Table 3. Relative intensities of major diacyl-glycero-PLs from CHO-K1 cells transfected with pCXN2.1 vector control and FLAG/mLPAAT3-pCXN2.1 expression plasmid. Mean values (n = 5-6) and standard error estimates are given; (*) P <0.05, (**) P <0.01 vs. vector control-transfected CHO-K1 cells

fatty acid composition	relative intensity of diacyl-glycero-PLs [% of PL subclass intensity]											
	control vector-transfected CHO cells						mLPAAT3-expressing CHO cells					
	PC	PE	PS	PI	PG	PA	PC	PE	PS	PI	PG	PA
14:0-16:0	0.87 ±0.03	n.d.	n.d.	n.d.	n.d.	n.d.	1.09 ±0.05**	n.d.	n.d.	n.d.	n.d.	n.d.
16:0-16:0	2.58 ±0.10	n.d.	n.d.	0.74 ±0.30	1.68 ±0.10	41.54 ±3.44	3.25 ±0.14**	n.d.	n.d.	0.71 ±0.31	1.61 ±0.10	41.30 ±3.47
16:0-18:0	0.60 ±0.04	n.d.	n.d.	n.d.	2.98 ±0.27	n.d.	0.73 ±0.05	n.d.	n.d.	n.d.	3.08 ±0.41	n.d.
16:0-16:1	8.63 ±0.07	1.86 ±0.11	1.34 ±0.04	n.d.	2.77 ±0.12	7.71 ±0.96	8.81 ±0.15	1.97 ±0.14	1.38 ±0.05	n.d.	2.77 ±0.18	7.86 ±0.94
14:0-18:1			n.d.	n.d.					n.d.	n.d.		
16:0-18:1	32.97 ±0.81	12.09 ±0.17	14.28 ±0.27	13.90 ±0.73	11.30 ±0.20	44.22 ±2.39	31.92 ±0.85	12.44 ±0.38	14.75 ±0.22	14.44 ±0.64	11.59 ±0.33	44.56 ±2.59
18:0-16:1	n.d.					n.d.	n.d.					n.d.
18:0-18:1	10.56 ±0.08	14.08 ±0.33	48.97 ±0.26	16.98 ±1.90	15.19 ±0.55	n.d.	9.75 ±0.29*	13.27 ±0.31	46.85 ±0.66	17.30 ±1.41	16.87 ±0.82	n.d.
16:0-18:2	11.98 ±0.16	8.97 ±0.14	1.73 ±0.12	3.14 ±0.21	n.d.	6.53 ±1.40	12.30 ±0.23	9.72 ±0.27*	1.91 ±0.05	3.66 ±0.25	n.d.	6.28 ±1.35
16:1-18:1					10.38 ±0.25						10.49 ±0.35	n.d.
18:1-18:1/18:0-18:2	21.54 ±0.20	25.29 ±0.47	11.10 ±0.12	13.72 ±0.29	36.33 ±1.19	n.d.	21.46 ±0.18	25.48 ±0.41	12.26 ±0.24**	15.00 ±0.46	35.28 ±0.95	n.d.
16:1-18:2	0.41 ±0.02	0.64 ±0.09	n.d.	n.d.	n.d.	n.d.	0.45 ±0.02	0.88 ±0.01*	n.d.	n.d.	n.d.	n.d.
16:0-18:3	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.

