Obesity resistance and deregulation of lipogenesis in Δ6- fatty acid desaturase (FADS2) deficiency

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The fads2 null mouse model

The generation and genotyping by PCR of the *fads2null* mouse has been described before [1]. Phenotypic characterization revealed the molecular basis of infertility of both genders and auxotrophy of the *fads2-/-*mutant. Hetero- (+/-) and homozygous (-/-) *fads2* mutant mice are viable.

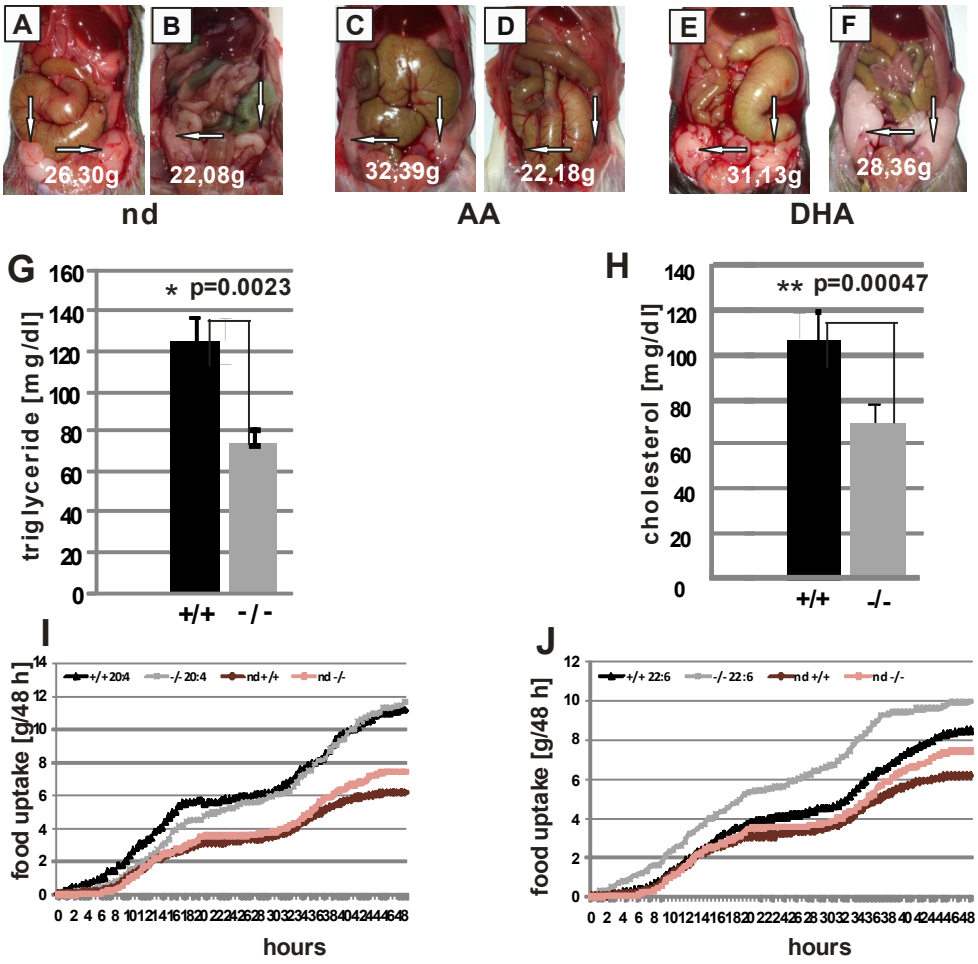


Figure S1 The *fads2null* mouse is lean and smaller than +/+ littermates
Situs of *nd-fads2+/+* and -/- (**A**) and (**B**), *AA+/+* and -/- (**C**) and (**D**) *DHA+/+* and *-/-* (**E**) and (**F**) male littermates (age 4months). The abdominal situs unveils strongly reduced subcutaneous, mesenteric and epididymal fat pads (white arrows) in the *nd-fads2-/-*male compared to +/+ littermate control, minimal fat depots in the *AA +/+* and *-/-*, but massive abdominal, mesenteric and epididymal adipose tissues in *DHA+/+* and -/- mice. Serum triglyceride concentration (**G**), serum total cholesterol concentration (**H**) of adult (>2months) *control +/+* and *fads2-/- mice*. Cohort sizes n=20. Phenomaster recordings document a similar food uptake of *fads2+/+* and *-/-* mice on *nd* and *AA*, (**I**), however, *DHA+/+* and *DHA-/-* mice were hyperphagic (**J**).

