to lysosomes in RAW264.7 cells. The Src family of protein tyrosine kinases is known to play key roles in receptor-mediated endocytosis and endosomal maturation via actin polymerization. Suppressed expression of Beclin1 was previously shown to reduce c-Src phosphorylation at Y416 (the active site) results of the present study showed that siRNA-mediated suppression of PGK1, Beclin1, or VPS34 inhibited c-Src phosphorylation at Y416. Thus, PGK1-Beclin1-VPS34 may play a role in c-Src activation in RAW264.7 cells (Fig. 8G).

The findings obtained here support an important role of sPLA₂-V in actin polymerization and endocytosis of AcLDL in primary cultures of mouse peritoneal macrophages. In addition, sPLA₂-V KO peritoneal macrophages had reductions in PGK1 expression, Beclin1 phosphorylation, and c-Src phosphorylation that were similar to those seen in sPLA₂-V KD RAW264.7 cells. Since primary macrophage cultures are difficult to be transfected with siRNA or cDNA constructs, the details of mechanisms associated with sPLA₂-V could not be defined. Nevertheless, similar mechanisms as those seen for RAW264.7 cells, a mouse monocyte/macrophage cell line, might be extended to explain the results obtained for peritoneal macrophages. Balestriери et al. showed an impairment of endocytosis and intracellular trafficking of zymosan and Candida albicans after binding to their cell-surface receptor, dectin-1 and/or Toll-like receptors, in sPLA₂-V KO murine macrophages in a manner that was independent of its enzymatic activity and inhibitor systems in sPLA₂-V KO macrophages in a
similar manner as observed in the present study. In addition, they showed that mice lacking sPLA2-V had an increased fungal burden in the kidney, liver, and spleen in an in vivo model of systemic candidiasis\(^\text{10}\). Thus, the findings at the cellular level were extended to those in an in vivo animal model. However, it remains unknown whether a decrease in PGK1 expression mediated by sPLA2-V deficiency may be involved in the mechanisms for the impairments of endocytosis and intracellular trafficking of zymosan and Candida albicans in in vitro macrophages and in vivo mice model.

It cannot be fully ruled out that enzymatic activity of sPLA2-V may be required for phagocytosis of microorganisms in macrophages. In human macrophages, ethanolamine lysophospholipid generated via sPLA2-V-mediated hydrolysis of membrane ethanolamine contributed to phagocytosis of microorganisms\(^\text{35}\). Moreover, studies using sPLA2-V-deficient mice have revealed that sPLA2-V-driven release of oleic and linoleic acids is crucial for polarization into M2-like macrophages, which have a greater phagocytic potential than M1-like macrophages\(^\text{36, 37}\), although the functions of M1 and M2 macrophages are context-specific.

It is unclear whether sPLA2-V-mediated endocytosis of modified LDL in macrophages has a beneficial or detrimental effect in atherosclerotic plaque progression and stability. Uptake of modified LDL protects macrophages against cytotoxicity of modified LDL but results in foam cell formation and plaque progression\(^\text{38}\). Although macrophage endocytosis/phagocytosis is necessary to remove pro-inflammatory apoptotic cells, strong proatherogenic properties are elicited upon endocytosis/phagocytosis of modified lipoproteins, erythrocytes and platelets\(^\text{39}\). Bostrom and his coworkers previously showed that sPLA2-V KO macrophages had less atherogenicity in LDL receptor-deficient mice\(^\text{39}\). In this regard, sPLA2-V-mediated endocytosis of modified LDL by macrophages may be proatherogenic. It remains to be defined whether cholesterol ester accumulation induced by Ac-LDL may be decreased in sPLA2-V KD RAW264.7 cells. If the decrease occurs, it will be required to be determined whether the decrease in cholesterol ester accumulation may be restored by sPLA2-V re-constitutive expression.

PGK1 translocates to mitochondria in response to hypoxic conditions and inhibits the pyruvate dehydrogenase complex, which in turn suppresses mitochondrial oxidative phosphorylation of pyruvate\(^\text{40}\). Thus, PGK1 could promote the Warburg effects in the lesion macrophages in the hypoxic milieu such as that found in thickened arterial walls with highly advanced atherosclerosis. sPLA2-V plays a role in the inflammatory innate immune response in macrophages as previously described\(^\text{9, 10}\). In this context, coordination of sPLA2-V and PGK1 might link the inflammatory innate immune response and changes in glucose metabolism in the lesion macrophages, thus providing a novel mechanism for immunometabolism\(^\text{41, 42}\).

