博士論文

Lysophospholipid acyltransferases are required for the regulation of phospholipid fatty acid composition

（リゾリン脂質アシル転移酵素はリン脂質の脂肪酸組成の調節に必要である）

Miki Eto
衛藤　樹

Contents

1. Abbreviations

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PS, phosphatidylserine

PI, phosphatidylinositol

PG, phosphatidylglycerol

PA, phosphatidic acid

CL, cardiolipin

PUFA, polyunsaturated fatty acid

G3P, glycerol-3-phosphate

LPA, lysophosphatidic acid

GPAT, glycerol-3-phosphate acyltransferase

LPAAT, lysophosphatidic acid acyltransferase

LPLAT, lysophospholipid acyltransferase

AGPAT, 1-acyl-glycerol-3-phosphate *O*-acyltransferase

MBOAT, membrane bound *O*-acyltransferase

LPCAT, lysophosphatidylcholine acyltransferase

LPEAT, lysophosphatidylethanolamine acyltransferase

LPSAT, lysophosphatidylserine acyltransferase

ER, endoplasmic reticulum

FBS, fetal bovine serum

mLPLAT, murine LPLAT

LC-MS/MS, liquid chromatography-tandem mass spectrometry

DPPC, dipalmitoyl-PC (16:0-16:0 PC)

POPC, palmitoyl-oleoyl PC (16:0-18:1 PC)

PLPC, palmitoyl-linoleoyl PC (16:0-18:2 PC)

PAPC, palmitoyl-arachidonoyl PC (16:0-20:4 PC)

PDPC, palmitoyl-docosahexaenoyl PC (16:0-22:6 PC)

LPC, lysophosphatidylcholine

LPE, lysophosphatidylethanolamine

LPS, lysophosphatidylserine

WT, wild type

2. Abstract

Glycerophospholipids, the major components of cellular membranes, are first synthesized in the *de novo* pathway (Kennedy pathway), and are matured through the remodeling pathway (Lands' cycle). Classically, it was considered that the remodeling pathway generates the diversity of glycerophospholipid composition, especially by incorporation of arachidonic acid. However, no detailed studies have been performed until recently to confirm the roles of the *de novo* pathway and the remodeling pathway for regulating glycerophospholipid composition. I studied how overexpressing lysophospholipid acyltransferase (LPLAT) enzymes affect phosphatidylcholine (PC) composition and acyl-CoA specificities of LPLAT activities, and studied the correlation of acyl-CoA specificities of LPLAT activities and PC composition using various tissues. The results suggested that the levels of 18:2-PC and 22:6-PC are maintained through the *de novo* pathway, whereas the levels of 16:0-PC and 18:1-PC are regulated through the remodeling pathway. I also identified a lysophosphatidic acid acyltransferase (LPAAT) enzyme, LPAAT4, with high specificity for polyunsaturated fatty acyl-CoAs, especially 22:6-CoA. This supports my speculation that 22:6-PC is predominantly maintained

through the *de novo* pathway.

Since my studies did not clarify how 20:4-PC is regulated, I performed further studies. I focused on LPCAT3, which has high specificity for 20:4-CoA. During differentiation of C3H10T1/2 cells, LPCAT3 mRNA was induced, concertedly with an increase in 20:4-containing glycerophospholipids. Generation of LPCAT3-deficient mice revealed that LPCAT3 deficiency decreases 20:4-containing glycerophospholipids *in vivo*. These data suggest that LPCAT3 is important for regulating 20:4-containing glycerophospholipids.

My study revealed the mechanism of how LPLAT enzymes regulate the acyl-chain composition of membrane glycerophospholipids.

3. Introduction

Glycerophospholipids (hereinafter referred to as phospholipids) are the major constituents of biological membranes, and are known to have not only structural roles, but also many important functional roles for cells [1,2]. Phospholipids are a class of lipids, which are composed of two fatty acids joined to glycerol, and contain charged head groups at their polar ends. They are classified into several groups according to their polar head groups: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA), and cardiolipin (CL) [3]. Not only the polar groups, but also the fatty acid compositon is diverse, resulting in over 1,000 molecular species of phospholipids. The *sn*-1 position is mainly comprised of saturated fatty acids, while the *sn*-2 position mainly contains polyunsaturated fatty acids (PUFAs). Phospholipid composition differs between tissues, and there are many studies showing altered phospholipid composition under pathological conditions, for example, cancer, nonalcoholic steatohepatitis, and Alzheimer's disease [4-6].

Phospholipids are first synthesized from glycerol-3-phosphate (G3P) through

the *de novo* pathway, also called the Kennedy pathway [7]. G3P is first transformed to lysophosphatidic acid (LPA) by glycerol-3-phosphate acyltransferases (GPATs), and are subsequently converted to PA by lysophosphatidic acid acyltransferases (LPAATs) [8]. All phospholipids are synthesized from the common intermediate PA, and are further reconstituted through the remodeling pathway (Lands' cycle) (Figure 1) [9]. In the Lands' cycle, phospholipase A_2s hydrolyze phospholipids at the *sn*-2 position producing lysophospholipids, which is used as a substrate for lysophospholipid acyltransferases (LPLATs) to form phospholipids again (Figure 2) [10,11]. Classically, it was considered that the Lands' cycle is important for generating the diversity of acyl-chain composition, especially for maintaining the level of arachidonic acid (20:4) in the *sn*-2 position [12]. However, recently our laboratory reported that LPAAT3, an enzyme functioning in the *de novo* pathway [13], prefers polyunsaturated fatty acyl-CoAs as substrates [14]. Although the Lands' cycle was proposed more than 50 years ago [9], the LPLAT enzymes functioning in the Lands' cycle were identified in the last 10 years, from the 1-acyl-glycerol-3-phosphate *O*-acyltransferase (AGPAT) family and the membrane bound *O*-acyltransferase (MBOAT) family [10]. Both families have important motifs

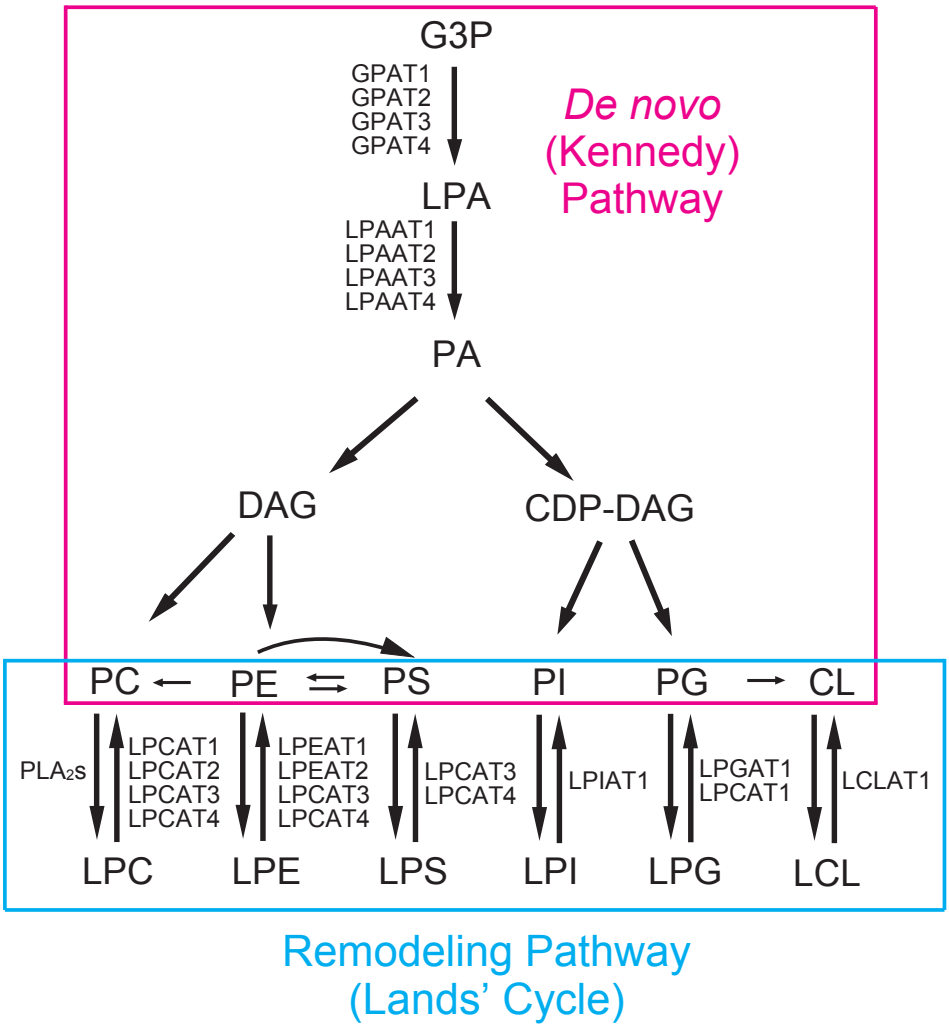


Figure 1. The phospholipid biosynthetic pathway

Phospholipids are first synthesized through the *de novo* pathway (Kennedy pathway) from G3P, and are further reconstituted through the remodeling pathway (Lands' cycle).

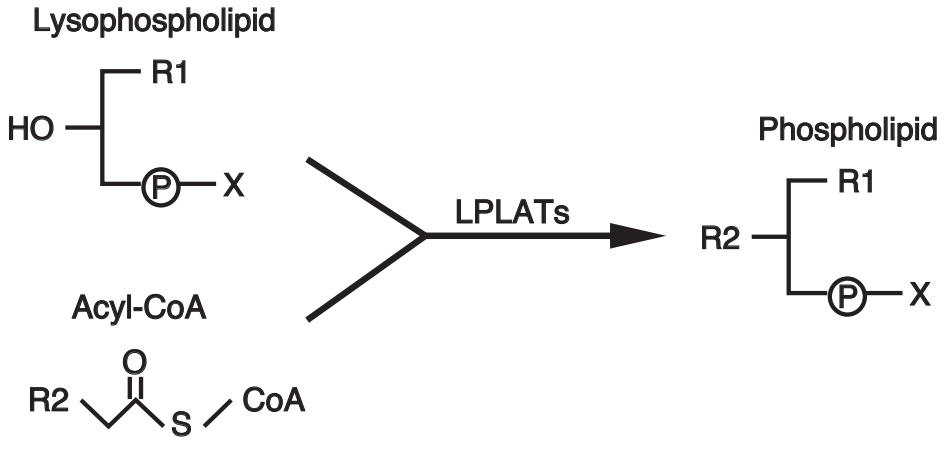


Figure 2. Enzymatic reaction of LPLAT enzymes

LPLAT enzymes synthesize phospholipids using lysophospholipid and acyl-CoA as substrates.

essential for LPLAT activities [15,16]. Individual LPLAT enzymes have different substrate specificities for both lysophospholipids (acceptor) and acyl-CoAs (donor). For example, LPCAT3 prefers 18:2-CoA and 20:4-CoA as acyl-CoA substrates, and possesses lysophosphatidylcholine acyltransferase (LPCAT), lysophosphatidylethanolamine acyltransferase (LPEAT), and lysophosphatidylserine acyltransferase (LPSAT) activities *in vitro*. LPLAT enzymes also have different tissue distributions, which may be important for the determination of the distinct phospholipid composition of each tissue. Most LPLAT enzymes are reported to be localized to the endoplasmic reticulum (ER), however, there are some reports which show mitochondrial localization [17] or localization to the Golgi [18]. Since many of these studies were performed using overexpression of LPLAT enzymes, further studies are needed to study their endogenous subcellular localization. The importance of some LPLAT enzymes has been shown using knockout mice. For example, lysophosphatidylinositol acyltransferase 1 deficient mice show atrophy of the cerebral cortex and the hippocampus, and die within a month [19]. LPCAT1 deficient mice have lower pulmonary surfactant functions [20], and LPCAT3 deficient mice are neonatally lethal, and accumulate triacylglycerol in the

enterocytes [21,22].

Although the classical theory that the Lands' cycle is important for incorporating arachidonic acid into phospholipids, generating the diversity of acyl-chain composition, was generally believed, no detailed studies have been performed to confirm the actual roles of the *de novo* pathway and the Lands' cycle. Several studies have been performed until now, to check the individual roles of LPLAT enzymes [23,24]. However, no comprehensive analyses comparing all the LPLAT enzymes have been performed. Here, I investigated how the LPAAT enzymes and the LPLAT enzymes of the remodeling pathway contribute to the determination of the fatty acid composition of phospholipids. I focused on PC, the major phospholipid species, and compared how LPAAT activities and LPCAT activities differentially regulate PC fatty acid composition.

This thesis is based upon the following papers: (1) Takeshi Harayama, Miki Eto, Hideo Shindou, Yoshihiro Kita, Eiji Otsubo, Daisuke Hishikawa, Satoshi Ishii, Kenji Sakimura, Masayoshi Mishina, and Takao Shimizu, Lysophospholipid Acyltransferases Mediate Phosphatidylcholine Diversification to Achieve the Physical

Properties Required In Vivo, Cell Metabolism, 2014 [20] (2) Miki Eto, Hideo Shindou, and Takao Shimizu, A novel lysophosphatidic acid acyltransferase enzyme (LPAAT4) with a possible role for incorporating docosahexaenoic acid into brain glycerophospholipids, Biochemical and Biophysical Research Communications, 2014 [25] (3) Miki Eto, Hideo Shindou, Andreas Koeberle, Takeshi Harayama, Keisuke Yanagida, and Takao Shimizu, Lysophosphatidylcholine Acyltransferase 3 Is the Key Enzyme for Incorporating Arachidonic Acid into Glycerophospholipids during Adipocyte Differentiation, International Journal of Molecular Sciences, 2012 [26]. Dr. Takeshi Harayama and Dr. Tomomi Hashidate-Yoshida helped me perform some of the experiments in this thesis.