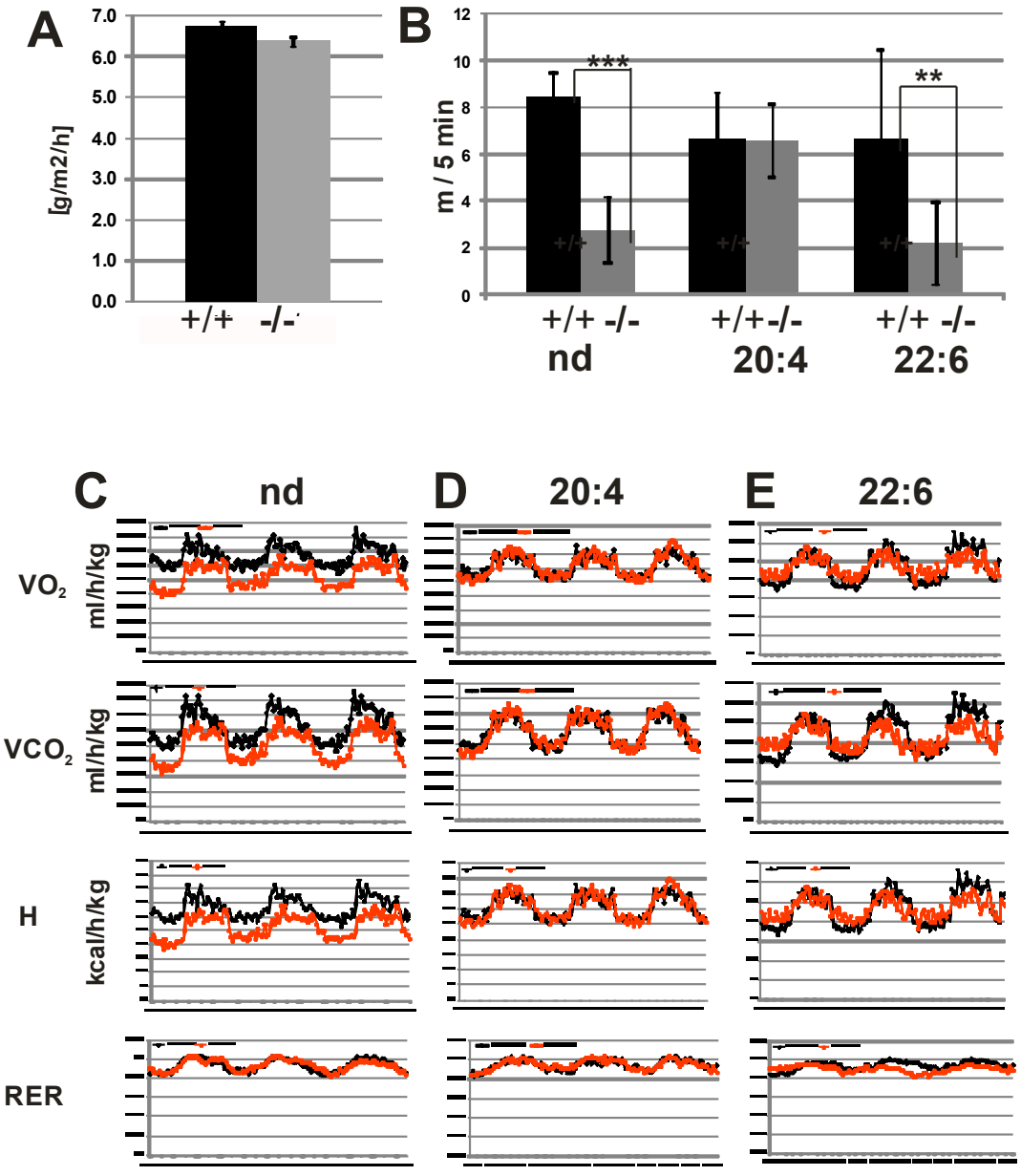


Figure S2

*Fads2-/-*mice have an unperturbed epidermal lipid barrier, locomotor activity in *fads2-/-*mice on nd, 20:4 and 22:6 supplemented diet. (**A**) Trans-epidermal water loss (TEWL) ($g*m^{-2}*h^{-1}$) of control +/+ and *fads2*-/- mice. (**B**) Locomotor activity (m/5min) in nd-, AA- and DHA+/+ and -/- mice. Metabolism and thermoregulation of *control fads2*+/+ and *fads2-/-* mice is reduced by about 20-30%. O_2-consumption VO_2, CO_2-production VCO_2, heat (**H**) dissipation and respiratory rates (RER) of *nd*- (**C**), *AA*- (**D**), and *DHA*- (**E**) *+/+* control (black tracing) and *-/-* mice (red tracing).