18:1-18:2	4.24 ±0.14	4.33 ±0.17	1.11 ±0.06	3.33 ±0.27	4.80 ±0.26	n.d.	4.30 ±0.13	4.72 ±0.20	1.25 ±0.09	3.38 ±0.30	4.65 ±0.28	n.d.
16:0-20:3		n.d.			n.d.	n.d.		n.d.			n.d.	n.d.
18:2-18:2	1.59 ±0.06	n.d.	n.d.	n.d.	n.d.	n.d.	1.70 ±0.04	n.d.	n.d.	n.d.	n.d.	n.d.
18:0-20:3	1.44 ±0.09	2.26 ±0.37	4.17 ±0.19	7.65 ±0.41	2.35 ±0.17	n.d.	1.47 ±0.06	2.21 ±0.31	4.57 ±0.21	7.27 ±0.56	2.18 ±0.25	n.d.
16:0-20:4	n.d.	3.24 ±0.11	0.39 ±0.04	5.86 ±0.22	n.d.	n.d.	n.d.	3.50 ±0.11	0.38 ±0.05	5.62 ±0.17	n.d.	n.d.
18:0-20:4	0.98 ±0.02	11.09 ±0.42	2.46 ±0.18	25.69 ±0.78	0.66 ±0.04	n.d.	1.08 ±0.03*	10.52 ±0.33	2.65 ±0.07	23.52 ±0.58*	0.78 ±0.03	n.d.
18:1-20:4	1.24 ±0.08	5.71 ±0.35	n.d.	4.89 ±0.26	1.14 ±0.04	n.d.	1.39 ±0.09	5.80 ±0.36	n.d.	5.03 ±0.54	1.13 ±0.05	n.d.
18:0-22:4	0.24 ±0.03	2.30 ±0.12	6.67 ±0.17	2.06 ±0.03	n.d.	n.d.	0.26 ±0.02	2.00 ±0.10	6.27 ±0.19	1.90 ±0.08	n.d.	n.d.
18:0-22:5	0.40 ±0.03	3.36 ±0.20	4.57 ±0.16	1.41 ±0.07	n.d.	n.d.	0.42 ±0.03	3.00 ±0.07	4.57 ±0.21	1.50 ±0.07	n.d.	n.d.
16:0-22:6	0.21 ±0.01	1.07 ±0.07	n.d.	n.d.	1.16 ±0.09	n.d.	0.25 ±0.01*	0.99 ±0.07	n.d.	n.d.	1.06 ±0.06	n.d.
18:2-20:4	n.d.		n.d.	n.d.		n.d.	n.d.		n.d.	n.d.		n.d.
18:1-20:5	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
18:0-22:6	0.16 ±0.01	2.52 ±0.16	3.19 ±0.19	0.64 ±0.05	5.47 ±0.71	n.d.	0.19 ±0.01*	2.35 ±0.13	3.16 ±0.11	0.69 ±0.06	5.62 ±0.58	n.d.
18:1-22:5			n.d.			n.d.			n.d.			n.d.
18:1-22:6	0.19 ±0.01	1.04 ±0.05	n.d.	n.d.	3.70 ±0.19	n.d.	0.25 ±0.01*	1.08 ±0.09	n.d.	n.d.	2.71 ±0.14	n.d.

n.d. = not detectable

Table 4. Total intensities of diacyl-glycero-PL subclasses from mLPAAT3-shRNA-transfected TM4 cells and mLPAAT3-expressing CHO-K1 cells. Mean values (n = 8) and standard error estimates are given as percentage of vector control; (*) P <0.05

PL subclass	total intensity of PL subclasses [% of vector control]	
	knockdown of LPAAT3 in TM4 cells	expression of LPAAT3 in CHO cells
PC	98.4 ± 1.7	101.0 ± 7.1
PE	97.8 ± 2.1	100.9 ± 5.1
PS	83.8 ± 5.1*	101.5 ± 5.2
PI	98.6 ± 3.3	105.6 ± 7.7
PG	103.1 ± 7.9	100.4 ± 8.0
PA	107.1 ± 7.9	104.7 ± 3.3

Table 5. Major free fatty acids from control-shRNA-transfected TM4 cells and control-vector-transfected CHO-K1 cells as relative intensities and from mLPAAT3-shRNA-transfected TM4 cells and FLAG/mLPAAT3-transfected CHO-K1 cells as percentage of control. Mean values (n = 5-6) and standard error estimates are given; (*) P <0.05 vs. vector control-transfected cells

