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

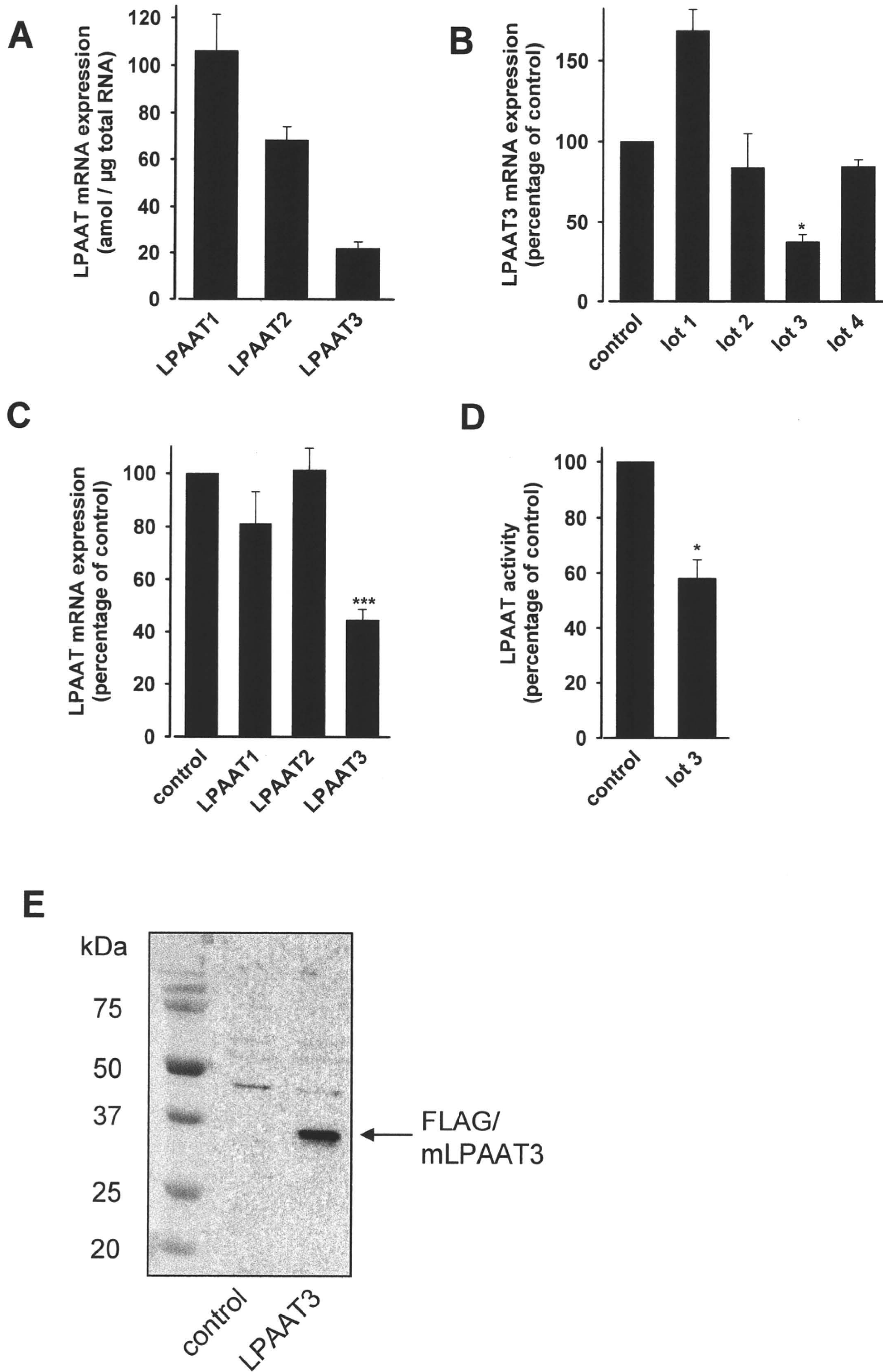
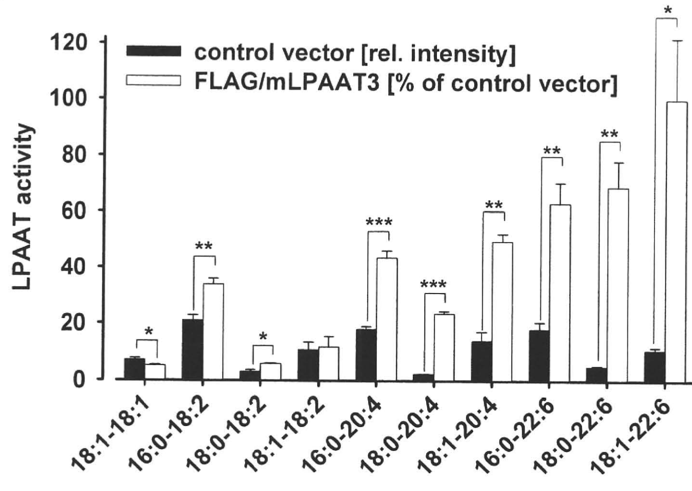