Table S1

Fatty acid composition of normal, 20:4 and 22:6 - supplemented diet

Table S1summarizes the fatty acid composition of diets used for the transformation of the nd-fads2-/- into the AA- (20:4) and DHA (22:6)-fads2-/- mouse lines. Arachidonic acid (AA) was supplemented as ARASCO and docosahexaenoic acid (DHA) as DHASCO trigycerides. These PUFAs are the only PUFA in the respective supplemented normal diet, which contains the two essential fatty acids (EFA) 18:2 and α-18:3 in concentrations to prohibit EFA deficiency.

FA	nd mg/kg	20:4 mg/kg	22:6 mg/kg
12:0			
14:0			
16:1			
ω9-16:1			
16:0	5246	9000	9000
18:0	1585	9000	35000
$18:1^{9}$	9218	31000	
$18:2^{9,12}$	**21800**	3000	
α-18:3	**3008**		
20:0	32		
$20:1^{11}$	40		
$20:2^{11,14}$			
ω6-20:4		**48000**	
ω3-20:5			
22:0			
ω3-22:5			
ω3-22:6			**56000**

Expression of genes involved in the regulation of lipid metabolism

Steady state mRNA concentrations in different tissues of the *fads2-/-* mouse was estimated by real time quantitative PCR of cDNAs of RNA of liver, muscle, brown (BAT) and white adipose tissue (WAT).

Total RNA was extracted from tissues of 6-wk-old wt and *fads2-/-* mutants using Trizol-reagent following the manufacturer´s instructions (GIBCO/BRL). For real time qRT-PCR analysis of expression levels, cDNAs were synthesized using Maloney murine leukemia virus reverse transcriptase, random hexamer primers and dNTPs. *hgprt* was used as internal marker.

Steady state levels of RNA of genes were quantified using the genes and primers listed in table S2.

Table S2

Sense and antisense primers used for qRT-PCR

d6-DS s	5'-tatggcaagaagaagctgaaatacctgccc-3'
d6-Ds as	5'-tgcttacctccataaatgaagctgccgtcc-3'
pparα s	5'-tggtggacacagagagccc-3'
pparα as	5'-gggtgtctgtgatgacagag-3'
pparβ s	5'-ggcctcatgaatgtgcccca-3'
pparβ as	5'-tccttgtagatttcctggagc-3'
pparγ s	5'-acttcggaatcagctctgtg-3'
pparγas	5'-taaggtggagatgcaggttc-3'
sreb1 s	5'-ggtggtgggcactgaagcaaagctgaatcc-3'
sreb1 as	5'-gattgcaggtcagacacagaaaggccagta-3'
hmgcoa s2 s	5'-ccaaggctgtcaaaacagtgctcatggaac-3'
hmgcoa s2 as	5'-ctgtttgtcactgctggatgacaggaagtc-3'
acs1 s	5'-atctacctgcggagtgaagccgtggcccag-3'
acs1 as	5'-acgttagatcttgatggtggcgtacagttc-3'
hnf1 α s	5'-tgaggtggcccagtacacgcacaccagcct-3'
hnf1 α as	5'-gaggaggccatgtgggtggtgataaaagtc-3'
hnf4 α s	5'-cccacgctgcagagcatcacctggcagatg-3'
hnf4 α as	5'-cttgcttggtgatcgttggctggggaatggcag-3'
cpt 1 s	5'-catgacgggcgctggcatcgaccgccacct-3'
cpt 1 as	5'-ggaattggcggtgaggccaaacaaggtgat-3'
cpt 2 s	5'-gaccgacacttgtttgctctacggtatctg-3'
cpt 2 as	5'-agttttgatggctttgccttcgagggcatc-3'
scd1 s	5'-tgcacctccctccggaaatgaacgagagaa-3'
scd2 s	5'-ttgaaaagagttctcaccactggggagcag-3'
scd3 s	5'-tattgagggcattggagccggagtccatcg-3'
scdxas	5'-cttgtagtacctcctctggaacatcaccag-3'
Δ5 s	5'-gggtggacttggcctggatgctcagcttct-3'
Δ5 as	5'-gctgctattggtgaaggtaagcgtccaacc-3'
elov1 s	5'-ttgctccaggaggaatgggctccttccatg-3'
elov1 as	5'-tccattttgctgaactgcacggggcagccg-3'