4. Materials and Methods

Materials

Fetal bovine serum (FBS) was obtained from Life technologies (Carlsbad, CA). 3-Isobutyl-1-methylxanthine, dexamethasone, insulin, and pioglitazone were purchased from Sigma (St. Louis, MO). 14:0/14:0 PE and 14:0/14:0 PC were purchased from NOF Corporation (Tokyo, Japan). All other phospholipids, acyl-CoAs, and lysophospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Methanol, chloroform, acetonitrile, and ammonium bicarbonate were obtained from Wako (Osaka, Japan).

Plasmids and vectors

Coding sequences of murine LPLATs (mLPLATs) were amplified with PCR, ligated into the pCXN2.1(+) vector and sequenced. FLAG epitopes (DYKDDDDK) were attached to the N-terminus.

Cell culture and transfection

Chinese hamster ovary-K1 (CHO-K1) cells (provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan) were cultured in Ham's F-12 medium (Nacalai Tesque) supplemented with 10% FBS. Transient transfection was performed with Lipofectamine 2000 (Invitrogen). Stable overexpression of LPLATs for the lipid analysis in Figure 4, was performed by Dr. Harayama. Transfection was performed using Amaxa Nucleofector II and nucleofector kit T (Lonza) continued with selection with G418 (2 mg/ml for 6 days, maintained in 0.3 mg/ml). Stable overexpression of mLPAAT4 was performed with Lipofectamine 2000 followed by selection with G418 (3 mg/ml for 4 days, maintained in 0.3 mg/ml). All the stably overexpressing cells are polyclonal.

Neuro 2A cells (ATCC, Manassas, VA) were cultured in minimal essential medium (MEM) (Nacalai Tesque) supplemented with 10% FBS. Transient transfection was performed using Lipofectamine 2000. siRNAs of mLPAAT4 (ON-TARGETplus SMARTpoolsiRNA, L-051038-01) and a negative control (D-001810-10) were purchased from Dharmacon. 5 nM siRNA was transfected with Lipofectamine RNAiMAX reagent (Life Technologies).

C3H10T1/2 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS at 37 °C. Cells were grown to confluence (day 0), and were differentiated into adipocytes by changing the medium to DMEM with 10 % FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 2.5 μM pioglitazone and 10 μg/ml insulin. On day 2, the medium was replaced with the same mixture. On day 4, the medium was changed to DMEM with 10 % FBS and 10 μg/ml insulin. From day 6, the cells were cultured in DMEM with 10 % FBS.

Preparation of membrane fractions

Cells were sonicated with a probe sonicator (Ohtake Works) 2 times for 30 seconds in a buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and 1× complete protease inhibitor cocktail. Tissues were homogenized with a physcotron homogenizer in 5 volumes of buffer containing 100mM Tris-HCl (pH 7.4), 300 mM sucrose, and 1× complete protease inhibitor cocktail. Samples were then centrifuged at 800 × *g* for 10 minutes, and the supernatants were ultracentrifuged at 100,000 × *g* for 1 hour. After ultracentrifugation, the resultant pellets were homogenized in a buffer

containing 20 mM Tris-HCl (pH 7.4), 300mM sucrose, and 1 mM EDTA. Protein concentration was measured by the Bradford method.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of phospholipids

Phospholipids were separated with Acquity UPLC system (Waters) and were detected with triple quadrupole TSQ Vantage (Thermo Scientific). BEH C8 columns (1 × 100 mm or 2.1 × 30 mm) obtained from Waters were used for the separation of PC. For the separation of PA, BEH amide columns (2.1 × 30 mm) were used. 20 mM ammonium bicarbonate and acetonitrile were used as solvents.

LPLAT activity measurement

LPLAT activities were measured by transferring acyl-CoA to lysophospholipids to form phospholipids. A modified method of Gijon et al. [27] was used. Protein from the membrane fraction was added to a reaction mixture containing 110 mM Tris-HCl (pH 7.4), 150 mM sucrose, and 0.5 mM EDTA (LPAAT activities, and

all LPLAT activities in figure 9), 110 mM Tris-HCl (pH 7.4), 150 mM sucrose, and 1.5 mM EDTA, 2mM $CaCl_2$, and 0.015 % Tween20 (LPCAT activities in figure 5 and 6), or 110 mM Tris-HCl (pH 7.4), 150 mM sucrose, and 0.5 mM EDTA, 1mM $CaCl_2$ (LPLAT activities for C3H10T1/2 cells), and substrates (acyl-CoAs and lysophospholipids). They were incubated at 37 °C for 10 minutes, unless otherwise stated. Reactions were stopped with chloroform:methanol (1:2), and lipids were extracted by Bligh and Dyer method after addition of internal standards (dimyristoyl-PA, dilauryl-PC, dimyristoyl-PC, dimyristoyl-PE, or 17:0/20:4 PS) [28]. Products were measured using LC-MS/MS and peak areas of chromatograms were compared with those of lipid standards.

The concentrations of substrates differ between the experiments, and are written in the "Results" section for each experiment.

Quantitative PCR analysis

Quantitative PCR analysis (LightCycler System; Roche Applied Science, Mannheim, Germany) was performed with FastStart DNA Master SYBR Green I

(Roche Applied Science). mRNA levels were normalized by a housekeeping gene, 36B4. The primers used for the experiments are listed in Table 1.

Confocal microscopy

To study the subcellular localization of LPAAT4, FLAG-tagged LPAAT4 was stably overexpressed in CHO-K1 cells. LPAAT4 was detected with anti-FLAG M2 antibody (Sigma). Anti-PDI antibody (Cell Signaling Technology, Danvers, MA), Mitotracker Red CMXRos (Life Technologies), and anti-GM130 antibody (Abcam, Cambridge, UK) were used as organelle markers for ER, mitochondria, and Golgi respectively. Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate and Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 546 conjugate (ThermoFisher Scientific, Waltham, MA) were used as secondary antibodies. Confocal microscopy was performed using LSM510 Laser Scanning Microscope (Carl Zeiss, Germany).

Table 1. Primers used for quantitative PCR analysis

LPAAT4 forward	CAAGATCAATGCCAGACTCTGCT
LPAAT4 reverse	AAACTTGTGATTGAGGACCACGA
LPCAT1 forward	GTGCACGAGCTGCGACT
LPCAT1 reverse	GCTGCTCTGGCTCCTTATCA
LPCAT2 forward	GTCCAGCAGACTACGATCAGTG
LPCAT2 reverse	CTTATTGGATGGGTCAGCTTTTC
LPCAT3 forward	TCAGGATACCTGATTTGCTTCCA
LPCAT3 reverse	GGATGGTCTGTTGCACCAAGTAG
LPCAT4 forward	TTCGGTTTCAGAGGATACGACAA
LPCAT4 reverse	AATGTCTGGATTGTCGGACTGAA
LPEAT1 forward	CTGAAATGTGTGTGCTATGAGCG
LPEAT1 reverse	TGGAAGAGAGGAAGTGGTGTCTG
PPARγ2 forward	TATGCTGTTATGGGTGAAACTCTGG
PPARγ2 reverse	GTCAAAGGAATGCGAGTGGTCT
36B4 forward	CTGAGATTCGGGATATGCTGTTG
36B4 reverse	AAAGCCTGGAAGAAGGAGGTCTT

Mice

All animal studies were approved and conducted in accordance with the guidelines of the Animal Research Committee of National Center for Global Health and Medicine (approval number 14045 and 15037) and the animal experimentation committee of The University of Tokyo (approval number 3).

Statistical analyses

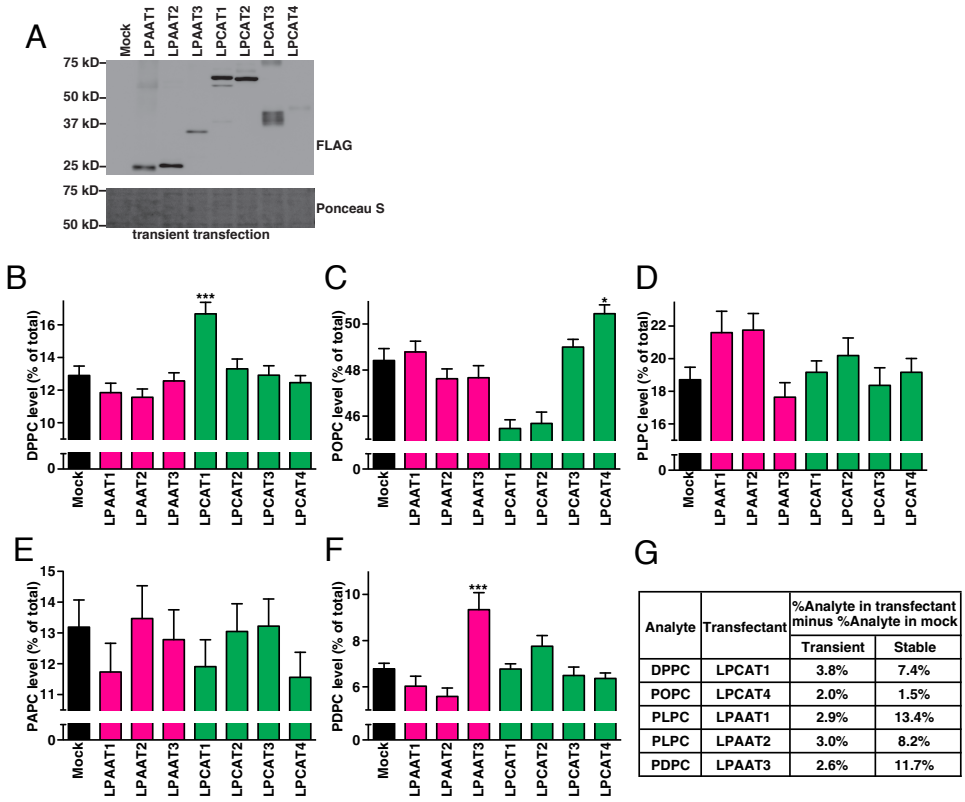
All statistical analyses were performed using Prism software (Graphpad). For comparisons of two groups, *t*-tests were performed. For multiple comparisons, one-way ANOVA or two-way ANOVA were performed, followed by Dunnett's multiple comparison tests or Tukey's multiple comparison tests.

5. Results

5.1 Roles of LPLAT enzymes on regulating PC fatty acid composition

Overexpression of LPLAT enzymes affects PC fatty acid composition

To investigate how LPLAT enzymes of the *de novo* and remodeling pathways regulate fatty acid composition of PC, I first transiently overexpressed various LPAATs (LPAAT1, 2, and 3) and LPCATs (LPCAT1, 2, 3, and 4) using CHO-K1 cells and studied the changes in PC fatty acid composition. Expression levels were confirmed by western blot analysis (Figure 3A). To simplify the analyses, we focused on five major PC species containing palmitic acid (16:0) at the *sn-1* position: dipalmitoyl (16:0-16:0)-PC (DPPC), palmitoyl-oleoyl (16:0-18:1)-PC (POPC), palmitoyl-linoleoyl (16:0-18:2)-PC (PLPC), palmitoyl-arachidonoyl (16:0-20:4)-PC (PAPC), and palmitoyl-docosahexaenoyl (16:0-22:6)-PC (PDPC). 10 μM each of linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6), essential fatty acids (EFAs) that cannot be synthesized endogenously, were supplemented to the medium 6 hours post-transfection, and cells were collected 18 hours later for lipid extraction using the method of Bligh and Dyer [28]. Dilauryl-PC was added before phase separation as an



Analyte	Transfectant	%Analyte in transfectant minus %Analyte in mock	
		Transient	Stable
DPPC	LPCAT1	3.8%	7.4%
POPC	LPCAT4	2.0%	1.5%
PLPC	LPAAT1	2.9%	13.4%
PLPC	LPAAT2	3.0%	8.2%
PDPC	LPAAT3	2.6%	11.7%

Figure 3. PC composition of CHO-K1 cells transiently overexpressing LPLAT enzymes

Modified from Cell Metabolism 20, 295-305, (2014) Harayama et al.

(A) Western blot analysis was performed with anti-FLAG M2 antibody using the membrane fraction to confirm protein expression of CHO-K1 cells transiently overexpressing LPLAT enzymes. Membranes were stained using Ponceau S before blocking with 5% skim milk overnight. (B-F) DPPC (B), POPC (C), PLPC (D), PAPC (E), and PDPC (F) levels of CHO-K1 cells transiently overexpressing LPLAT enzymes, supplemented with essential fatty acids (10 μM each of 18:2, 20:4, and 22:6). (G) Increases in the levels of PC species by transient and stable overexpression of LPLAT enzymes. *, $p < 0.05$; and ***, $p < 0.001$ vs. mock transfectants. Statistical values are illustrated only for increases. Error bars show the SEM.

internal standard. Fatty acid composition of PC was analyzed using LC-MS/MS.

Overexpression of LPCAT1 and LPCAT4 increased levels of DPPC and POPC, respectively (Figures 3B, 3C, and 3G). On the other hand, LPCAT2 and LPCAT3 overexpression did not change the level of any of the five PC species. LPAAT1 and LPAAT2 tended to elevate PLPC levels with reproducibility (Figures 3D and 3G), although no statistical significance was observed. LPAAT3 overexpression upregulated PDPC levels (Figures 3F and 3G). None of the seven enzymes changed the level of PAPC (Figure 3E).