fatty acid	relative intensity of free fatty acids [% of total fatty acids]		level of free fatty acids [% of control]	
	control-shRNA-transfected TM4 cells	control-vector-transfected CHO-K1 cells	knockdown of LPAAT3 in TM4 cells	expression of LPAAT3 in CHO-K1 cells
14:0	2.58±0.23	0.73±0.08	102.4±2.3	104.9±7.7
16:0	21.64±1.03	20.44±1.87	97.5±3.7	98.3±4.1
16:1	2.26±0.40	0.68±0.18	110.5±7.2	138.8±13.0
18:0	35.02±3.70	30.51±2.82	95.7±5.6	98.7±4.0
18:1	18.17±1.24	5.61±0.53	100.9±6.7	110.6±6.0
18:2	2.09±0.42	0.54±0.08	89.3±8.9	116.2±5.1
18:3	0.62±0.10	n.d.	77.5±6.8*	n.d.
20:0	2.16±0.13	3.47±0.13	93.7±6.3	96.3±1.0
20:1	0.08±0.00	0.38±0.09	103.7±3.7	104.6±5.3
20:3	n.d.	0.04±0.02	n.d.	119.5±5.6*
20:4	4.83±1.79	2.87±0.20	88.5±7.4	109.5±2.6
20:5	2.20±0.22	0.82±0.06	93.9±7.8	121.4±6.4*
22:0	5.34±0.19	11.70±0.56	88.9±6.6	97.3±1.9
22:5	0.44±0.12	n.d.	78.0±5.4*	n.d.
22:6	0.67±0.04	1.01±0.06	96.3±7.4	107.2±11.7
24:0	3.44±0.44	8.31±0.40	91.2±11.5	98.4±4.0
26:0	2.96±0.23	6.24±0.35	103.5±4.6	93.0±4.2

n.d. = not detectable

Figure legends

Figure 1. mLPAAT3 knockdown in TM4 cells and expression in CHO-K1 cells. (A-C) Expression levels of mRNA were analyzed using quantitative real-time PCR as described in Materials and Methods. (**A**) Expression levels of mLPAAT1, -2 and -3 mRNA in control-shRNA-transfected TM4 cells. (**B**) mLPAAT3 mRNA expression in TM4 cells transfected with control-shRNA (control) or shRNA sequences 'lot 1-4'. (**C**) Expression of mLPAAT1, -2 and -3 mRNA in TM4 cells transfected with control-shRNA or 'lot 3'-shRNA (LPAAT1-3). (**D**) The conversion of *sn*-1-18:1-LPA (50 μM) with 20:4-CoA (25 μM) to *sn*-1-18:1-*sn*-2-20:4-PA by microsomal preparations (1 μg protein, incubation for 30 min at 37°C) of stable control- or 'lot 3'-shRNA-transfected TM4 cells was analysed by LC-MS as described in Materials and Methods. Data are given as mean ± S.E., $n = 2$-5, $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. vector shRNA control, ANOVA + Tukey HSD post-hoc tests (**B**, **C**) or student's *t* test (**D**). (**E**) Expression of mLPAAT3 in CHO-K1 cells. CHO-K1 cells were transfected with control vector (control) or vector encoding for FLAG-tagged mLPAAT3 (LPAAT3). Expression of FLAG/mLPAAT3 was analyzed at the protein level by Western Blotting using anti-FLAG antibody. Microsomal preparations corresponding to 5 μg protein were loaded in each lane. The data is representative of 2 independent experiments.

Figure 2. Effect of the expression of mLPAAT3 in CHO-K1 cells on microsomal LPLAT activities. The conversion of *sn*-1-lyso-PLs with acyl-CoA substrates to diacyl-PLs by microsomal preparations of CHO-K1 cells was compared between cells transfected with control-vector and a FLAG/mLPAAT3 vector construct. LPAAT (**A**), LPCAT, LPSAT, LPEAT and LPGAT (**B**) activities were examined using 1 μg microsomal protein (100,000 × g pellet), 25 μM acyl-CoA and 50 μM lyso-PLs for selected substrate combinations (acyl-CoA/lyso-PL). After incubation for 30 min at 37°C (total volume = 100 μl), the reaction was

stopped by addition of 375 µl methanol/chloroform (2:1, v/v) supplemented with internal standard (0.8 nmol 1,2-di-*sn*-glycero-3-14:0-PE). To reveal the effects of the knockdown of mLPAAT3 on microsomal LPAAT (**A**), LPCAT, LPSAT, LPEAT and LPGAT activities (**B**), formed PLs were extracted and analysed by LC-MS (selective ion monitoring mode) as described in Materials and Methods. Data are given as mean ± S.E., n = 2-3, *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, student's t test.