elov2 s	5'-tggataccttgtggtcaaagcttctttgga-3'
elov2 as	5'-attcttcacttctttctcttgcagctcttt-3'
elov3 s	5'-gaacaaagtgccttcgggtggctggttcat-3'
elov3 as	5'-cattggctcttggatgcaactttgcccttg-3'
elov4 s	5'-gtgggtggctggaggccaagcgtttttcgg-3'
elov4 as	5'-attcgtggccgtctttccggtttttgactg-3'
elov5 s	5'-atacaagagtcaaaggatggttcctcctgg-3'
elov5 as	5'-catcacaaaccaccagatgttgagcatggt-3'
elov6 s	5'-agacatggtcgctgggggtggttggttcat-3'
elov6 as	5'-ctcagccttcgtggctttcttcactttgcc-3'
pepck s	5'-cagcagccaagttgcccaagatcttccatg-3'
pepckas	5'-ttacatctggctgattctctgtttcagggc-3'
glc-6-p s	5'cggagactggttcaacctcgtcttcaagtg-3
glc-6-p as	5'-tgtggctgaaagtttcagccacagcaatgc-3'
fruct-1,6-bp s	5'-ctatggtatcgctggctcaaccaatgtgac-3'
fruct-1,6-bp as	5'-cacgtcccagtccaccataatgaattctcc-3'

Western blotting of protein lysates of subcellular fractions

Liver nuclei, mitochondria and microsomes were isolated by standard cell fractionation. Protein aliquots were treated in lysis buffer containing protease – and phosphatase inhibitors, and separated by gradient (4-12% Bis-Tris gel) polyacrylamide-SDS electrophoresis (SDS-PAGE) using the Invitrogen NuPAGE system (Invitrogen life technologies, Karlsruhe, Germany). Proteins were transferred by capillary electroblotting to a nitrocellulose membrane for Western blot analysis.

For **densitometric analyses** Western blots of liver proteins of *wt* and *fads2-/-* mice, were separated by PAGE andquantified using the IMAGE J2X program.

Steps in phospholipidomic analysis and characterization of the novel non mammalian eicosa-5,11,14-trienoic acid

Total lipids were extracted from liver, brain, kidney, serum, ovary, testis and muscle by homogenizing tissue in an Ultraturrax in 10 volumes of chloroform/methanol C/M 2:1 (v/v), and re-extracted using C/M 1:1 (v/v) and then C/M 1:2 (v/v) for 1 h each at 37°C under a stream of nitrogen. The combined extracts of total lipids were dissolved in C/M 2:1 (v/v), washed with 2 M KCl, and concentrated. Phospho- and sphingolipids were separated by HPTLCin solvent system C/M/2N NH_4OH 65:25:4 (v/v/v) and using 2% borate impregnated HPTLC plates (Merck, Germany) in chloroform/ethanol/triethylamine/water 60/70/70/14 (v/v/v/v). Bands were identified by primuline (2mg/dl acetone- water 5:1) and by Zinzadze reagent for phospholipids, anthrone reagent for carbohydrate-containing sphingolipids, and by charring with

50% H_2SO_4/H_2O for all lipid classes. Lipid bands visualized with primuline for further fatty acid analysis, were transferred into Sovirel tubes for extraction collected on small-fritted glass filters and eluted with C/M 2:1(v/v).

Steps in the analysis of the phospho-lipidome of liver, muscle, BAT and WAT of adult (4mo) *fads2*+/+and-/- mice are lipid extraction, HPTLC, MS/MS, transesterification or saponification and GC/MS of FAME.