In conclusion, sPLA2-V in the nucleus of RAW264.7 cells binds to the promoter region of the Pglk1 gene and increases its transcriptional activity. sPLA2-V regulates endocytosis of AcLDL through PGK1-Beclin1 in a manner that is independent of its enzymatic activity. Although a non-enzymatic action of sPLA2s through the sPLA2 receptor on the plasma membrane has been demonstrated in previous studies\(^\text{43, 44}\), the present study provides a novel functional role for sPLA2-V as a transcriptional regulator in the nucleus.

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**Conflict of Interest**

Kiyotaka Kugiyama has received scholarship donations from Takeda, Daiichi Sankyo, Astellas, Boehringer Ingelheim, MSD, Boston Scientific Japan, Abbott, Medtronic, Biotronik Japan, and St Jude Medical. The remaining authors declare no conflicts of interest.

**Author Contribution**

DF and KK contributed to design, analysis and interpretation of data and drafted the paper. YW performed experiments. TY, TN, MM and KM designed and supervised the study.

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Supplementary Table 1. Oligonucleotide sequences for qPCR, siRNA, and mutagenesis

| qPCR primers | sense | antisense | cat. no. |
|--------------|-------|-----------|----------|
| Pla2g1b      | 5’-cga ctt aga cag gtg ctc cca g-3’ | 5’-ggg gct gca ggr gat ctc g-3’ |          |
| Pla2g2a      | 5’-cct tgt gct caa tac agg tcc-3’ | 5’-gtc arg aat aca aca gca cgc gtc-3’ |          |
| Pla2g2e      | 5’-cct tgt gct caa tac agg tcc-3’ | 5’-ccc agc acc acc tgt ctc g-3’ |          |
| Pla2g2f      | 5’-gac tgt gtc ctc ctc gtt ccc ctc-3’ | 5’-ctc cgg tgt tca gaa ctc g-3’ |          |
| Pla2g3       | 5’-gac tgt tgt tat ggg caa-3’ | 5’-agg agt cgr gtc aga tga-3’ |          |
| Pla2g5       | 5’-gac tgt tgt tat ggg caa-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Pla2g10      | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Pla2g12b     | 5’-gga agg gat gac tat gtt cag arg-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Src          | 5’-gga agg gat gac tat gtt cag arg-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Gapdh        | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Pgk1         | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| Becn1        | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| ChIP-binding site | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |
| ChIP-unrelated site | 5’-gta gca cga cgg atc gaa gc-3’ | 5’-ggg gct gca cca gca gtc-3’ |          |

| siRNA          | Sense (5’-3’) | Anti-sense (5’-3’) | Cat. no. |
|----------------|---------------|-------------------|----------|
| Pla2g2d #1     | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00195777 |          |
| Pla2g2d #2     | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00195777 |          |
| Pla2g2e #1     | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Pla2g2e #2     | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Pla2g12a #1    | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00195777 |          |
| Pla2g12a #2    | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00195777 |          |
| Pgk1 #1        | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Pgk1 #2        | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Becn1 #1       | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Becn1 #2       | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Pik3c3 #1      | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Pik3c3 #2      | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |

| mutagenesis primers | Sense (5’-3’) | Anti-sense (5’-3’) | Cat. no. |
|---------------------|---------------|-------------------|----------|
| avoidance of shRNA 1st | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| avoidance of shRNA 2nd | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| Myc deletion        | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |
| H48Q                | 5’-cag acu ggu gcu gcu aga a-3’ | SASI_Mm01_00067977 |          |

Pre-designed siRNAs were obtained from Sigma. Pik3c3 corresponds to vacuolar protein sorting 34 (VPS34).
Supplementary Fig. 1. Immunofluorescence Microscopic Study of the Isolated Nuclei