Fig. 2

A



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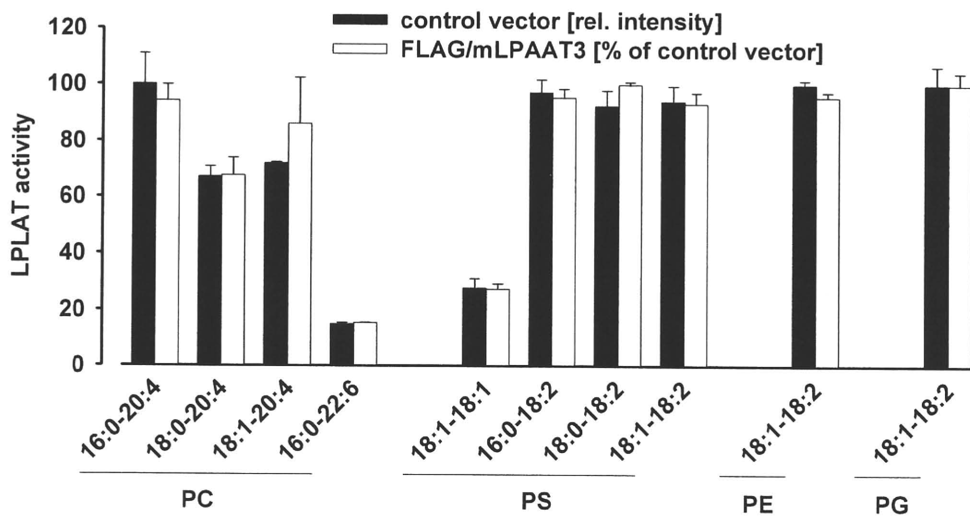
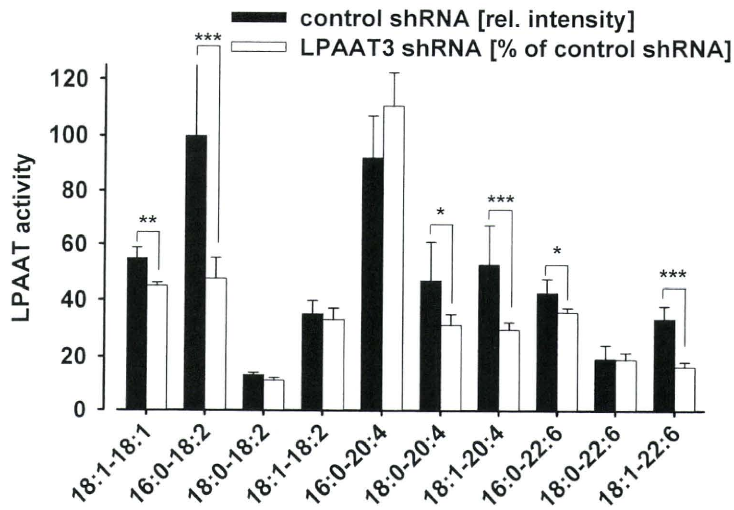