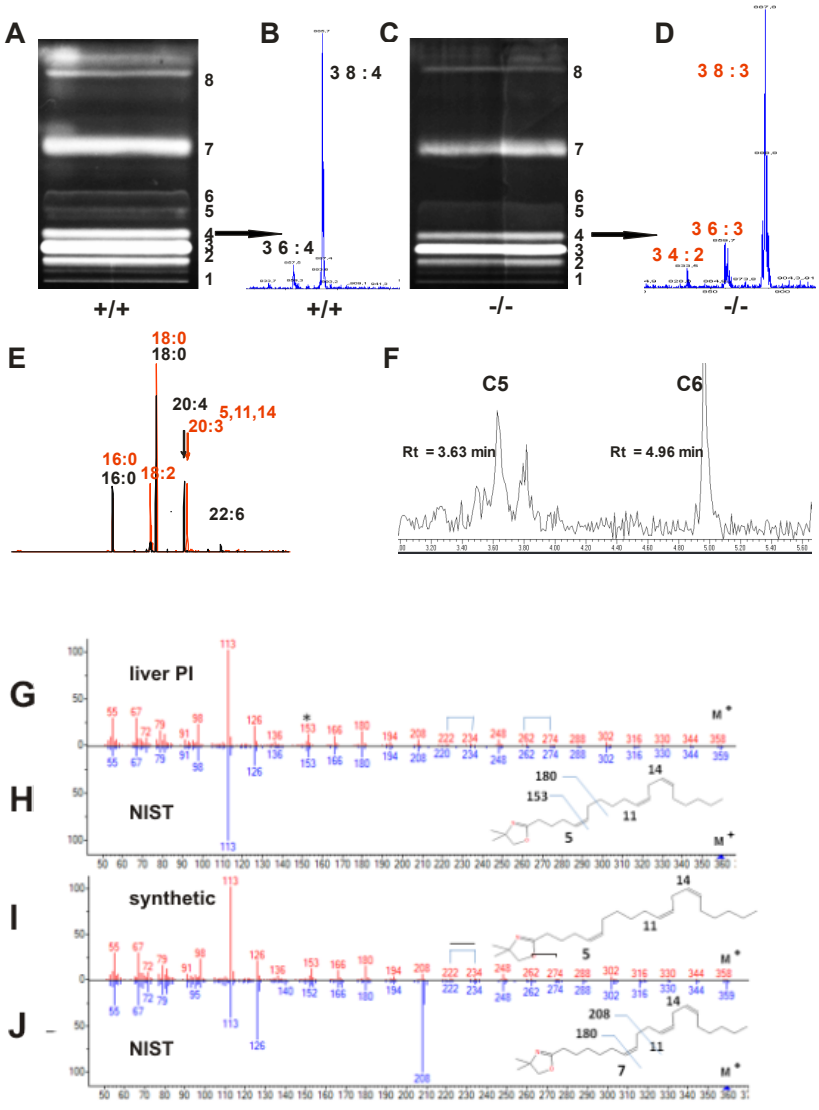


Figure S3

Paradigmatic scheme of analytical procedures of PI-species of liver of control (+/+) and *fads2-/-* liver. HPTLC separation of total lipid extract of control *fads2+/+* (**A**) and *fads2-/-* mice (**C**) into start (1), SM (2), PC (3), PI (4), PS (5), PS(6), PE (7) and cardiolipin (8). MS/MS of PI-species (band 4) of *fads2+/+* (**B**) and *fads2-/-* (**D**) liver using the CAMAG TLC-interphase to the mass spectrometer, PL bands were eluted

for transmethylation. Overlay of tracings of GC-MS-analyses of fatty acid methylesters of PI fractions of control (black tracing) and *fads2-/-* (red tracing) liver (**E**). Ozonolysis of fatty acid residues of PI of liver of *fads2-/-*mouse and GC/MS of methylesters **(F)**.Two dominant fragments were released from FAMEs of the liver PI fraction of the f*ads2-/-*mouse by oxidative ozonolysis: dimethylester of glutaric (C5) representing the carboxy-end and adipic acid (C6) the carbon chain between the Δ5 and Δ11 double bond of $20:3^{5,11,14}$. GC/MS of 2,2-dimethyloxazoline (DMOX) derivatives of liver PI as $20:3^{5,11,14}$ (**G**), of$20:3^{5,11,14}$ taken from NIST library (**H**),of synthetic $20:3^{5,11,14}$ (**I**) and of $20:3^{7,11,14}$ (NIST-library) (**J**).

Analysis of derivatives fatty acid methylesters and DMOX derivatives by combined gas-liquid-chromatography - mass spectrometry (GC-MS)

FAME were separated, identified and quantified by combined gas-liquid-chromatography - electrospray ionization mass spectrometry (ESI-MS) on an Agilent (Waldbronn, Germany) 6890/5973N instrument equipped with a HP-5MS fused silica column (length 17 m, i.d. 0.25 mm, film thickness 0.25 µm) or on a Carlo Erba Instrument Model GC8000. Samples of fatty acid methylesters and 2,2-dimethyloxazoline derivatives were injected in a 10:1 split mode into the mass spectrometer, which was operated in full scan mode over a mass range of 50-500 u, and electron ionization (EI) was utilized at 70 eV. The injector temperature was set to 300°C.

Oxidative ozonolysis

Micro-scale oxidative ozonolysis for the characterization of double bond systems of FAME of the individual phospholipid classes was developed. Fatty acid mixtures of individual phospholipid classes, separated by HPTLC and eluted with $CHCl_3/CH_3OH$ (1/1v/v) were dissolved in glacial acetic acid/methylacetate 1:2 (v/v) (1ml), cooled to -65°C and ozone bubbled through the solution for 5min. H_2O_2 (50%) (50-100µl) solution was added and the reaction mixture incubated for 24h at 35°C. Excess H_2O_2 was destroyed by traces of PtO_2. Solvents were evaporated in a stream of nitrogen and the residue esterified with 5%HCl-methanol (1ml, 60min at 80°C). Samples were taken to dryness in a stream of nitrogen and the residue dissolved in CH_2Cl_2 for GC-MS. A linear temperature program (10/min) from 60°C to 250°C was used.