Nuclei were visualized with 4',6-diamidino-2-phenyldindole (DAPI) (blue). Scale bars were 10 µm. Images of the nucleus (A, B, C, and E) were taken consecutively with 1 µm intervals from the bottom edge to the top edge of the nucleus. Among them, the single image that represents the most center of the nucleus was shown. A and B, Immunoreactivity (green) of sPLA_2-V and Myc-sPLA_2-V was detected in the nuclei isolated from sPLA_2-V KD RAW264.7 cells with re-constitutive expression of sPLA_2-V and Myc-sPLA_2-V, respectively. No immunoreactivity was detected in the nuclei isolated from sPLA_2-V KD RAW264.7 cells transfected with empty vector (lower panels). C, Immunoreactivity of endogenous sPLA_2-V was detected in the nuclei isolated from primary peritoneal macrophages of sPLA_2-V wild-type (WT) mice but not from sPLA_2-V knockout (KO) mice. D, In the nuclei isolated from peritoneal macrophages of sPLA_2-V WT mice, confocal microscopic images were taken consecutively at 1 µm intervals from the bottom edge to the top edge of the nucleus. E, The immunoreactivity (green) of GAPDH (cytoplasmic marker) was not detected in the isolated nuclei, suggesting little contamination by cytosolic proteins during procedure of nucleus isolation.
The table below contains ChIP-Seq peak annotations. ChIP-Seq peaks were called using the MACS2 algorithm with a $P$ value of < 0.05, then annotated using ChipPeeker. The input sample was used by MACS and PeakSeq as a negative control when scoring significant peaks.

| chr | start | end   | width | length | abs_summit | pileup | (−)log10 (p-value) | fold_enrichment | (−)log10 (q-value) | MACS_peakID | annotation               |
|-----|-------|-------|-------|--------|------------|--------|---------------------|------------------|---------------------|-------------|--------------------------|

**sPLA2-V-KD cells transfected with empty vector**

| chr  | start | end   | width | length | abs_summit | pileup | (−)log10 (p-value) | fold_enrichment | (−)log10 (q-value) | MACS_peakID | annotation               |
|------|-------|-------|-------|--------|------------|--------|---------------------|------------------|---------------------|-------------|--------------------------|

**sPLA2-V-KD cells with re-constitutive expression of sPLA2-V**

| chr  | start | end   | width | length | abs_summit | pileup | (−)log10 (p-value) | fold_enrichment | (−)log10 (q-value) | MACS_peakID | annotation               |
|------|-------|-------|-------|--------|------------|--------|---------------------|------------------|---------------------|-------------|--------------------------|

**sPLA2-V-KD cells with expression of sPLA2-V-H4KQ**

| chr  | start | end   | width | length | abs_summit | pileup | (−)log10 (p-value) | fold_enrichment | (−)log10 (q-value) | MACS_peakID | annotation               |
|------|-------|-------|-------|--------|------------|--------|---------------------|------------------|---------------------|-------------|--------------------------|