Next, in collaboration with Dr. Harayama, LPAAT and LPCAT enzymes were stably overexpressed using CHO-K1 cells, to see if more significant changes in PC fatty acid composition could be observed. Expression levels of enzymes were confirmed by western blot analysis with anti-FLAG M2 antibody using protein from the membrane fraction (Figure 4A). Stable transfectants were seeded on 96 well plates, were cultured for 1 or 2 days, and were supplemented with EFAs. Lipids were extracted with methanol. Dilauryl-PC was added to each sample, which were dried using a centrifugal evaporator. Samples were then redissolved in methanol for LC-MS/MS analysis.

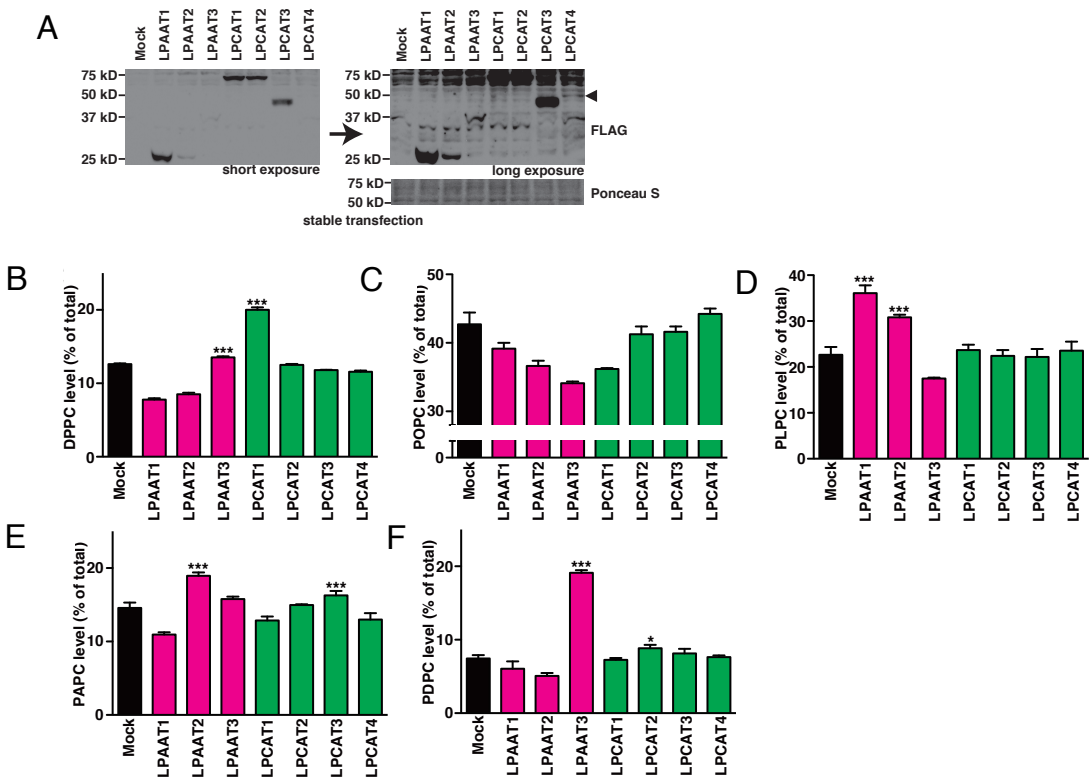


Figure 4. PC composition of CHO-K1 cells stably overexpressing LPLAT enzymes

Modified from Cell Metabolism 20, 295-305, (2014) Harayama et al.

(A) Western blot analysis was performed with anti-FLAG M2 antibody using the membrane fraction to confirm protein expression of CHO-K1 cells stably overexpressing LPLAT enzymes. Membranes were stained using Ponceau S before blocking with 5% skim milk overnight. The membrane was exposed for a longer time period to detect LPCAT4. (B-F) DPPC (B), POPC (C), PLPC (D), PAPC (E), and PDPC (F) levels of CHO-K1 cells stably overexpressing LPLAT enzymes, supplemented with essential fatty acids. *, $p < 0.05$; and ***, $p < 0.001$ vs. mock transfectants. Statistical values are illustrated only for increases. Data are shown by the mean ± SD of triplicate measurements.

Except for LPCAT4, stable overexpression changed PC composition more significantly compared to transient expression (Figures 3G, 4B-4D, and 4F). The reason why we could not see significant changes by LPCAT4 stable overexpression might be due to the low expression level of LPCAT4. In addition, LPAAT2 and LPCAT3 stable overexpression led to an increase in PAPC (Figure 4E).

Enzymatic activities of LPLAT enzymes of the de novo and remodeling pathway

To investigate whether the changes in PC profiles by overexpression of LPAAT and LPCAT enzymes are due to their substrate specificity, LPAAT and LPCAT activities of transient and stable transfectants were measured. The experiments were performed in collaboration with Dr. Harayama. 0.01 μg of membrane protein was incubated with a reaction mixture containing substrates (25 μM deuterium-labeled LPA or lysophosphatidylcholine (LPC), and 1 μM each of 16:0-, 18:1-, 18:2-, 20:4-, and 22:6-CoA) for 10 minutes at 37 °C. Products were analyzed by LC-MS/MS, and the ratio of the five products was calculated. The linearity of enzymatic product accumulation was confirmed up to 10 minutes (Figure 5A). Overexpression of LPAAT1

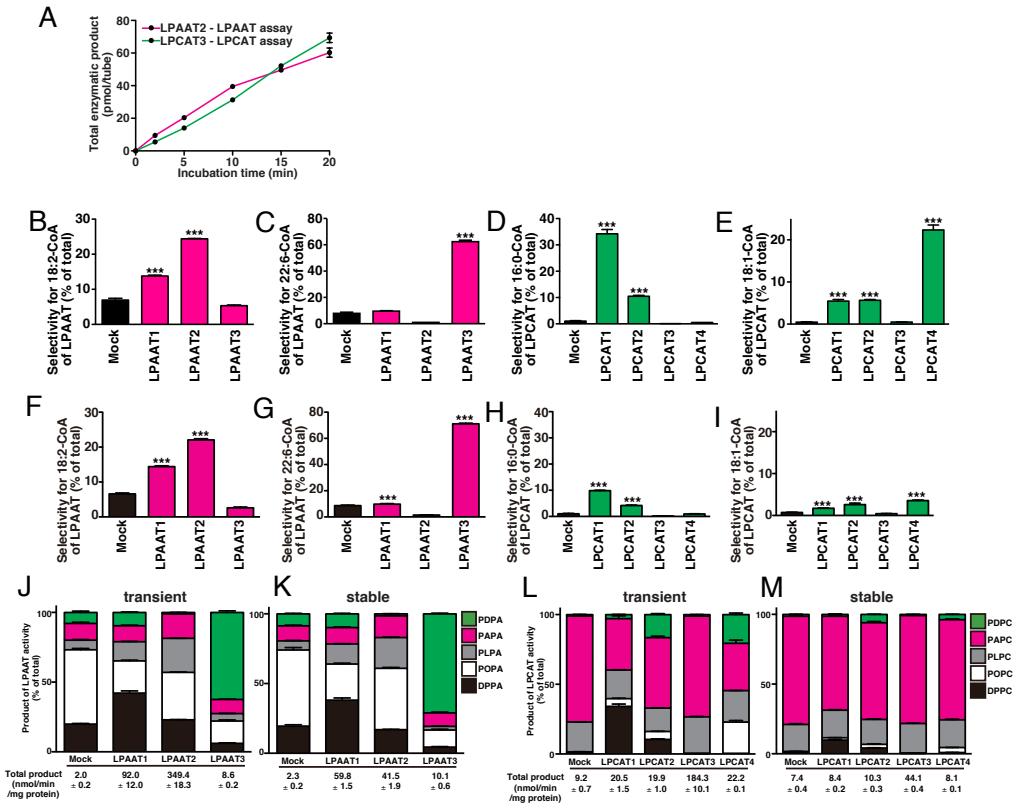


Figure 5. Changes in acyl-CoA specificities of LPLAT activities by overexpression of LPLAT enzymes

Modified from Cell Metabolism 20, 295-305, (2014) Harayama et al.

(A) Products of LPCAT and LPAAT activity assays accumulated linearly for at least 10 minutes. (B and C) Selectivity towards 18:2-CoA (B) and 22:6-CoA (C) of LPAAT activities in CHO-K1 cells transiently overexpressing LPAAT enzymes. (D and E) Selectivity towards 16:0-CoA (D) and 18:1-CoA (E) of LPCAT activities in CHO-K1 cells transiently overexpressing LPCAT enzymes. (F and G) Selectivity towards 18:2-CoA (F) and 22:6-CoA (G) of LPAAT activities in CHO-K1 cells stably overexpressing LPAAT enzymes. (H and I) Selectivity towards 16:0-CoA (H) and 18:1-CoA (I) of LPCAT activities in CHO-K1 cells stably overexpressing LPCAT enzymes. (J and K) Transient (J) and stable (K) transfection of LPAAT enzymes change the acyl-CoA specificities of LPAAT activities. (L and M) Transient (L) and stable (M) transfection of LPCAT enzymes change the acyl-CoA specificities of LPCAT activities. ***, $p < 0.001$ vs. mock transfectants. Statistical values are illustrated only for increases. (B-E, J and L) Error bars show the SEM. (A, F-I, K and M) Error bars show the SD.

and LPAAT2 increased selectivity towards 18:2-CoA, and LPAAT3-overexpression enhanced selectivity towards 22:6-CoA (Figures 5B, 5C, 5F, 5G, 5J, and 5K). Overexpression of LPCAT1 increased selectivity towards 16:0-CoA, and LPCAT4-overexpression led to an increase in selectivity towards 18:1-CoA (Figure 5D, 5E, 5H, 5I, 5L, and 5M). These results suggest that the changes in PC profiles by overexpression of LPLAT enzymes (Figures 3B-3G, 4B-4F) are due to the changes in acyl-CoA selectivity of the individual LPAAT and LPCAT enzymes. The activities of the enzymes functioning in *de novo* pathway seem to affect the levels of PLPC and PDPC, and the activities of the enzymes of the remodeling pathway seem to change the levels of DPPC and POPC.

Correlation between tissue PC composition and LPLAT activities

I next studied whether there is a correlation between PC profiles and acyl-CoA selectivity of LPAAT and LPCAT activities in mouse tissues in collaboration with Dr. Harayama. I extracted membrane fractions of various tissues, and analyzed the PC composition (Figure 6A), LPAAT activities (Figure 6D), and LPCAT activities (Figure 6E). The ratio of the enzymatic products was calculated (Figures 6B and 6C).

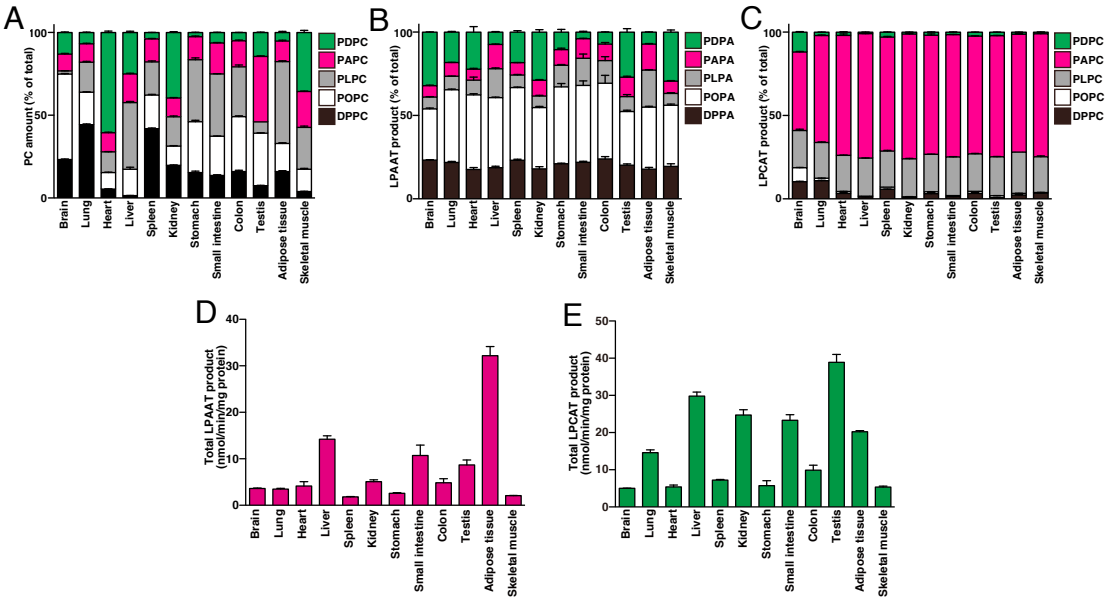
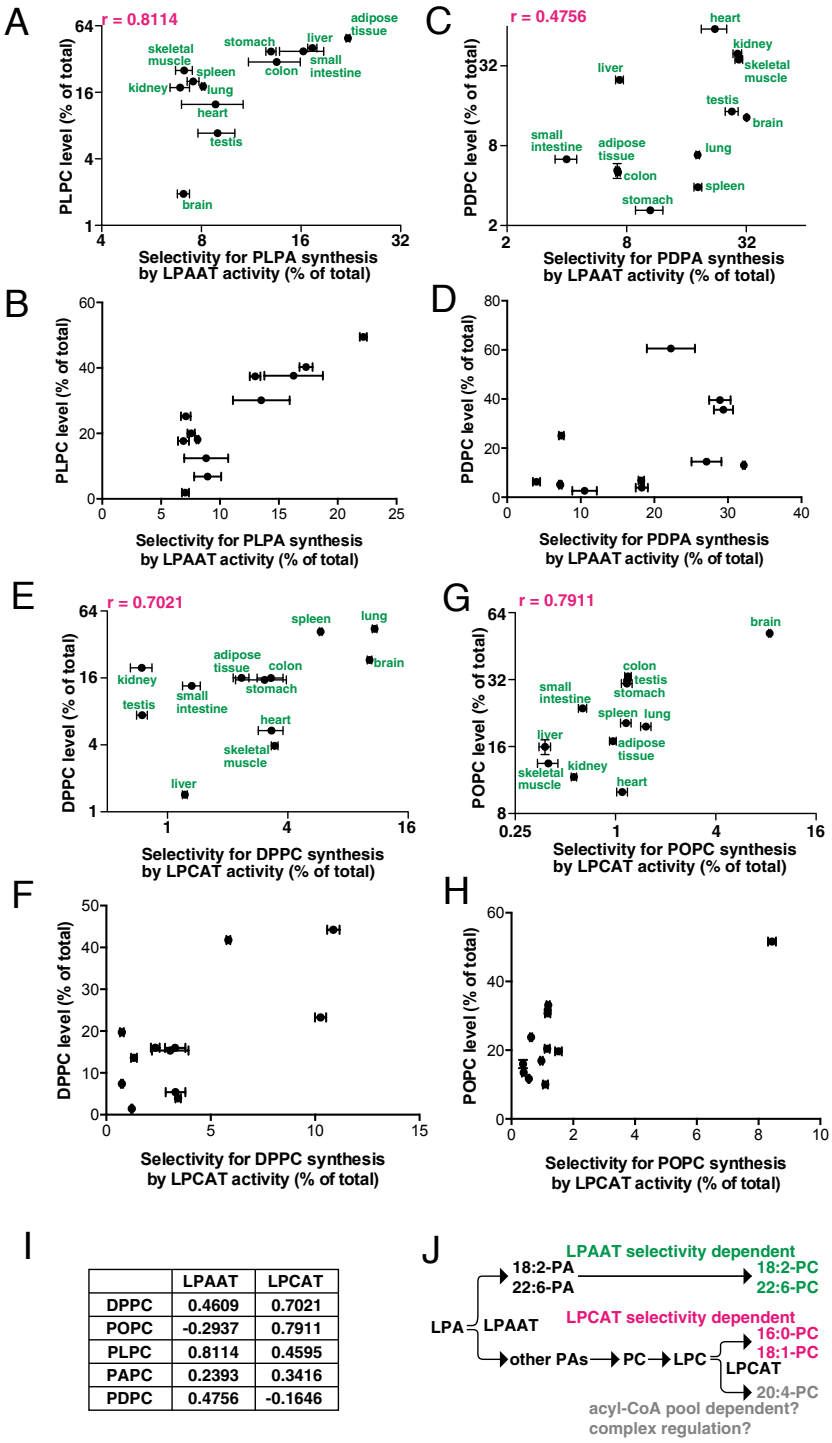