Targeted substitution of DAG-structures of fads2-/- membrane PL of muscle, BAT and WAT by dietary 20:4 and 22:6 supplements.

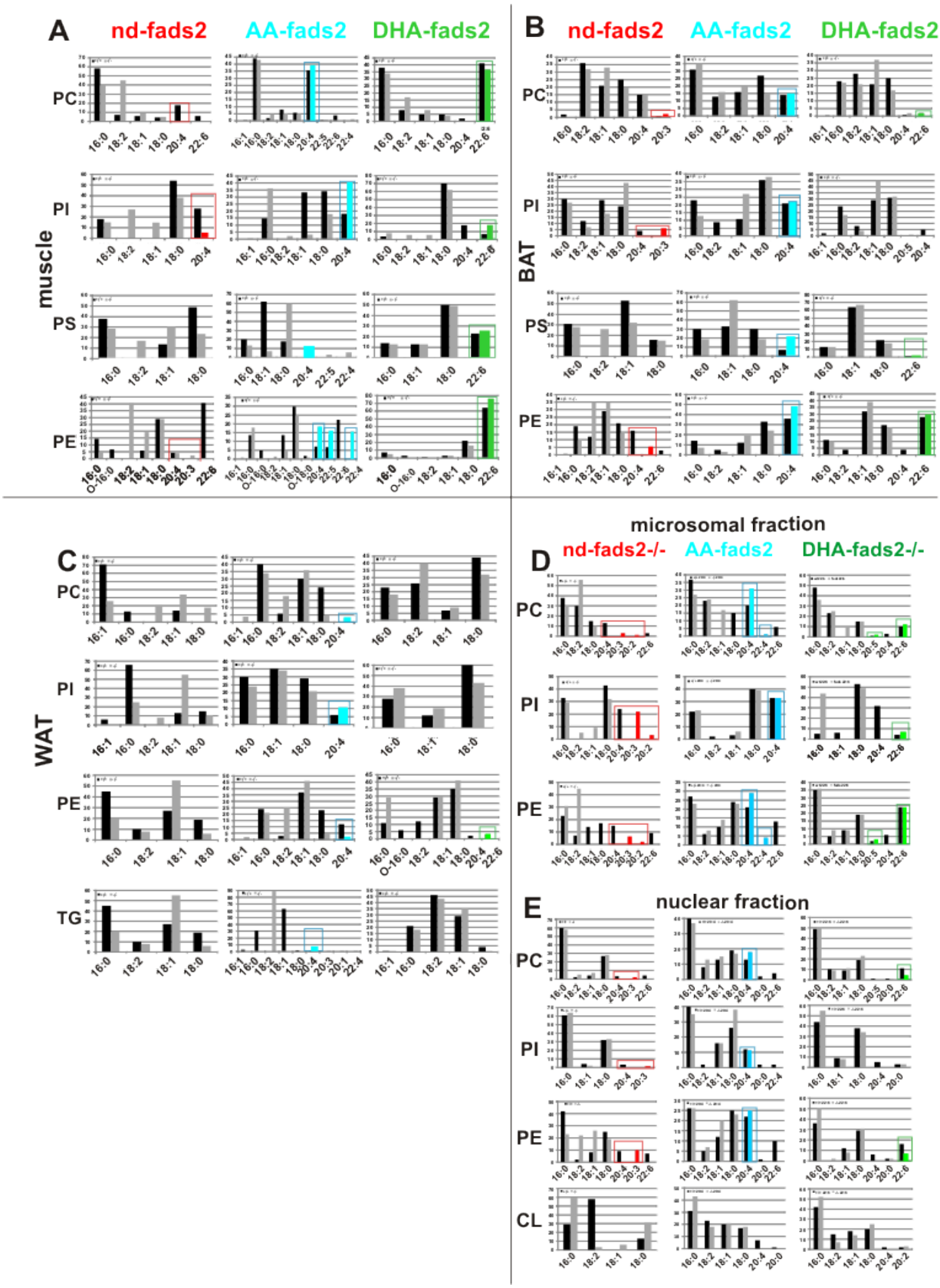
Phospholipid classes were separated by HPTLC, and after elution,species of each PL-class were analyzed by MS/MS using the Applied Biosystems (Darmstadt, Germany) QTrap analyzer. Samples were injected directly into the ion source or eluted and transferred from the HPTLC-plate using the CAMAG HPTLC- MS interface. Analytes were dissolved in methanol / 5mM ammonium acetate and injected into the ion source by means of a Hamilton syringe pump at 3µL/min. The ESI source was operated at room temperature using a spray voltage of 5500V. Nitrogen was used as the sheath and collision gas and was obtained from a Peak Scientific (Inchinnan, Scotland) MN30L nitrogen generator. Declustering and collision offset potentials were adjusted to 50V in precursor as well as product ion scan experiments. A mass range of 200-750 u and 50-700 u was recorded, respectively. Ester lipids were saponified in 0.5N KOH in methanol for 2 h at 40°C. Unsaponifiable lipids were extracted with dichloromethane, the aqueous phase was acidified with 2 N HCl and fatty acids were extracted with hexane/ether 1:1 (v/v), concentrated, and esterified with 5% HCl-methanol at 80°C for 1 h. One volume of water was added and fatty acid methylesters (FAME) extracted with hexane and concentrated under nitrogen for GC-MS and GC analysis.