Fig. 3

A



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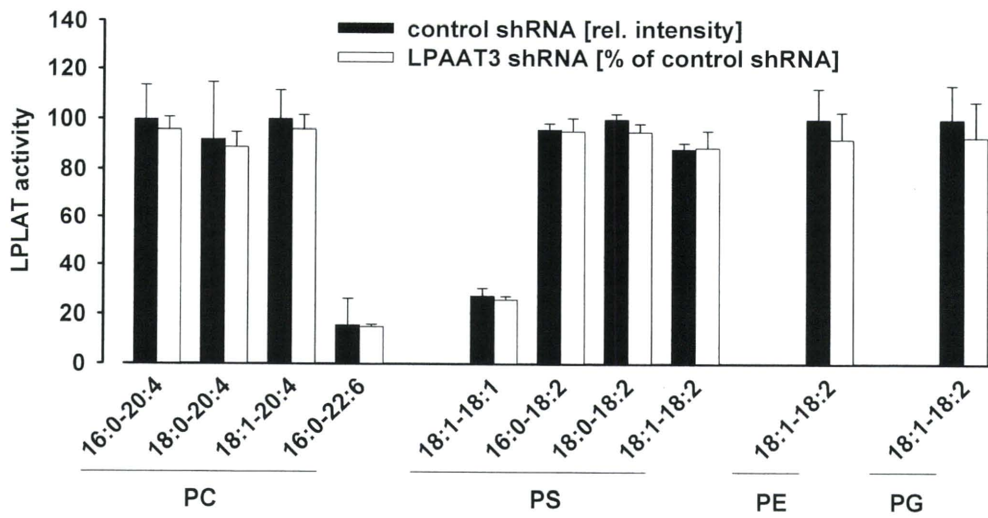
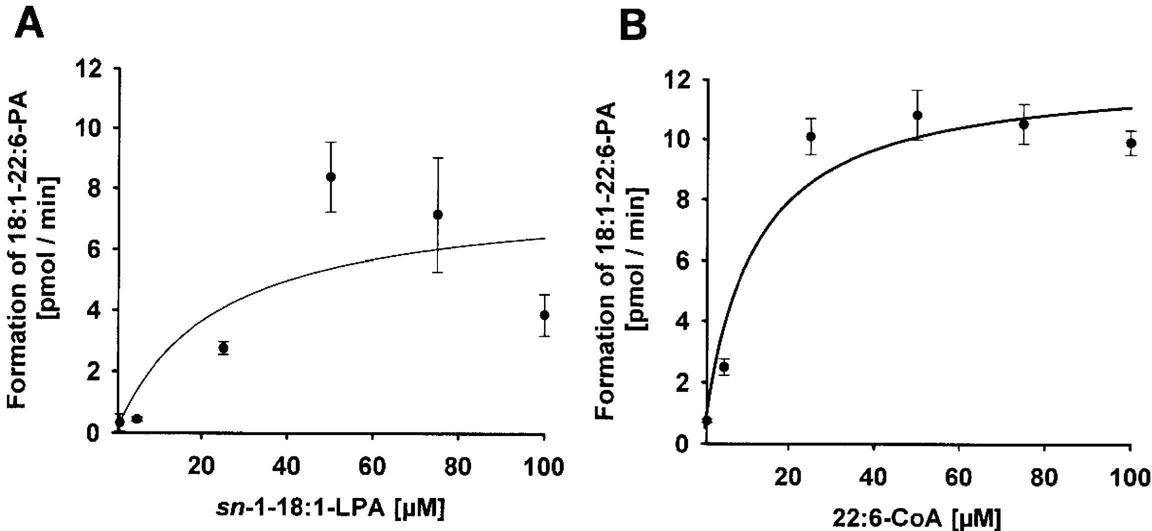


Supplemental Fig. 1



Supplemental Fig. 2