Figure 6. PC composition and LPLAT activities in various tissues

Modified from Cell Metabolism 20, 295-305, (2014) Harayama et al.

(A) The percentages of PDPC, PAPC, PLPC, POPC, and DPPC were analyzed using different mouse tissues. Error bars show the SEM (n=3). (B and C) Acyl-CoA specificity of LPAAT (B) and LPCAT (C) activities were measured with the membrane fraction of different tissues. Error bars show the SEM (n=3). (D and E) The total amounts of LPAAT (D) and LPCAT (E) products.

Each tissue had different acyl-CoA preference for LPAAT activity (Figure 6B). However, the acyl-CoA selectivity for LPCAT activity was quite similar among the tissues. All tissues besides the brain showed high selectivity towards 18:2-CoA and 20:4-CoA, and exhibited very weak selectivity towards other substrates. To study whether the acyl-CoA selectivity of LPLAT activities can determine PC fatty acid composition, acyl-CoA selectivity of LPAAT and LPCAT activities were plotted against PC profiles, shown in both log scale and linear scale (Figures 7A-7H). Correlation coefficients were calculated (Figure 7I). There was a correlation between LPAAT activity towards 18:2- and 22:6-CoA, with 18:2- and 22:6-containing PC, respectively (Figure 7A-7D). LPCAT selectivity for 16:0- and 18:1-CoA correlated with 16:0- and 18:1- containing PC, respectively (Figure 7E-7H). These results are consistent with the results obtained from overexpression studies using CHO-K1 cells. Although there are still many problems with these experiments, which will be discussed later, my results suggest a possibility that the levels of 18:2 and 22:6 are regulated through the *de novo* pathway when LPA is converted to PA (LPAAT), and that the levels of 16:0 and 18:1 are regulated by the remodeling pathway (LPCAT) (Figure 7F).



	LPAAT	LPCAT
DPPC	0.4609	0.7021
POPC	-0.2937	0.7911
PLPC	0.8114	0.4595
PAPC	0.2393	0.3416
PDPC	0.4756	-0.1646

Figure 7. Correlation between LPLAT activity and PC composition

Modified from Cell Metabolism 20, 295-305, (2014) Harayama et al.

Figure 7. Correlation between LPLAT activity and PC composition

(A and B) Using various tissues, LPAAT activities towards 18:2-CoA were plotted against the amount of PLPC. Data are shown in log scale (A) or linear scale (B). (C and D) LPAAT activities towards 22:6-CoA were plotted against the amount of PDPC. Data are shown in log scale (C) or linear scale (D). (E and F) LPCAT activities towards 16:0-CoA were plotted against the amount of DPPC. Data are shown in log scale (E) or linear scale (F). (G and H) LPCAT activities towards 18:1-CoA were plotted against the amount of POPC. Data are shown in log scale (G) or linear scale (H). (I) Pearson's correlation coefficients of the relationship between acyl-CoA specificities of LPAAT and LPCAT activities, and the corresponding PC species of different tissues. (J) We speculate that 18:2-PC and 22:6-PC are mainly incorporated through the *de novo* pathway, and that 16:0-PC and 18:1-PC are mainly synthesized through the remodeling pathway.

5.2 Identification of a novel LPAAT enzyme with preference for 22:6-CoA

Identification of a novel LPAAT enzyme

As mentioned above, my results suggested that 18:2 and 22:6 are regulated mainly through the *de novo* pathway. From the studies using CHO-K1 cells, I found that LPAAT1 and LPAAT2 seem to be important for the regulation of 18:2, and LPAAT3 seems to be the main regulator for 22:6. It is known that LPAAT3 is mainly expressed in the testis [13]. However, not only the testis, but also tissues such as brain, liver, heart, kidney, and skeletal muscle contained a relatively large amount of 22:6-containing PC (Figure 6A). I speculated that there might still be an unidentified LPAAT enzyme with specificity for 22:6-CoA.

AGPAT4 (also referred LPAATδ) is a member of the AGPAT family, which has high homology with LPAAT3 (Figure 8). Although there is a study about its structure and tissue distribution [29], there are no detailed studies about its biochemical functions. I decided to investigate whether this enzyme possesses LPAAT activity. Mouse AGPAT4 (mAGPAT4) (NCBI accession number NM_026644) cDNA was

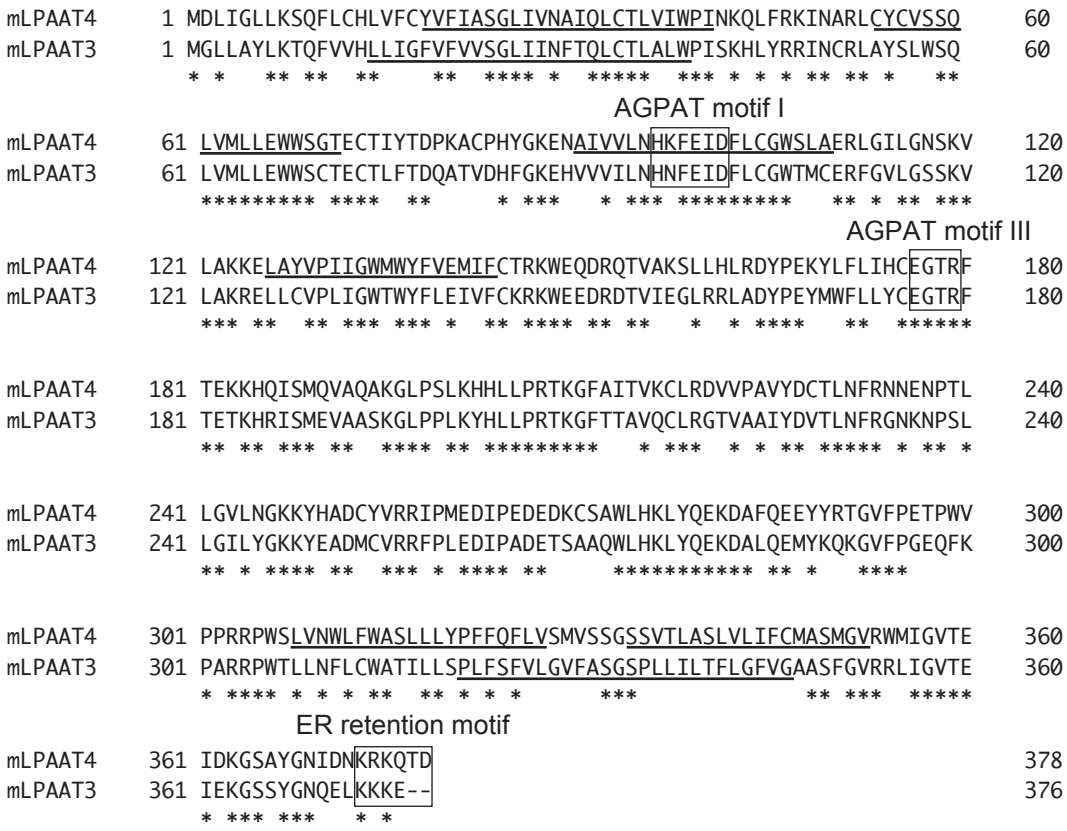


Figure 8. Amino acid sequence alignment of mouse LPAAT4 (mLPAAT4) and mLPAAT3

AGPAT motif I (NHX_4D motif), AGPAT motif III (EGTR motif) and ER retention-like motifs are *boxed*. Putative transmembrane domains predicted by HMMTOP are underlined. Conserved amino acids are shown with *asterisks*. Alignments were made using GENETYX-MAC (version 17.0.2).

cloned using mouse brain RNA as a template. I also constructed AGPAT4 H96A with a mutation in an important acyltransferase motif for catalysis [15]. Vector control, AGPAT4, and AGPAT4 H96A were transiently overexpressed into CHO-K1 cells, and LPAAT activities were measured using 0.5 μg of protein from the membrane fractions. Expression levels were quantified by western blot analysis (Figure 9A). 25 μM 16:0 LPA (not deuterium labeled) and a mixture of 5 different acyl-CoAs (1 μM each of 16:0-, 18:1-, 18:2, -20:4, and 22:6-CoA) were used as mixed substrates. We found that AGPAT4 possesses LPAAT activity with high specificity for polyunsaturated fatty acyl-CoA, especially DHA-CoA (Figure 9B). I renamed AGPAT4 to LPAAT4 according to the proposed LPLAT nomenclature [10]. LPAAT4 H96A showed similar activities to the control, suggesting that the motif is essential for the enzyme activity of LPAAT4. LPAAT4 possessed similar acyl-CoA specificities with 18:0 LPA and 18:1 LPA (Figures 9C and 9D). I also studied whether LPAAT4 possesses LPLAT activities of the Lands' cycle. LPCAT, LPEAT, LPSAT, LPIAT, and LPGAT activities were measured with 25 μM lysophospholipid (16:0) and a mixture of 5 different acyl-CoAs

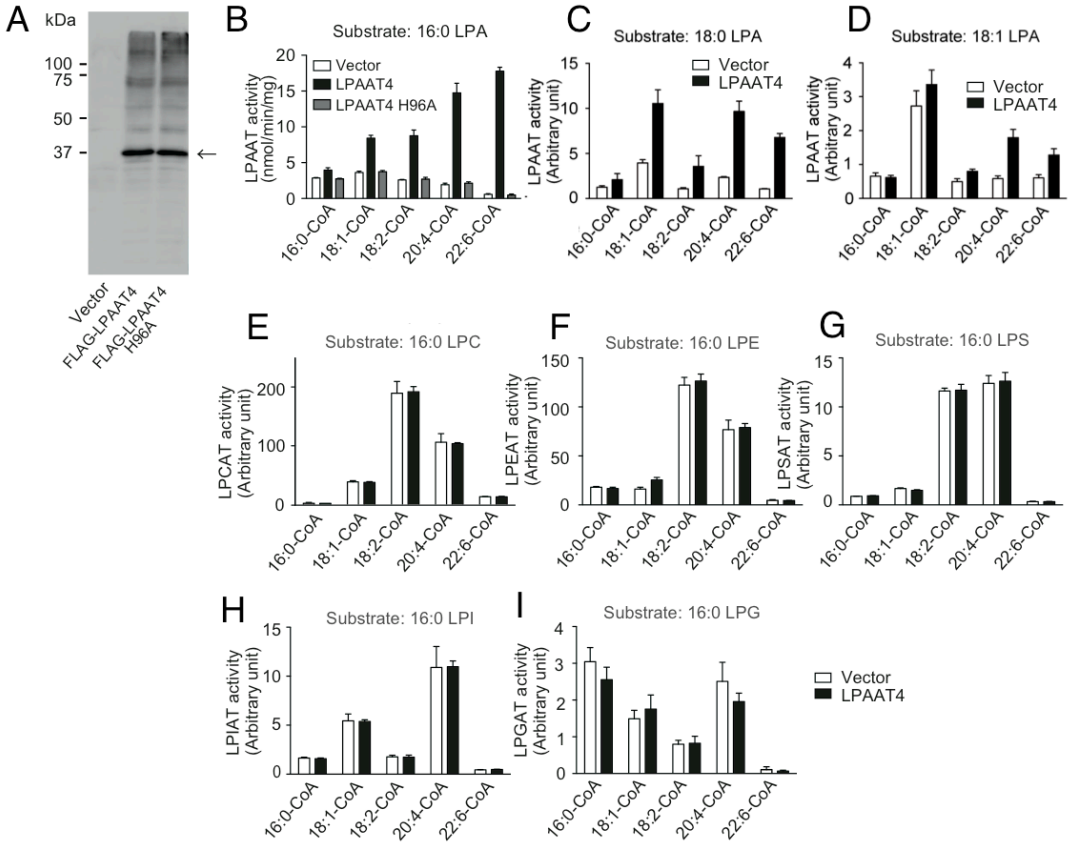


Figure 9. Substrate specificity of LPAAT4

Modified from Biochem. Biophys. Res. Commun. 443, 718-724, (2014) Eto et al.