Chemical synthesis of 20:3^{5,11,14}

[^{14}C]-labelled and unlabeled **20:3^{5,11,14}** have been synthesized in this laboratory by acetylenic chemistry, via the following intermediates:1,7-octadiin,1-chloro-4,10-undecadiyne,1-chloro-4,10,13-nonadecatriyne, [1-^{14}CN]- 4,10,13-nonadecatriyne, reduction with Lindlar catalyst to [1-^{14}CN]-all cis 4,10,13-nonadecatriene,[1-^{14}C]eicosa-all-cis5,11,14-trienoic acid, sa.0.25µCi/µmole [2-4]**.**

Figure S4.

Comparison of fatty acid pattern of total PLs fatty acids of PC, PI, PS, PE, cardiolipin (not shown) of muscle (**A**), BAT (**B**),WAT (**C**), liver microsomal (**D**) and nuclear membranes (**E**). Fatty acids of control *fads2+/+* black, *fads2-/-* grey and **20:3^{5,11,14}** red bars. AA blue and DHA green bars in *AA-/-* and *DHA-/-* mice. PL-classes were separated by HPTLC and PL-species of classes identified by MS/MS. Fatty acids of DAGs of individual Pl classes were identified as methylesters by GC/MS as described under figure S4.


A
nd-fads2
AA-fads2
DHA-fads2
muscle
PC
PI
PS
PE
B
BAT
C
WAT
TG
microsomal fraction
D
nd-fads2-/-
DHA-fads2-/-
nuclear fraction
E
CL

Separation and identification of phosphatidyl inositol and its phosphate esters

We followed the procedure described [5] with minor modifications. Liver or muscle tissue samples were either immediately extracted by homogenizing (ultra turrax) in 5 volumes (w/v) $CHCl_3$-CH_3OH 2:1. The sediment was suspended in 2ml water after centrifugation. The aqueous phase was alkalized by 150µl 5mM LiOH, followed by extraction with 4ml $CHCl_3$-CH_3OH. The upper phase with the acidic phospholipids was acidified with 20µl 2N HCl and extracted with $CHCl_3$-CH_3OH 2:1 (v/v) and $CHCl_3$.The lower phase was concentrated under N_2 for derivatization. Derivatization: 50µl TMS-diazomethane (2M in hexane) were added to 1ml lipid extract to give a yellow solution and incubated for 10 min at RT. The reaction was quenched with acetic acid until the color cleared, washed twice with 700µl upperphase of $CHCl_3$:CH_3OH:H_2O (2:1: 0.75 v/v/v), centrifuged. The lower phase was concentrated under N_2 and the residue dissolved in 100µl methanol for MS.

HPLC - Mass spectroscopy

90µl sample injection. Gradient: 67% to 90% acetonitrile, 0.1% formic acid in water on 1mm x 100mm Waters AcquityBEH 300 C4 1,7µl column ,over 20 min. Scanning with parent ion masses[5] PI -4,5-P2 18:0/20:4 1,117 and daughter ion 1,117 – 490 amu (for loss of derivatized PI P2 head group), 627.5347 diacyl-glycerol (DAG); 341.23 amu MH+ of permethylated PI-3,4,5-P3 1225.5729;neutral loss of exact mass 598.0383 permethylated head group of PI-3,4,5- P3, 627.5347 exact mass of DAG.