Figure 3. Effect of the knockdown of mLPAAT3 in TM4 cells on microsomal LPLAT activities. The conversion of *sn*-1-lyso-PLs with acyl-CoA substrates to diacyl-PLs by microsomal preparations of TM4 cells was compared between stable control-shRNA and LPAAT3-shRNA ('lot 3') transfected cells. Acyltransferase activity was examined using 25 µM acyl-CoA, 50 µM lyso-PLs and microsomal protein (100,000 g pellet, 1 µg, exception: 62.5 ng for the formation of PC) for selected substrate combinations (acyl-CoA/lyso-PL). After 30 min at 37°C, the reaction was stopped, and PLs formed were extracted and analysed by LC-MS (selective ion monitoring mode) as described in Materials and Methods. Data are given as mean ± S.E., n = 3-5, *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, student's t test.

Supplemental data

The entire coding region of mLPAAT2 [DNA Data Bank of Japan (DDBJ) accession number NM 026212] was identified in the National Center for Biotechnology Information (NCBI) database. A 0.9 kb cDNA clone encoding the full-length mLPAAT2 was obtained by PCR amplification using a forward primer designed to encode FLAG epitope (DYKDDDDK) in frame with the start codon of target DNA coding region (5′-CTAGCTAGCCACCATGGAT-TACAAGGATGACGATGACAAGGACCCGTGGCCATGGCTGACGGCG) and a reverse primer (CCGCTCGAGCTACTGGGCTGGCAAGACCCCAGGC-3′). Mouse adipose tissue cDNA was used as a template. Amplified PCR products were cloned into the pCXN2.1 vector and sequenced.

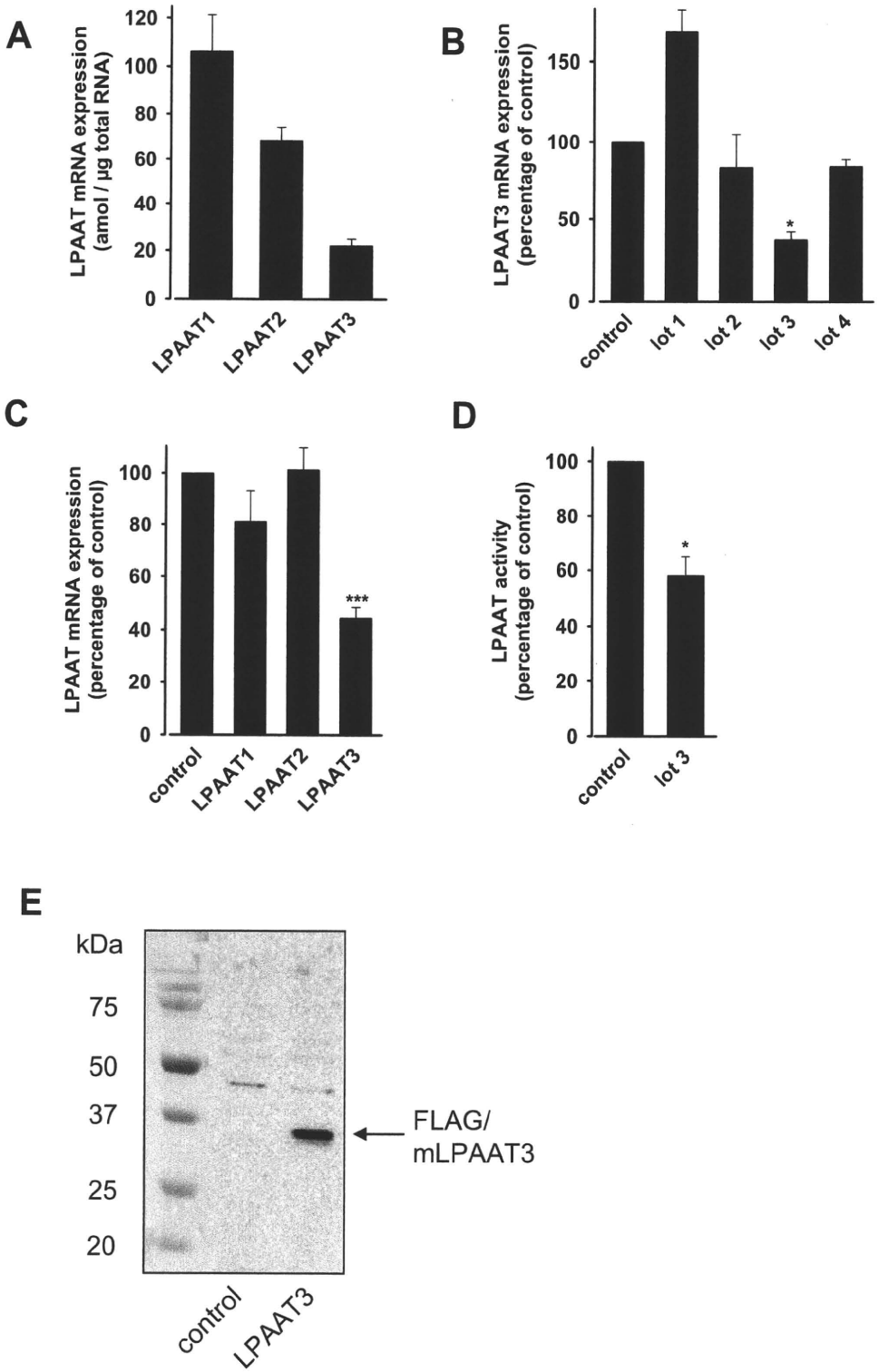
[Supplemental Figure 1]