(A) Western blot analysis was performed to check transfection efficiencies of proteins used for LPLAT activity assays, using anti-FLAG M2 antibody (5 μg/lane). (B-D) LPAAT activities were measured with 0.5 μg of microsomal protein of vector, LPAAT4, or LPAAT4 H96A overexpressing CHO-K1 cells, using 25 μM LPA (16:0, 18:0, or 18:1) and a mixture of 5 different acyl-CoAs (1 μM each of 16:0-, 18:1-, 18:2-, 20:4-, and 22:6-CoA). Data are shown by the mean ± SD of triplicate measurements. (E-I) LPCAT (E), LPEAT (F), LPSAT (G), LPIAT (H), and LPGAT (I) activities were measured with 0.5 μg of microsomal protein of vector or LPAAT4 overexpressing CHO-K1 cells, using 25 μM lysophospholipid and a mixture of 5 different acyl-CoAs (1 μM each of 16:0-, 18:1-, 18:2-, 20:4-, and 22:6-CoA). Data are shown by the mean ± SD of triplicate measurements.

(1 μM each of 16:0-, 18:1-, 18:2, -20:4, and 22:6-CoA). Under these conditions, mLPAAT4 did not possess any of the LPLAT activities of the remodeling pathway (Figures 9E-9I).

Kinetics of LPAAT4 as an LPAAT enzyme

I next studied the substrate binding affinities of LPAAT4 by measuring LPAAT activities with different concentrations of 22:6-CoA and LPA. First, I studied LPAAT activities with 25 μM 16:0-LPA and 0-10 μM 22:6-CoA (Figure 10A). I next measured LPAAT activities using 10 μM 22:6-CoA and 0-40 μM 16:0 LPA (Figure 10B). LPAAT assays were also performed with 10 μM 22:6-CoA and 0-30 μM 18:0 LPA (Figure 10C). Activities were inhibited at higher concentrations of substrates (22:6-CoA>10μM, 16:0 LPA>40 μM, 18:0-LPA>30 μM). The maximum velocities and *Km* values calculated from three independent experiments, and are shown in the figures.

Lipid composition of LPAAT4 overexpressing cells

I was interested in whether LPAAT4 has a role for regulating the 22:6 content

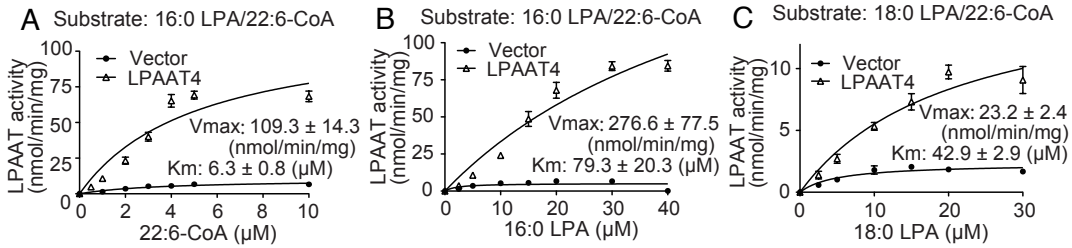


Figure 10. Kinetics of LPAAT4 as an LPAAT enzyme

Modified from Biochem. Biophys. Res. Commun. 443, 718-724, (2014) Eto et al.

(A-C) LPAAT activities were measured with microsomal protein of vector or LPAAT4 overexpressing CHO-K1 cells, using 25 μM 16:0 LPA and 0-10 μM 22:6-CoA (A), 10 μM 22:6-CoA and 0-40 μM 16:0 LPA (B), or 10 μM 22:6-CoA and 0-30 μM 18:0 LPA. The maximum velocities (Vmax) and *km* values were calculated from three independent experiments, and are shown in the insets as the mean ± SEM.

of cellular phospholipids. LPAAT4 or LPAAT4 H96A transiently overexpressing CHO-K1 cells were treated with 50 μM DHA in 2% FBS medium 48 h after transfection, and lipids were extracted at indicated time points (0, 1, 3, 6, 9, 12, 24 h) using methanol. The fatty acid profiles of PC and PE, and PS were analyzed with LC-MS/MS. The major species of PC, PE and PS (16:0, 18:0, or 18:1 at the *sn*-1 position, 16:0, 18:1, 18:2, 20:4, or 22:6 at the *sn*-2 position) were analyzed with LC-MS/MS. I analyzed signal intensities for individual species, and the ratio of each signal was calculated. LPAAT4 overexpression caused an increase in the percentage of 18:0-22:6 PC (Figure 11B). However, the amounts of 16:0-22:6 PC and 18:1-22:6 PC were not changed significantly (Figures 11A and 11C). This was unexpected because LPAAT4 possessed clear *in vitro* activity towards 22:6-CoA with 16:0 LPA as a substrate (Figure 9B). I also analyzed 22:6-containing PE and PS species (16:0-22:6, 18:0-22:6, and 18:1-22:6). However, none of them were altered by the overexpression of LPAAT4 (data not shown). This is either due to the low catalytic activity of LPAAT4, or endogenous other enzymes with similar activities, or both.

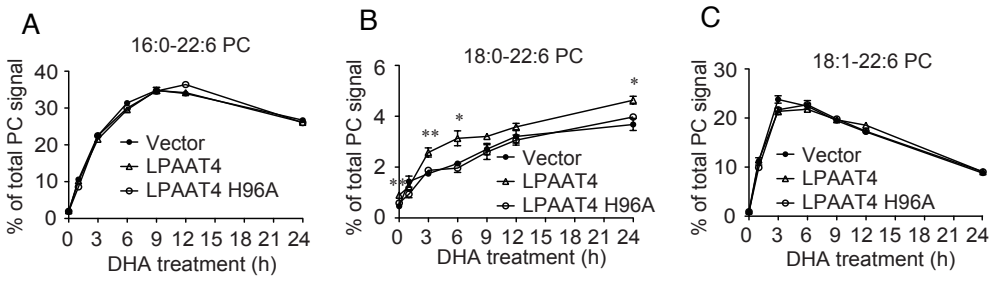


Figure 11. DHA incorporation into LPAAT4 overexpressing CHO-K1 cells

Modified from Biochem. Biophys. Res. Commun. 443, 718-724, (2014) Eto et al.

(A-C) CHO-K1 cells overexpressing vector, LPAAT4, or LPAAT4 H96A were treated with 50 μM DHA, and PC composition was analyzed at the indicated time points. Signal intensities of 16:0-22:6 PC (A), 18:0-22:6 PC (B), and 18:1-22:6 PC (C) were calculated as the percentage of the total PC signal. Data are shown by the mean ± SEM of triplicate measurements. Statistics were calculated with one-way analysis of variance (ANOVA) followed by post hoc Tukey's test; *$p<0.05$, **$p<0.01$.

Knockdown of LPAAT4 using Neuro 2A cells

To study the endogenous activity of LPAAT4, I performed siRNA-mediated knockdown of LPAAT4 using Neuro 2A cells. siRNA efficiency was confirmed by quantitative PCR analysis (Figure 12A) and western blot analysis (Figure 12B). I measured LPAAT activity using 25 μM 16:0 LPA and 10 μM DHA, with 0.5 μg protein from the membrane fraction of control or LPAAT4 siRNA treated cells. LPAAT4 knockdown caused an approximately 40% decrease in LPAAT activity (Figure 12C), suggesting that endogenous LPAAT4 has enzymatic activity.

I next investigated whether LPAAT4 knockdown affects fatty acid profiles of phospholipids. Neuro 2A cells transfected with control or LPAAT4 siRNA were treated with 50 μM DHA in 2% FBS medium, and lipids were extracted at indicated time points (0, 1, 3, 6, 9, 12, 24 h). The major PC, PE, and PS species (16:0, 18:0, or 18:1 at the *sn*-1 position, 16:0, 18:1, 18:2, 20:4, or 22:6 at the *sn*-2 position) were analyzed using LC-MS/MS, and the ratio was calculated in the same procedure as mentioned in the overexpression experiments. The percentage of 18:0-22:6 PC (Figure 12E) and 18:1-22:6 PC (Figure 12F) were decreased by LPAAT4 knockdown, however, the

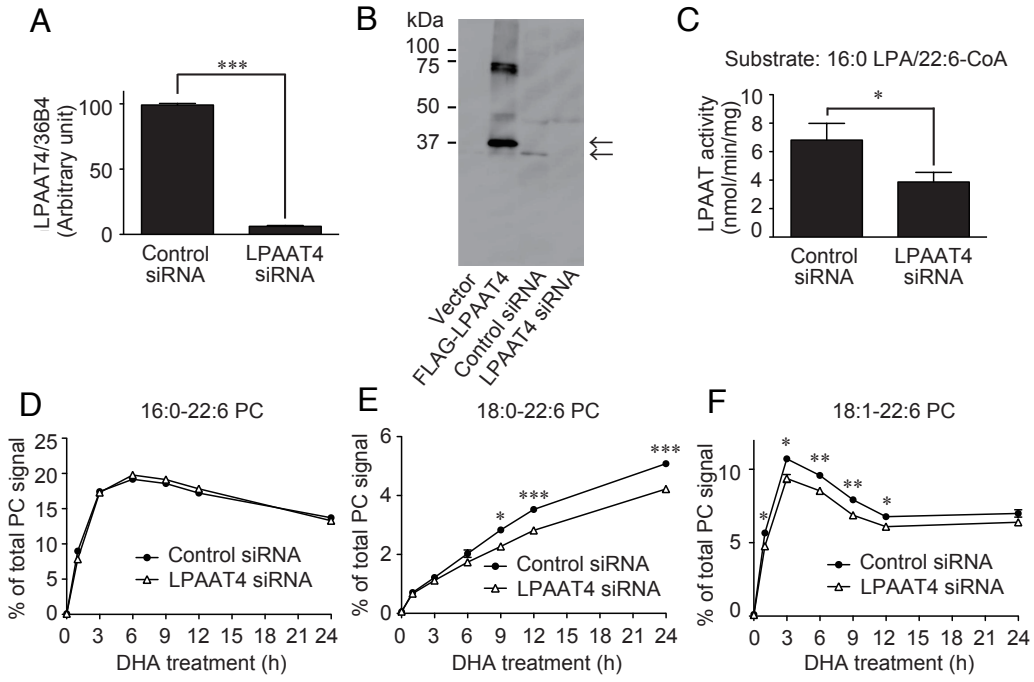


Figure 12. siRNA-mediated knockdown using Neuro 2A cells

Modified from Biochem. Biophys. Res. Commun. 443, 718-724, (2014) Eto et al.

(A) Quantitative PCR analysis of Neuro 2A cells treated with control or LPAAT4 siRNA. Expression level of LPAAT4 was normalized by expression level of 36B4. (B) Western blot analysis of control or LPAAT4 siRNA treated cells. FLAG-tagged LPAAT4 was used as a positive control (5 μg/lane). (C) LPAAT activities were analyzed with 0.5 μg microsomal proteins from Neuro 2A cells treated with control or LPAAT4 siRNA. 25 μM 16:0 LPA and 10 μM 22:6-CoA were used as substrates. (D-F) Control or LPAAT4 knockdown Neuro 2A cells were treated with 50 μM DHA, and the fatty acid composition of PC was analyzed at the indicated time points. Signal intensities of 16:0-22:6 PC (D), 18:0-22:6 PC (E), and 18:1-22:6 PC (F) were calculated as the percentage of total PC signal. Data are shown by the mean ± SEM of triplicate measurements. Statistics were calculated with Student's *t*-test; *p<0.05, **p<0.01, *** p<0.001

percentage of 16:0-22:6 PC did not change (Figure 12D). Major PE and PS species (16:0-22:6, 18:0-22:6, and 18:1-22:6) were also analyzed, however, LPAAT4 knockdown did not have any effect on their profiles (data not shown). These results are also inconsistent with the *in vitro* activity of LPAAT4. However, they are consistent with the results from the lipid analysis of LPAAT4 overexpressing CHO-K1 cells. I need further studies to reveal the reason why the ratio of 16:0-22:6 PC was not affected. One possible reason is that other LPAAT enzymes, for example LPAAT3, might be mainly maintaining the level of 16:0-22:6 PA in these cells.