Analysis for anandamide

Tissue extracts were analyzed for anandamide using an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) interfaced via electrospray ionization to an Applied BiosystemsQTrap 2000 mass spectrometer (Darmstadt, Germany). The liquid chromatograph was equipped with a Macherey&Nagel (Duren, Germany) C-18 Pyramid column (4 x 70 mm, particle size 5 µm), and the eluents used were A: 5 mM ammonium acetate/0.1% acetic acid (pH 3.5) and B: 100% acetonitrile. A gradient was employed from 10% B increasing linearly to 100% B within 10 min. The mass spectrometer was operated in the positive mode using an electrospray voltage of 5500 V and multiple reaction monitoring (MRM). Characteristic product ions were

generated from the protonated molecule of anandamide using collision-induced dissociation (CID) yielding the ion transitions 348 to105, 348 to 91, 348 to 81, and 348 to 77, which were employed to unambiguously identify anandamides.

Histology and immunohistochemistry

Two months-old control *(+/+),(+/-)* and *(-/-) fads2* mice were perfused from the left ventricle with PBS and PBS-buffered 4% paraformaldehyde for cryo- and paraffin embedding.

Sections were either stained with hematoxylin-eosin for transmission microscopy or immunostained with affinity purified rabbit polyclonal or monoclonal antibodies recognizing Calnexin, Lamin A/C, SREBP1c specific antibodies and Cy3 conjugated anti-rabbit IgG secondary antibodies for fluorescence microscopy using the Zeiss Image M1 and for confocal images the inverted Leica TCS-SP laser scanning microscope with a 10x, 25 or 63x PLFluotar 1.32-0.6 oil immersion objective. The 488nm argon ion laser line was used forexcitation of GFP and the 568nm krypton ion laser line for excitation of the Cy3-fluorophor.

Pulse chase experiments

Comparison of arachidonic and 20:3^{5,11,14} metabolism in primary hepatocytes of *fads2*+/+ and -/- mice

Primary hepatocyte monolayers were cultured for 16 hours in fatty acid-albumin complex supplemented serum free D4-linoleic acid supplemented basal medium for 8 hours. Stock solutions:fatty acid free serum albumin was vortexed with the sodium salts of the respective unlabelled and labelled [1-^{14}C] arachidonic acid (spec.act.0.50µCi/µmole), [1-^{14}C] eicosa-5,11,14trienoic acid (spec.act.0.22µCi/µmole). Molar ratio of albumin to fatty acid 1:10 in PBS, adjusted to 0.1mM fatty acid concentration. The culture medium was 1mM in the respective fatty acid. Cells were washed twice with Ca^{2+} and Mg^{2+} free MEM containing 5% defatted serum albumin

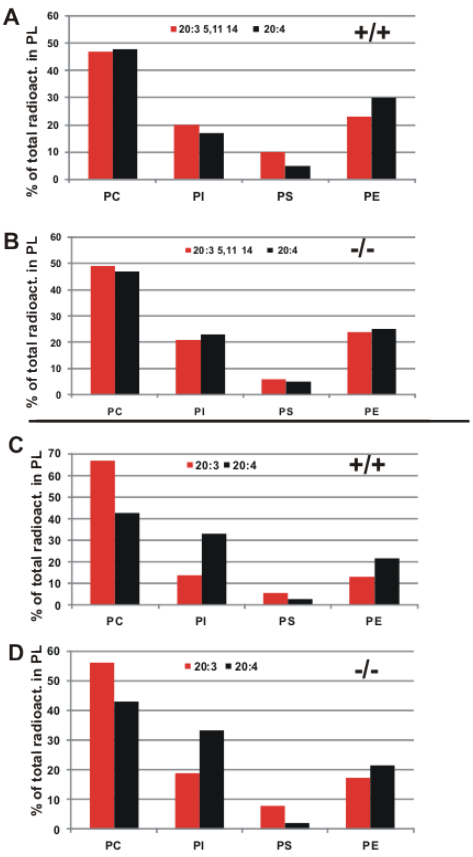


Figure S5

Comparative incorporation of [1-^{14}C] 20:3 ^{5,11,14} (red bars), and [1-^{14}C] 20:4^{5,8,11,14} (black bars) in phospholipid classes of primary hepatocytes in culture of control *fads2+/+* (**A**) and 2months old male *fads2-/-* mice (**B**) in separate culture dishes. Competitive incorporation of [1-^{14}C] 20:3^{5,11,14} (red bars) in the presence of equimolar concentrations of unlabelled 20:4^{5,8,11,14}, and of [1-^{14}C] 20:4^{5,8,11,14} (black bars) and equimolar concentrations of unlabelled 20:3^{5,11,14} in individual phospholipd classes of hepatocytes of *fads2+/+* (**C**) and *fads2-/-*mice (**D**). HPTLC separation of total lipid extracts of hepatocytes, isolation and quantification of distribution of radioactivity.

References

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