Supplemental Figure 1. Dependence of the formation of 18:1-22:6-PA by mLPAAT3 on *sn*-1-18:1-LPA and 22:6-CoA concentrations. Microsomal preparations of CHO-K1 cells transfected with FLAG/mLPAAT3 vector were used as source of LPAAT3. Microsomes (corresponding to 1 μg protein) were incubated for 30 min at 37°C either with varying concentrations of *sn*-1-18:1-LPA (1-100 μM) and a fixed concentration of 22:6-CoA (25 μM) (**A**) or with a fixed concentration of *sn*-1-18:1-LPA (50 μM) and varying concentrations of 22:6-CoA (1-100 μM) (**B**). For the calculation of the turnover rate in pmol/min, the LC-MS system was calibrated using *sn*-1-17:0-*sn*-2-20:4-PA as standard. The solid lines represent the nonlinear fit to the Michaelis-Menten equation. Data are given as mean ± S.E., $n = 3$.

[Supplemental Figure 2]

Supplemental Figure 2. Effect of the expression of mLPAAT3 in CHO-K1 cells on microsomal LPIAT activity. The conversion of crude *sn*-1-lyso-PI with arachidonoyl-CoA by microsomal preparations of CHO-K1 cells was compared between cells transfected with control-vector and a FLAG/mLPAAT3 vector construct. LPIAT activities were examined using 2 μg microsomal protein (100,000 × g pellet), 25 μM arachidonoyl-CoA and 50 μM lyso-PI. After 10 min at 37°C, the reaction was stopped, and PI species formed were extracted and analysed by LC-MS (selective ion monitoring mode) as described in Materials and Methods. Data are given as mean ± S.E., $n = 2$, $^*p < 0.05$, student's t test.

Fig. 1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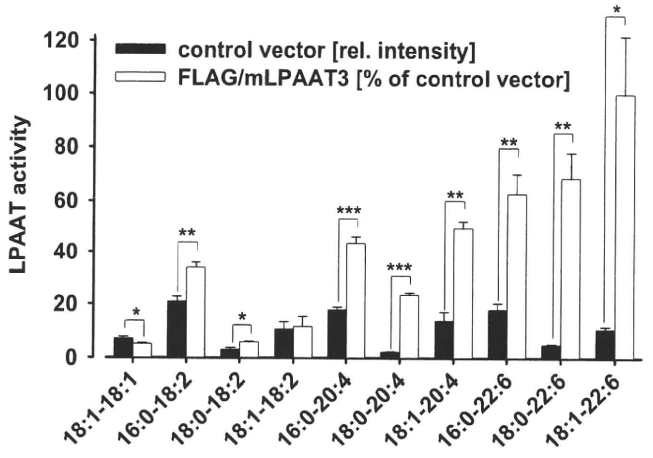
100
80
60
40
20
0
LPAAT1
LPAAT2
LPAAT3
B
LPAAT3 mRNA expression (percentage of control)
150
100
50
0
*
control
lot 1
lot 2
lot 3
lot 4
C
LPAAT mRNA expression (percentage of control)
100
80
60
40
20
0
***
control
LPAAT1
LPAAT2
LPAAT3
D
LPAAT activity (percentage of control)
100
80
60
40
20
0
*
control
lot 3
E
kDa
75
50
37
25
20
FLAG/
mLPAAT3
control
LPAAT3