**Supplementary Table 2.** ChIP-Seq peak annotations (genome build mouse NCBI38/mm10)
(Cont. Supplementary Table 2)

| gene Chr | gene Start | gene End | gene Length | gene Strand | gene Translcript | distance ToTSS | ENSEMBL | SYMBOL | GENENAME |
|----------|------------|----------|-------------|-------------|-----------------|--------------|---------|---------|----------|
| 12       | 91880640   | 91841573 | 31135       | 2           | 20338 ENSMUST00010178462.7 | -32247       | ENSMUSG00000020964 | Sel1l    | sel-1 suppressor of lin-12-like (C. elegans) |
| 13       | 96838294   | 96417737 | 5206       | 1           | 67463 ENSMUST0000992955.5 | -28335       | ENSMUSG00000021671 | Poc5     | POC5 centriolar protein |
| 14       | 15994139   | 16002581 | 14405       | 2           | 432825 ENSMUST0000961211.11 | 183500      | ENSMUSG00000092094 | Gnm5458  | predicted gene 5458 |
| 12       | 120772700  | 120886691| 118295      | 2           | 320739 ENSMUST0001820991.1 | 169195      | ENSMUSG0000028457 | Arb85    | ATPase, class I, type 8B, member 5 |
| 5        | 42627191   | 42375650 | 11455       | 1           | 320571 ENSMUST00001025393.3 | 33408       | ENSMUSG0000028457 | Mapk10   | mitogen-activated protein kinase 10 |
| 8        | 102097500  | 103008001| 212132      | 2           | 264141 ENSMUST0001128488.7 | -28675      | ENSMUSG0000046709 | Mapk10   | mitogen-activated protein kinase 10 |
| 8        | 806107200  | 80658356 | 42877       | 1           | 333315 ENSMUST0000369596.5 | 29252       | ENSMUSG0000042353 | Frem3    | Fasl related extracellular matrix protein 3 |
| 8        | 81342556   | 81745902 | 40347       | 1           | 234515 ENSMUST0001721678.7 | -19074      | ENSMUSG0000037940 | Inpp4b   | inositol polyphosphate-4-phosphatase, type II |
| 12       | 91880640   | 91841573 | 31135       | 2           | 20338 ENSMUST00010178462.7 | -322436     | ENSMUSG00000020964 | Sel1l    | sel-1 suppressor of lin-12-like (C. elegans) |
| 13       | 96838294   | 96417737 | 5206       | 1           | 67463 ENSMUST0000992955.5 | -28335       | ENSMUSG00000021671 | Poc5     | POC5 centriolar protein |
| 14       | 15994139   | 16002581 | 14405       | 2           | 432825 ENSMUST0000961211.11 | 183500      | ENSMUSG00000092094 | Gnm5458  | predicted gene 5458 |
| 12       | 120772700  | 120886691| 118295      | 2           | 320739 ENSMUST0001820991.1 | 169195      | ENSMUSG0000028457 | Arb85    | ATPase, class I, type 8B, member 5 |
| 8        | 1206107200 | 120610736 | 105    | 2           | 18655 ENSMUST0000815293.12 | 0          | ENSMUSG0000062070 | Pkg1     | phosphoglycerate kinase 1 |
| 12       | 91880640   | 91841573 | 31135       | 2           | 20338 ENSMUST00010178462.7 | -322436     | ENSMUSG00000020964 | Sel1l    | sel-1 suppressor of lin-12-like (C. elegans) |
| 13       | 96838294   | 96417737 | 5206       | 1           | 67463 ENSMUST0000992955.5 | -28335       | ENSMUSG00000021671 | Poc5     | POC5 centriolar protein |
| 14       | 15994139   | 16002581 | 14405       | 2           | 432825 ENSMUST0000961211.11 | 183500      | ENSMUSG00000092094 | Gnm5458  | predicted gene 5458 |
| 12       | 120772700  | 120886691| 118295      | 2           | 320739 ENSMUST0001820991.1 | 169195      | ENSMUSG0000028457 | Arb85    | ATPase, class I, type 8B, member 5 |
| 5        | 42627191   | 42375650 | 11455       | 1           | 320571 ENSMUST00001025393.3 | 33408       | ENSMUSG0000028457 | Mapk10   | mitogen-activated protein kinase 10 |
| 8        | 102097500  | 103008001| 212132      | 2           | 264141 ENSMUST0001128488.7 | -28675      | ENSMUSG0000046709 | Mapk10   | mitogen-activated protein kinase 10 |
| 8        | 806107200  | 80658356 | 42877       | 1           | 333315 ENSMUST0000369596.5 | 29252       | ENSMUSG0000042353 | Frem3    | Fasl related extracellular matrix protein 3 |
| 8        | 81342556   | 81745902 | 40347       | 1           | 234515 ENSMUST0001721678.7 | -19074      | ENSMUSG0000037940 | Inpp4b   | inositol polyphosphate-4-phosphatase, type II |

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Supplementary Fig. 2. Effects of siRNA-mediated knockdown of sPLA₂-IID, -IIE, or -XIIA on Intracellular Translocation of AcLDL to Lysosomes and PGK1 Expression in sPLA₂-V WT RAW264.7 Cells

A and B, siRNA-mediated reduction of expression of sPLA₂-IID, -IIE, or -XIIA did not change translocation of pHrodo-conjugated AcLDL to lysosomes (A) and expression of \textit{Pgk1} mRNA at baseline (B). MFI indicates mean fluorescence intensity. \(n=5\) in each experiment. Values in B were normalized to that of control siRNA (1). C, Reduction of expression of sPLA₂-IID, -IIE, or -XIIA mRNA by their respective siRNAs (#1 and #2). \(n=5\) in each experiment. Values were normalized to that of control siRNA (1).