Tissue distribution and subcellular localization of LPAAT4

I next analyzed the tissue distribution of LPAAT4 using quantitative PCR analysis, normalized by a housekeeping gene, 36B4. Expression of LPAAT4 mRNA was highest in brain, followed by lung, stomach, and colon (Figure 13A). I also performed western blot analysis to check its tissue distribution in protein level. LPAAT4 was detected using anti-LPAAT4 antibody (Scrum Inc.) against a C-terminal peptide (IDNKRKQTD). FLAG-tagged LPAAT4 was used as a positive control.

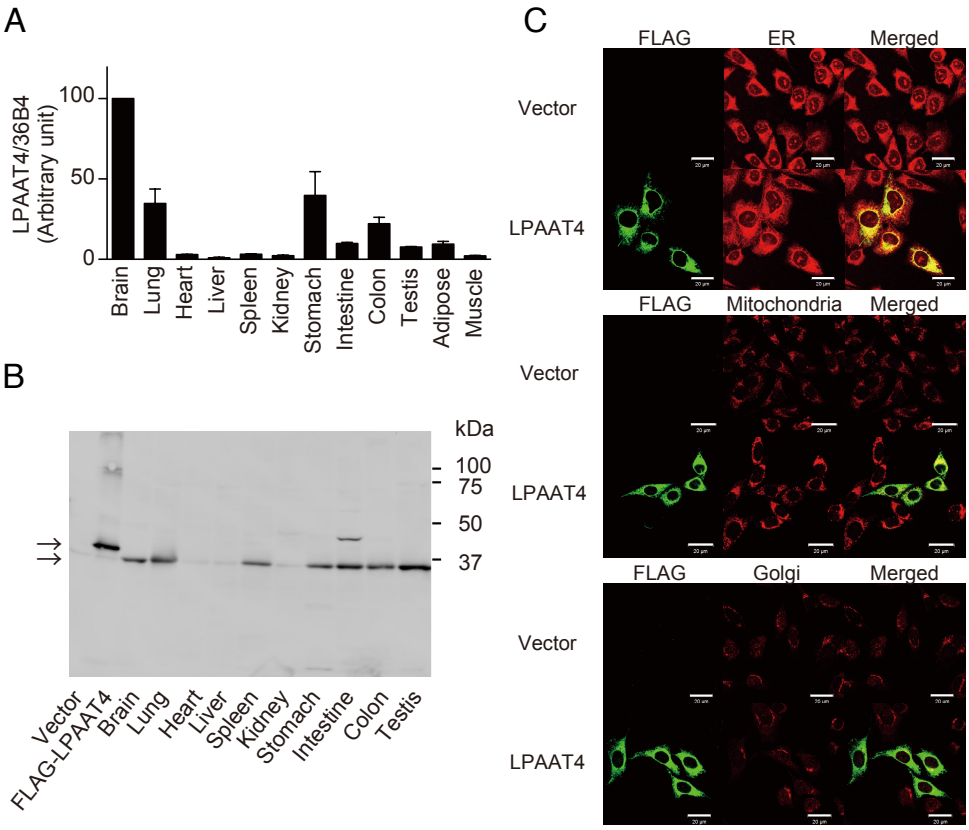


Figure 13. Tissue distribution and subcellular localization of LPAAT4

Modified from Biochem. Biophys. Res. Commun. 443, 718-724, (2014) Eto et al.

(A) LPAAT4 tissue distribution was analyzed by quantitative PCR analysis, using mice at 8 weeks of age. Data are shown by the mean ± SEM (n=3). (B) Western blot analysis of microsomal protein from various tissues was performed with anti-LPAAT4 antibody (10 μg/lane). (C) CHO-K1 cells stably overexpressing vector or FLAG-tagged LPAAT4 were stained with anti-FLAG M2 antibody to detect LPAAT4 (green), and with organelle markers (red) for ER (anti-PDI antibody), mitochondria (MitoTracker Red), or Golgi (anti-GM130 antibody). Scale bars indicate 20 μm.

LPAAT4 was detected at a molecular weight of approximately 37 kDa, which is inconsistent with the calculated molecular weight. This is also observed in other acyltransferases, and may be caused by multiple membrane spanning domains [13,23]. Highest protein expression was observed in the testis, which was inconsistent with the mRNA expression levels (Figure 13B). I further analyzed the subcellular localization of LPAAT4. CHO-K1 cells stably overexpressing LPAAT4 was stained with anti-FLAG M2 antibody to detect LPAAT4, and were co-stained with organelle markers: anti-PDI antibody for ER, MitoTracker Red CMXRos to detect mitochondria, and anti-GM130 for detection of Golgi. LPAAT4 showed a similar expression pattern to the ER marker (Figure 13C). These results suggest that mLPAAT4 is mainly localized to the ER under these conditions. I still need further studies to confirm the endogenous localization.

5.3 LPCAT3 is involved in regulating 20:4-containing phospholipids

LPCAT3 might be involved in maintaining the amount of 20:4-containing phospholipids

The results from the first part of our study suggested that the amounts of 18:2- and 22:6-containing PC are mainly regulated through the *de novo* pathway, and that the amounts of 16:0- and 18:1-containing PC are regulated mainly by the remodeling pathway. However, I could not find out how the level of 20:4-containing PC is maintained. My results showed that endogenous LPCAT activities in CHO-K1 cells (Figures 5L and 5M) and in all tissues (Figure 6C) have high selectivity for 20:4-CoA remodeling pathway, while endogenous LPAAT activity showed quite low selectivity towards 20:4-CoA. I also found that the stable overexpression of LPCAT3 resulted in a slight increase in the level of PAPC (Figure 4E). Thus, I speculated that LPCAT3, which shows strong activity towards 20:4-CoA [23] might be one of the key enzymes for maintaining 20:4-containing PC.

LPCAT3 expression is upregulated during differentiation of C3H10T1/2 cells into adipocytes

To begin with, I decided to find a condition in which LPCAT3 expression level changes. I tried differentiating C3H10T1/2 cells [30], a mesenchymal stem cell line into adipocytes *in vitro* [31]. mRNA was extracted at days 0, 2, 4, 6, and 8 after starting differentiation, and expression levels of LPCAT3 was examined using quantitative PCR analysis. Values were normalized with the expression of 36B4. LPCAT3 mRNA expression was increased during differentiation (Figure 14A). I also studied expression levels of LPCAT1, LPCAT2, LPCAT4, and LPEAT1 during differentiation. LPCAT1, LPCAT2, and LPCAT4 mRNAs were not detected. LPEAT1 expression was dramatically decreased on day 2, and increased after that (Figure 14B). The expression level of PPARγ2, a marker for adipocyte differentiation, was elevated during cell culture (Figure 14C). To check if the increase in LPCAT3 mRNA expression was due to adipocyte differentiation, and not due to the confluency of the cells, I analyzed C3H10T1/2 cells cultured for 8 days, with or without the differentiation mixture. LPCAT3 expression was only elevated when cells were treated with the differentiation mixture (Figure 14D), suggesting that the increase in LPCAT3 mRNA was caused by the differentiation of C3H10T1/2 cells into adipocytes.

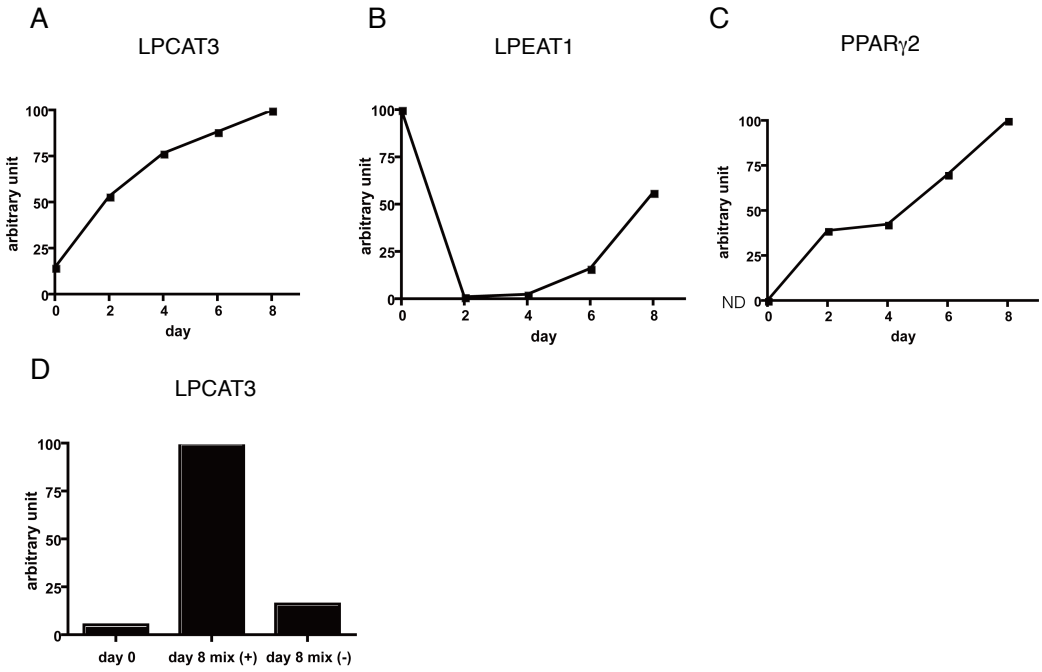


Figure 14. LPCAT3 mRNA expression is induced during adipocyte differentiation

Modified from Int. J. Mol. Sci. 13, 16267-16280 (2012) Eto et al.

(A-C) Quantitative PCR analysis was performed to analyze mRNA expression of LPCAT3 (A), LPEAT1 (B), and PPARγ2 (C) during differentiation of C3H10T1/2 cells into adipocytes. mRNA expression levels were normalized by 36B4 expression. (D) mRNA expression of LPCAT3 in day 0 and day 8 cells. mix (+) indicates cells cultured with the induction mixture, and mix(-) indicates cells cultured without the mixture. mRNA expression levels were normalized by 36B4. Data shown are the mean ± SEM of three independent experiments.

Changes in LPLAT activities and phospholipid fatty acid composition during differentiation

Next, LPLAT activities of C3H10T1/2 cells before and after differentiation were measured to see if the increase in LPCAT3 mRNA expression caused a change in LPLAT activities. In this study, I will term the cells before differentiation (day 0) "preadipocytes", and the cells after differentiation (day 8), "adipocytes". LPCAT3 is known to possess LPCAT, LPEAT, and LPSAT activities with preference for 18:1-CoA, 18:2-CoA, and 20:4-CoA [23]. LPCAT, LPEAT, and LPSAT activities were measured with a reaction mixture containing 5 μM each of 16:0-, 18:1-, 18:2-, 20:4, and 22:6-CoA, and multiple lysophospholipids (25 μM each of 16:0 deuterium labeled LPC, 16:0 lysophosphatidylethanolamine (LPE), and 16:0 lysophosphatidylserine (LPS)), using 0.5 μg protein from the membrane fraction of preadipocytes and adipocytes. I found that adipocytes showed stronger LPCAT, LPEAT, and LPSAT activities compared to preadipocytes, especially with 18:1-CoA, 18:2-CoA, and 20:4-CoA as acyl-donors (Table 2). These results are consistent with the substrate specificity of LPCAT3, suggesting that LPCAT3 might have caused the increase in the activities. To

Table 2. LPLAT activities of preadipocytes and adipocytes

Int. J. Mol. Sci. 13, 16267-16280 (2012) Eto et al.

LPCAT activity

Substrate	Preadipocyte (relative units)	Adipocyte (relative units)	*P* value
16:0-CoA	7.50 ± 0.29	12.36 ± 0.68	$P = 0.0008$
18:1-CoA	3.13 ± 0.19	18.24 ± 0.61	$P < 0.0001$
18:2-CoA	198.50 ± 13.60	1821.54 ± 80.62	$P < 0.0001$
20:4-CoA	267.49 ± 11.45	1882 ± 63.12	$P < 0.0001$
22:6-CoA	0.56 ± 0.04	2.30 ± 0.18	$P = 0.0002$

LPEAT activity

Substrate	Preadipocyte (relative units)	Adipocyte (relative units)	*P* value
16:0-CoA	0.20 ± 0.02	0.82 ± 0.06	$P = 0.0001$
18:1-CoA	0.09 ± 0.01	3.10 ±0.17	$P < 0.0001$
18:2-CoA	1.36 ± 0.04	14.14 ± 0.63	$P < 0.0001$
20:4-CoA	2.44 ± 0.12	14.44 ± 0.40	$P < 0.0001$
22:6-CoA	0.02 ±0.01	0.20 ± 0.02	$P = 0.0002$

LPSAT activity

Substrate	Preadipocyte (relative units)	Adipocyte (relative units)	*P* value
16:0-CoA	0.61 ± 0.07	1.73 ± 0.05	$P < 0.0001$
18:1-CoA	0.35 ± 0.03	1.93 ± 0.03	$P < 0.0001$
18:2-CoA	2.65 ± 0.08	17.25 ± 0.24	$P < 0.0001$
20:4-CoA	6.97 ± 0.07	28.97 ± 0.88	$P < 0.0001$
22:6-CoA	0.043 ± 0.002	0.151 ± 0.007	$P < 0.0001$

Data shown are the signal intensity of products, normalized by the signal intensity of internal standards. The data represent the mean ± SD of triplicate measurements. Statistical analyses were performed using *t*-test.

see if the increase in LPCAT3 expression caused a change in phospholipid composition, the profiles of PC, PE and PS between preadipocytes and adipocytes were compared. Lipids were extracted from the membrane fraction of preadipocytes and adipocytes, and the phospholipid composition was measured using LC-MS/MS, by detecting head groups. Signal intensities for each species were summed up, and the percentage of each species was calculated. Many changes were observed in the profiles of PC and PE, including an increase in species probably containing 20:4, such as 36:4 PC, 38:4 PC, and 36:4 PE (Figure 15A and 15B). Since detection by polar head groups does not determine the acyl-chain composition of phospholipids, (for example, 16:0-20:4 PC, 18:1-18:3 PC, and 18:2-18:2 PC are all 36:4 PC), I further analyzed fatty acid profiles of PC and PE by detecting fatty acid anions to confirm that the increased species (36:4 PC, 38:4 PC, and 36:4 PE) actually contained 20:4 (data not shown). Although there are many possible explanations for these results, these increases in 20:4-containing phospholipids might be caused by LPCAT3.