Fig. 2

A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FLAG/mLPAAT3 [% of control vector]
LPAAT activity
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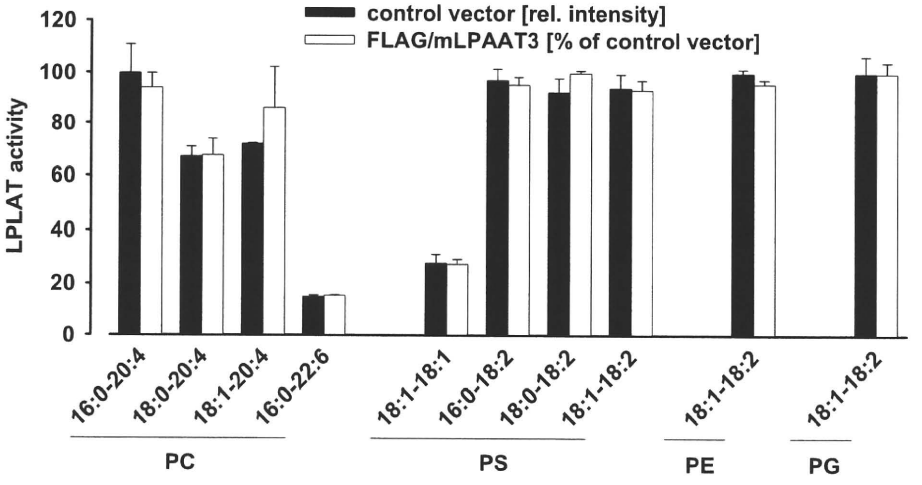
100
80
60
40
20
0
*
**
*
***
***
**
**
**
*
18:1-18:1
16:0-18:2
18:0-18:2
18:1-18:2
16:0-20:4
18:0-20:4
18:1-20:4
16:0-22:6
18:0-22:6
18:1-22:6

B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FLAG/mLPAAT3 [% of control vector]
LPLAT activity
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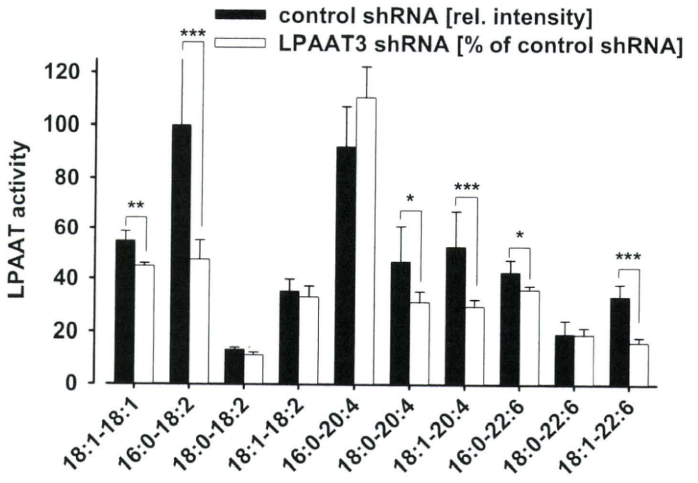
100
80
60
40
20
0
16:0-20:4
18:0-20:4
18:1-20:4
16:0-22:6
18:1-18:1
16:0-18:2
18:0-18:2
18:1-18:2
18:1-18:2
18:1-18:2
PC
PS
PE
PG

Fig. 3

A


control shRNA [rel. intensity]
LPAAT3 shRNA [% of control shRNA]
LPAAT activity
120
100
80
60
40
20
0
**
***
*
***
*
***
18:1-18:1
16:0-18:2
18:0-18:2
18:1-18:2
16:0-20:4
18:0-20:4
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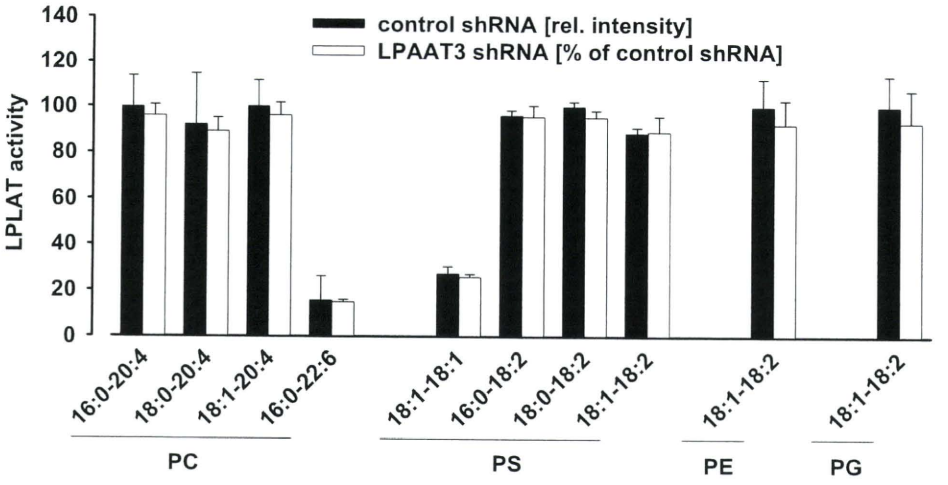
16:0-22:6
18:0-22:6
18:1-22:6

B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LPAAT3 shRNA [% of control shRNA]
LPLAT activity
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120
100
80
60
40
20
0
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18:1-20:4
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18:1-18:1
16:0-18:2
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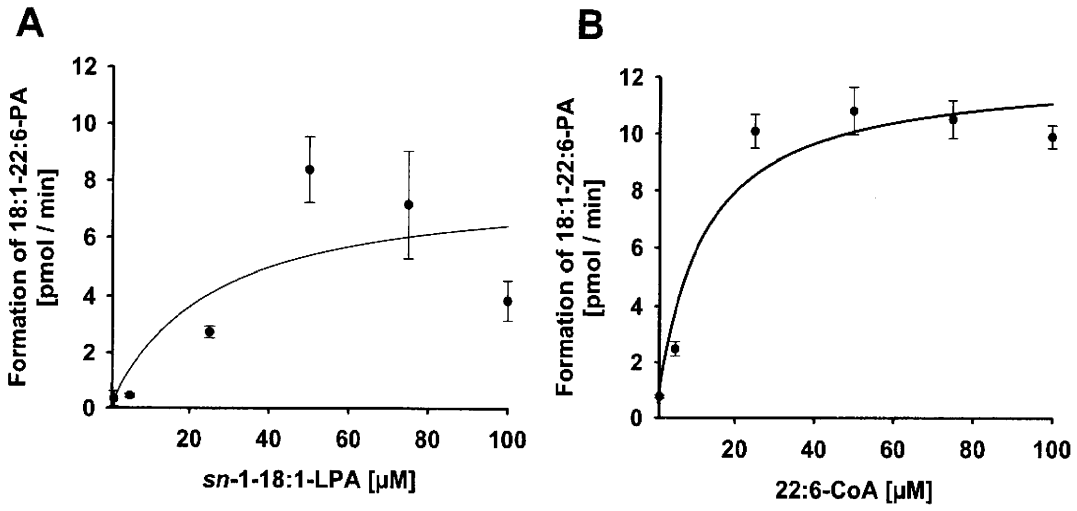
18:1-18:2
18:1-18:2
PC
PS
PE
PG

Supplemental Fig. 1



Supplemental Fig. 2