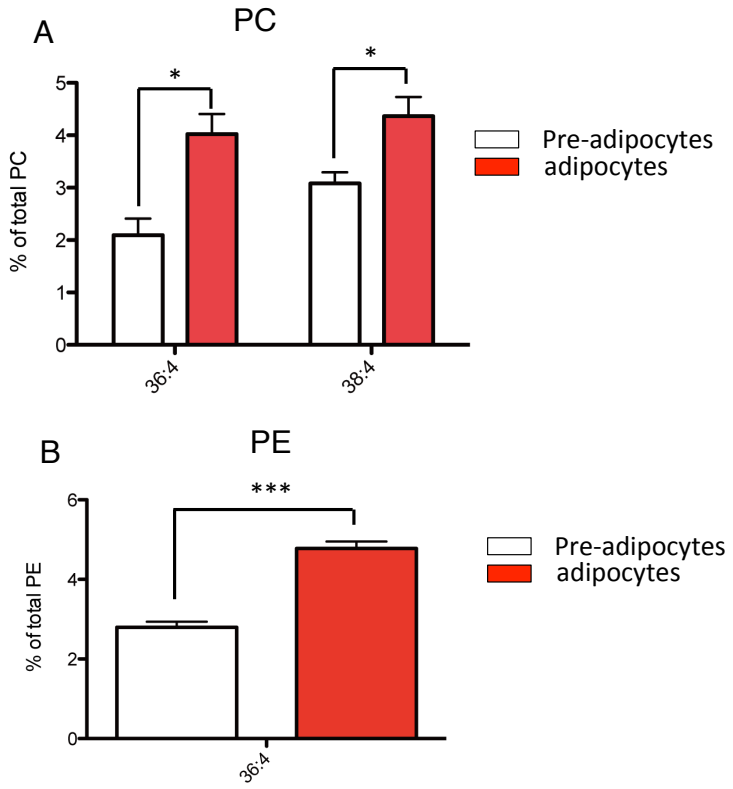


Figure 15. Phospholipid compositions of preadipocytes and adipocytes

Int. J. Mol. Sci. 13, 16267-16280 (2012) Eto et al.

(A) The signal intensities of each PC species were summed up and the percentages were calculated. The graph shows the percentage of 36:4 PC and 38:4 PC, which probably contain 20:4. (B) The signal intensities of each PE species were summed up and the percentages were calculated. The graph shows the percentage of 38:4 PE, which probably contains 20:4. Data show the mean ± SEM of three independent experiments. Statistical analyses were performed using *t*-test. *, P <0.05; ***, P <0.001.

To study further if LPCAT3 is actually involved in regulating the levels of 20:4-containing phospholipids, LPCAT3-deficient mice were produced. Studies performed by researchers in our laboratory showed that LPCAT3 deficient mice have decreased 20:4-containing phospholipids, suggesting that LPCAT3 is important for regulating their levels [21].

6. Discussion

It was classically considered that the remodeling pathway is important for making the acyl-chain diversity of phospholipids, by incorporation of PUFAs, especially arachidonic acid. However, no detailed studies have been performed until now, to confirm this classical theory. In this study, I investigated how LPLAT enzymes maintain phospholipid fatty acid composition.

The results from the analysis of the PC composition and LPLAT activities of different tissues showed that LPAAT activities towards 18:2- and 22:6-CoA correlate with the amount of 18:2- and 22:6-containing PC, respectively, and that LPCAT activities towards 16:0- and 18:1-CoA correlate with the amount of 16:0- and 18:1-containing PC respectively. The studies using CHO-K1 cells overexpressing LPLAT enzymes showed that LPAAT activity towards 18:2-CoA was increased by LPAAT1 and LPAAT2, and LPAAT activity towards 22:6-CoA was increased by LPAAT3. LPCAT activity towards 16:0-CoA was increased by transfection of LPCAT1, and LPCAT activity towards 18:1-CoA was increased by that of LPCAT4. I also found that overexpression of these LPLAT enzymes changed PC fatty acid profiles, which

correlated with the change in activities. From these results I speculated that the levels of 18:2- and 22:6-containing phospholipids are mainly maintained through the *de novo* pathway (at the step of LPA to PA), and that the levels of 16:0- and 18:1-containing phospholipids are regulated through the remodeling pathway (LPC to PC). The results provide a new view on how phospholipid composition is produced and maintained.

However, there are still many limitations to this study. I used overexpression for many of my experiments, which is very different from natural conditions. Also, the expression efficiencies differ between enzymes, making it difficult to compare between enzymes. The differences in the endogenous expressions or activities between enzymes, is also a problem. Also, the experiments using different tissues only give information about correlations between LPLAT activities and PC composition under a certain condition, which might differ when the assay condition changes, for example by changing the concentrations of substrates. I still need further studies to get more direct evidence on the speculation.

Although there are still problems to be solved, my new theory suggested that 22:6 is mainly incorporated through the *de novo* pathway. The only LPAAT enzyme

identified until now with preference for 22:6-CoA was LPAAT3. However, LPAAT3 is not highly expressed in every tissues rich in 22:6-containing phospholipids [13]. I was interested if there were still unidentified LPAAT enzymes. My study newly revealed that LPAAT4, previously known as AGPAT4, possesses LPAAT activity with preference towards 22:6-CoA. AGPAT4 was recognized as a member of the AGPAT family from its structure, but the previous study only showed very weak LPAAT activity towards 18:1-CoA, and the biologically relevant substrates were unknown [29]. LPAAT4 knockdown using Neuro 2A cells decreased LPAAT activity with 22:6-CoA, suggesting that endogenous LPAAT4 possesses enzymatic activity. While the knockdown efficiency was quite high, the decrease in LPAAT activity was only around 40 %. Other LPAAT enzymes, for example LPAAT3, or still unidentified enzymes might be functioning. LPAAT4 seemed to affect the amount of some 22:6-containing PC species; LPAAT4 overexpression in CHO-K1 cells increased the ratio of 18:0-22:6 PC, and LPAAT4 knockdown in Neuro 2A cells decreased the ratio of 18:0-22:6 PC and 18:1-22:6 PC. However I could not observe a change in 16:0-22:6 PC and all 22:6-containing PE and PS species. It remains unclear why significant changes were not

seen in these phospholipids. One possibility is that, other LPAAT enzymes, such as LPAAT3, or LPLAT enzymes of the remodeling pathway might be involved. Although I found that the level of 22:6-containing PC is mainly maintained through the *de novo* pathway in the first part of our study, I did not investigate how 22:6-containing PE and PS are regulated, so there is still a possibility that the remodeling pathway has some function for producing them. PS synthases and PS decarboxylases might have have roles in regulating the fatty acid composition of PE and PS. Another possibility is that phospholipid-degrading enzymes might be involved. Calcium independent phospholipase $A_2\beta$ knockout mice is reported to have increased 16:0-22:6 PC in the spinal cord [33], and secretory phospholipase A_2 III knockout showed decrease in 22:6-constaining PCs in the testis [34]. LPAAT4 mRNA was expressed highest in the brain, which is a tissue known to be rich in 22:6-containing PE and PS, mainly in the form of 18:0-22:6 [35]. However, recently, a study using LPAAT4 knockout mice showed that LPAAT4 deficiency does not change the levels of 22:6-containing phospholipids in the brain [17]. Although the main function of LPAAT4 might not be to maintain the 22:6 levels of phospholipids, it still might have some roles for synthesizing 22:6-containing

phospholipids locally. There are a number of reports showing the importance of 22:6 for the nervous system [36], and its relation to nervous disorders such as Alzheimer's disease [37,38]. However, it is still controversial whether 22:6 is effective as a treatment for cognitive impairment or Alzheimer's Disease [39-41]. If LPAAT4 has a contribution for synthesizing 22:6-containing phospholipids in the nervous system, it might be a good tool for studying the true role of 22:6 for the brain.

In the first part of our study, I speculated that the levels of 18:2- and 22:6-containing PC are maintained through the de novo pathway, and that 16:0- and 18:1-containing PC are regulated through the remodeling pathway. However, my results in the first part of the study did not reveal how the level of 20:4-containing phospholipids are determined. LPCAT3 is known to have strong LPCAT, LPEAT, and LPSAT activities with high selectivity towards 20:4-CoA [23], and LPCAT3 stable overexpression slightly increased the level of PAPC (Figure 4E). I thought LPCAT3 might be the key enzyme to determine the level of 20:4-containing phospholipids. In the study using C3H10T1/2 cells, I found that LPCAT3 mRNA levels are increased during adipocyte differentiation. I observed increases in LPCAT, LPEAT, and LPSAT

activities, especially with 18:2-CoA and 20:4-CoA, which correlates with the activities of LPCAT3. I also found several changes in fatty acid profiles of phospholipids between preadipocytes and adipocytes, including an increase in 20:4-containing species. These data suggest a possibility that increased LPCAT3 expression enhanced LPLAT activities with 20:4-CoA, resulting in an increase in 20:4-containing phospholipids. There are several reports showing the role of 20:4-containing phospholipids for adipocyte function. For example, there is a positive correlation between BMI and 20:4-containing adipose tissue in children [42], and another report shows that high content of 20:4 in adipose tissue increases metabolic syndrome in Costa Rican adults [43]. Also, some eicosanoids, which are produced from 20:4-containing phospholipids, are known to act as inflammatory mediators [2], and endogenous ligands for PPARγ [44].

However, many other interpretations can be made from the results of my study. For example, the induction of LPCAT3 mRNA expression might be caused by the direct stimulation with the reagents, and not by the differentiation of C3H10T1/2 cells into adipocytes. Also, the increases in LPLAT activities and 20:4-containing phospholipids might be caused by other enzymes. Although further studies are needed

to clarify the actual role of LPCAT3, my results suggest a possibility that LPCAT3 might have an important role for adipocyte differentiation, or the function of adipocytes.

Since the studies with C3H10T1/2 cells only showed a correlation between LPCAT3 expression level and the amount of 20:4-containing phospholipids, LPCAT3 deficient mice were produced to gain more evidence to confirm that LPCAT3 actually has a role for maintaining the level of 20:4-containing phospholipids. Studies by researchers in our laboratory showed that LPCAT3 deficient mice died a few days after birth, which is probably due to hypoglycemia caused by malabsorption of nutrients [21]. LPCAT3 deficient mice at E18.5-19.5 showed reduced phospholipids containing C20 fatty acids, especially 20:4, which suggested that LPCAT3 has a role for maintaining 20:4-containing phospholipids. On the other hand, LPCAT3 deficiency caused an increase in phospholipids with C22 and C24 n-6 PUFAs. The increase in 20:4-CoA caused by LPCAT3 deficiency might have enhanced its elongation into C22 and C24 n-6 PUFAs, which are further incorporated into phospholipids [45]. From the studies of LPCAT3 deficient mice it became clear that LPCAT3 has a role for synthesizing 20:4-containing phospholipids *in vivo* [21]. Since LPCAT3 deficiency did not completely

deplete 20:4-containing phospholipids, other LPLAT enzymes might be involved in maintaining their levels.

In conclusion, I newly found a possibility that the levels of 18:2- and 22:6-containing phospholipids are maintained through the *de novo* pathway, whereas 16:0- and 18:1-containing phospholipids are regulated through the remodeling pathway. This was different from the classical theory that the remodeling pathway is important for the incorporation of polyunsaturated fatty acids, especially arachidonic acid. I newly identified LPAAT4, which might have a function for maintaining the levels of 22:6-containing phospholipids through the *de novo* pathway together with LPAAT3. I found that during differentiation of C3H10T1/2 cells in to adipocytes, LPCAT3 expression, LPLAT activities that match the substrate specificities of LPCAT3, and 20:4-containing phospholipids are increased. LPCAT3 deficient mice showed decreased 20:4-containing phospholipids, suggesting that LPCAT3 has a role for maintaining 20:4-containing phospholipids. My study revealed a new understanding of how LPLAT enzymes modulate the acyl-chain composition of phospholipids. Aberrant acyl-chain composition is observed in various diseases, for example metabolic disorders, cancer

and Alzheimer's disease [4,6]. Therefore, this study might possibly lead to a new cure or diagnosis for these diseases.

7. Acknowledgements

First, I would like to specially thank Professor Takao Shimizu (Department of Lipid Signaling, Research Institute, National Center for Global Health and Medicine) for supervising my study, and Associate Professor Yoshihiro Kita (Department of Lipidomics, Graduate School of Medicine, The University of Tokyo) for giving me advice on my research and my thesis. I am deeply grateful to Dr. Hideo Shindou (Department of Lipid Signaling, Research Institute, National Center for Global Health and Medicine) for instructing my research. I greatly thank Dr. Takeshi Harayama (University of Geneva) for great contribution to this study, and also for giving me many valuable suggestions on my study and on my thesis. I am also greatly thankful to Dr. Tomomi Hashidate-Yoshida (Department of Lipid Signaling, Research Institute, National Center for Global Health and Medicine) for great contribution to the work on LPCAT3 deficient mice, and also for advice on my research. I also thank Drs. Suzumi Tokuoka, Fumie Hamano (Department of Lipidomics, Graduate School of Medicine, The University of Tokyo), Daisuke Hishikawa, Ryo Morimoto (Department of Lipid Signaling, Research Institute, National Center for Global Health and Medicine),

Keisuke Yanagida (Weill Cornell Medical College) and Andreas Koeberle (Friedrich Schiller University Jena) for contribution to this work, and for discussions. I am grateful to Dr. Keizo Waku (Teikyo University) for valuable suggestions. I thank Dr. J.-i. Miyazaki (Osaka University) for supplying pCXN2. As to collaboration, I thank Professor Masayoshi Mishina (Brain Science Laboratory, The Research Organization of Science and Technology, Ritsumeikan University), Professor K. Sakimura (Department of Cellular Neurobiology, Brain Research Institute, Niigata University), Professor Satoshi Ishii (Department of Immunology, Graduate School of Medicine, Akita University), Drs. Yoshiko Mukumoto and Hiroshi Kiyonari (Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology), Dr. Eiji Otsubo (Advanced Medical Research Department, Research Division, Mitsubishi Tanabe Pharma Corporation), Drs. Tadashi Okamura and Rieko Yanobu-Takahashi (Department of Laboratory Animal Medicine, Research Institute, National Center for Global Health and Medicine), and Dr. Miwa Tamura-Nakano (Communal Laboratory, Research Institute, National Center for Global Health and Medicine). I deeply thank Professor Takeshi Iwatsubo and Dr. Tadafumi Hashimoto (Department of

Neuropathology, Graduate School of Medicine, The University of Tokyo) for valuable suggestions and discussions. I am deeply grateful to Takeda Science Foundation for the scholarship. I also thank the MD student course program for the support. Finally, I thank all the members in the laboratory for helpful discussions and technical support.

8. References

1. Van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **9**, 112-124 (2008).
2. Shimizu, T. Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu Rev Pharmacol Toxicol* **49**, 123-150 (2009).
3. Yamashita, A., Hayashi, Y., Nemoto-Sasaki, Y., Ito, M., Oka, S., Tanikawa, T., Waku, K. & Sugiura, T. Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms. *Prog Lipid Res* **53**, 18-81 (2013).
4. Morita, Y., Sakaguchi, T., Ikegami, K., Goto-Inoue, N., Hayasaka, T., Hang, V. T., Tanaka, H., Harada, T., Shibasaki, Y., Suzuki, A., Fukumoto, K., Inaba, K., Murakami, M., Setou, M. & Konno, H. Lysophosphatidylcholine acyltransferase 1 altered phospholipid composition and regulated hepatoma progression. *J Hepatol* **59**, 292-299 (2013).
5. Puri, P., Wiest, M. M., Cheung, O., Mirshahi, F., Sargeant, C., Min, H. K., Contos, M. J., Sterling, R. K., Fuchs, M., Zhou, H., Watkins, S. M. & Sanyal, A. J. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **50**, 1827-1838 (2009).
6. Mapstone, M., Cheema, A. K., Fiandaca, M. S., Zhong, X., Mhyre, T. R., Macarthur, L. H., Hall, W. J., Fisher, S. G., Peterson, D. R., Haley, J. M., Nazar, M. D., Rich, S. A., Berlau, D. J., Peltz, C. B., Tan, M. T., Kawas, C. H. & Federoff, H. J. Plasma phospholipids identify antecedent memory impairment in older adults. *Nat Med* **20**, 415-418 (2014).
7. Kennedy, E. P. & Weiss, S. B. The function of cytidine coenzymes in the biosynthesis of phospholipides. *J Biol Chem* **222**, 193-214 (1956).
8. Coleman, R. A. & Mashek, D. G. Mammalian triacylglycerol

metabolism: synthesis, lipolysis, and signaling. *Chem Rev* **111**, 6359-6386 (2011).

9. Lands, W. E. Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis. *J Biol Chem* **231**, 883-888 (1958).
10. Shindou, H. & Shimizu, T. Acyl-CoA:lysophospholipid acyltransferases. *J Biol Chem* **284**, 1-5 (2009).
11. Shindou, H., Hishikawa, D., Harayama, T., Eto, M. & Shimizu, T. Generation of membrane diversity by lysophospholipid acyltransferases. *J Biochem* **154**, 21-28 (2013).
12. Macdonald, J. I. & Sprecher, H. Phospholipid fatty acid remodeling in mammalian cells. *Biochim Biophys Acta* **1084**, 105-121 (1991).
13. Yuki, K., Shindou, H., Hishikawa, D. & Shimizu, T. Characterization of mouse lysophosphatidic acid acyltransferase 3: an enzyme with dual functions in the testis. *J Lipid Res* **50**, 860-869 (2009).
14. Koeberle, A., Shindou, H., Harayama, T. & Shimizu, T. Role of lysophosphatidic acid acyltransferase 3 for the supply of highly polyunsaturated fatty acids in TM4 Sertoli cells. *FASEB J* **24**, 4929-4938 (2010).
15. Harayama, T., Shindou, H., Ogasawara, R., Suwabe, A. & Shimizu, T. Identification of a novel noninflammatory biosynthetic pathway of platelet-activating factor. *J Biol Chem* **283**, 11097-11106 (2008).
16. Shindou, H., Eto, M., Morimoto, R. & Shimizu, T. Identification of membrane O-acyltransferase family motifs. *Biochem Biophys Res Commun* **383**, 320-325 (2009).
17. Bradley, R. M., Marvyn, P. M., Aristizabal Henao, J. J., Mardian, E. B., George, S., Aucoin, M. G., Stark, K. D. & Duncan, R. E. Acylglycerophosphate acyltransferase 4 (AGPAT4) is a mitochondrial lysophosphatidic acid acyltransferase that regulates brain phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol levels. *Biochim Biophys Acta* **1851**, 1566-1576 (2015).
18. Schmidt, J. A. & Brown, W. J. Lysophosphatidic acid acyltransferase 3 regulates Golgi complex structure and function. *J Cell Biol* **186**,

211-218 (2009).

19. Lee, H. C., Inoue, T., Sasaki, J., Kubo, T., Matsuda, S., Nakasaki, Y., Hattori, M., Tanaka, F., Udagawa, O., Kono, N., Itoh, T., Ogiso, H., Taguchi, R., Arita, M., Sasaki, T. & Arai, H. LPIAT1 regulates arachidonic acid content in phosphatidylinositol and is required for cortical lamination in mice. *Mol Biol Cell* **23**, 4689-4700 (2012).
20. Harayama, T., Eto, M., Shindou, H., Kita, Y., Otsubo, E., Hishikawa, D., Ishii, S., Sakimura, K., Mishina, M. & Shimizu, T. Lysophospholipid acyltransferases mediate phosphatidylcholine diversification to achieve the physical properties required in vivo. *Cell Metab* **20**, 295-305 (2014).
21. Hashidate-Yoshida, T., Harayama, T., Hishikawa, D., Morimoto, R., Hamano, F., Tokuoka, S. M., Eto, M., Tamura-Nakano, M., Yanobu-Takanashi, R., Mukumoto, Y., Kiyonari, H., Okamura, T., Kita, Y., Shindou, H. & Shimizu, T. Fatty acid remodeling by LPCAT3 enriches arachidonate in phospholipid membranes and regulates triglyceride transport. *Elife* **4**(2015).
22. Rong, X., Wang, B., Dunham, M. M., Hedde, P. N., Wong, J. S., Gratton, E., Young, S. G., Ford, D. A. & Tontonoz, P. Lpcat3-dependent production of arachidonoyl phospholipids is a key determinant of triglyceride secretion. *Elife* **4**(2015).
23. Hishikawa, D., Shindou, H., Kobayashi, S., Nakanishi, H., Taguchi, R. & Shimizu, T. Discovery of a lysophospholipid acyltransferase family essential for membrane asymmetry and diversity. *Proc Natl Acad Sci U S A* **105**, 2830-2835 (2008).
24. Koeberle, A., Shindou, H., Harayama, T., Yuki, K. & Shimizu, T. Polyunsaturated fatty acids are incorporated into maturating male mouse germ cells by lysophosphatidic acid acyltransferase 3. *FASEB J* **26**, 169-180 (2011).
25. Eto, M., Shindou, H. & Shimizu, T. A novel lysophosphatidic acid acyltransferase enzyme (LPAAT4) with a possible role for incorporating docosahexaenoic acid into brain glycerophospholipids. *Biochem Biophys Res Commun* **443**, 718-724 (2014).

26. Eto, M., Shindou, H., Koeberle, A., Harayama, T., Yanagida, K. & Shimizu, T. Lysophosphatidylcholine acyltransferase 3 is the key enzyme for incorporating arachidonic acid into glycerophospholipids during adipocyte differentiation. *Int J Mol Sci* **13**, 16267-16280 (2012).
27. Gijon, M. A., Riekhof, W. R., Zarini, S., Murphy, R. C. & Voelker, D. R. Lysophospholipid acyltransferases and arachidonate recycling in human neutrophils. *J Biol Chem* **283**, 30235-30245 (2008).
28. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917 (1959).
29. Lu, B., Jiang, Y. J., Zhou, Y., Xu, F. Y., Hatch, G. M. & Choy, P. C. Cloning and characterization of murine 1-acyl-sn-glycerol 3-phosphate acyltransferases and their regulation by PPARalpha in murine heart. *Biochem J* **385**, 469-477 (2005).
30. Reznikoff, C. A., Brankow, D. W. & Heidelberger, C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res* **33**, 3231-3238 (1973).
31. Feldman, B. J., Streeper, R. S., Farese, R. V., Jr. & Yamamoto, K. R. Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. *Proc Natl Acad Sci U S A* **103**, 15675-15680 (2006).
32. Ntambi, J. M., Buhrow, S. A., Kaestner, K. H., Christy, R. J., Sibley, E., Kelly, T. J., Jr. & Lane, M. D. Differentiation-induced gene expression in 3T3-L1 preadipocytes. Characterization of a differentially expressed gene encoding stearoyl-CoA desaturase. *J Biol Chem* **263**, 17291-17300 (1988).
33. Beck, G., Sugiura, Y., Shinzawa, K., Kato, S., Setou, M., Tsujimoto, Y., Sakoda, S. & Sumi-Akamaru, H. Neuroaxonal dystrophy in calcium-independent phospholipase A2beta deficiency results from insufficient remodeling and degeneration of mitochondrial and presynaptic membranes. *J Neurosci* **31**, 11411-11420 (2011).
34. Sato, H., Taketomi, Y., Isogai, Y., Miki, Y., Yamamoto, K., Masuda, S.,

Hosono, T., Arata, S., Ishikawa, Y., Ishii, T., Kobayashi, T., Nakanishi, H., Ikeda, K., Taguchi, R., Hara, S., Kudo, I. & Murakami, M. Group III secreted phospholipase A2 regulates epididymal sperm maturation and fertility in mice. *J Clin Invest* **120**, 1400-1414 (2010).

35. Kim, H. Y. Novel metabolism of docosahexaenoic acid in neural cells. *J Biol Chem* **282**, 18661-18665 (2007).
36. Salem, N., Jr., Litman, B., Kim, H. Y. & Gawrisch, K. Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36**, 945-959 (2001).
37. Marszalek, J. R. & Lodish, H. F. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. *Annu Rev Cell Dev Biol* **21**, 633-657 (2005).
38. Teng, E., Taylor, K., Bilousova, T., Weiland, D., Pham, T., Zuo, X., Yang, F., Chen, P. P., Glabe, C. G., Takacs, A., Hoffman, D. R., Frautschy, S. A. & Cole, G. M. Dietary DHA supplementation in an APP/PS1 transgenic rat model of AD reduces behavioral and Abeta pathology and modulates Abeta oligomerization. *Neurobiol Dis* **82**, 552-560 (2015).
39. Freund-Levi, Y., Eriksdotter-Jonhagen, M., Cederholm, T., Basun, H., Faxen-Irving, G., Garlind, A., Vedin, I., Vessby, B., Wahlund, L. O. & Palmblad, J. Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. *Arch Neurol* **63**, 1402-1408 (2006).
40. Quinn, J. F., Raman, R., Thomas, R. G., Yurko-Mauro, K., Nelson, E. B., Van Dyck, C., Galvin, J. E., Emond, J., Jack, C. R., Jr., Weiner, M., Shinto, L. & Aisen, P. S. Docosahexaenoic acid supplementation and cognitive decline in Alzheimer disease: a randomized trial. *JAMA* **304**, 1903-1911 (2010).
41. Chew, E. Y., Clemons, T. E., Agron, E., Launer, L. J., Grodstein, F., Bernstein, P. S. & Age-Related Eye Disease Study 2 Research, Group. Effect of Omega-3 Fatty Acids, Lutein/Zeaxanthin, or Other Nutrient Supplementation on Cognitive Function: The AREDS2 Randomized Clinical Trial. *JAMA* **314**, 791-801 (2015).

42. Savva, S. C., Chadjigeorgiou, C., Hatzis, C., Kyriakakis, M., Tsimbinos, G., Tornaritis, M. & Kafatos, A. Association of adipose tissue arachidonic acid content with BMI and overweight status in children from Cyprus and Crete. *Br J Nutr* **91**, 643-649 (2004).
43. Williams, E. S., Baylin, A. & Campos, H. Adipose tissue arachidonic acid and the metabolic syndrome in Costa Rican adults. *Clin Nutr* **26**, 474-482 (2007).
44. Harmon, G. S., Lam, M. T. & Glass, C. K. PPARs and lipid ligands in inflammation and metabolism. *Chem Rev* **111**, 6321-6340 (2011).
45. Schmitz, G. & Ecker, J. The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res* **47**, 147-155 (2008).