

Supplementary information to

Δ 6-desaturase (FADS2) deficiency unveils the role of ω 3-and ω 6-polyunsaturated fatty acids

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Targeting the *fads2* gene

For the construction of the targeting construct, two fragments were obtained by PCR amplification of 129/SvEv mouse genomic DNA using the forward primer 5'-GCG GCC GCT GTC AGT CAT CCC TCT GAG CTT TAA AAC-3' and the reverse primer 5'-CCC GGA TGC CTC GAG CGC TAC GGC CTC CGG-3' for the 5'-fragment, and the forward primer 5'-CTT CCG TTG GGA GGG AAT TCA GAA GCA CAA-3' and reverse primer 5'-ATG CAA ATA TAA GGG CTG GCA TTG GAA TTC-3' for the 3'-fragment. The fragments were cloned into the two respective restriction sites flanking the *Neo^r* gene in the pPNT vector. The NotI-linearized targeting vector was used for electroporation of HM mouse embryonic stem cells. G418 and gancyclovir-resistant ES cell clones were genotyped by PCR amplification of the genomic DNA of selected clones following established procedures (Bradley et al., 1984). The start codon of Δ 6-desaturase in exon I was deleted by the mutated forward primer used for the generation of the short arm 5' fragment. The NotI-linearized targeting vector was electroporated into HM mouse embryonic stem cells (129/ola). G418 and gancyclovir-resistant ES-cell clones were selected. CJ7Bl/6 blastocyst injection resulted in three chimeric males which passed the mutant *fads* allele onto their offsprings. They were crossed to homozygosity. Hetero- (+/-) and homozygous (-/-) *fads2* mutant mice were viable. For PCR genotyping of ES-cell clone genomic DNA and chimeric mice tail DNA, we used the forward primer *fads2* 5'UTRs 5'-CCTTCCTTGTTCCAGACACGGTCTCAAGAG-3' located 5' of the insert and 3' neo reverse-primer *fads2* exI 3' 5'-CGTAGCATCTTCTCCCGAATAGTGTCCGAT-3' located downstream at the 3' end of exon I.

Multitissue expression of *fads2*

Expression of FADS2 in different tissues of the *fads2*^{-/-} mouse was estimated by semiquantitative RT-PCR of multi-tissue RNA (liver, white adipose tissue (WAT), kidney, brain, spleen, muscle, heart, intestine).

Total RNA was extracted from tissues of 6-wk-old wt and *fads2*^{-/-} mutants using Trizol-reagent following the manufacturer's instructions (GIBCO/BRL). DNaseI treatment (25 units/10 µg) of total RNA removed contaminating genomic DNA. For semiquantitative analysis of expression levels, cDNA was synthesized using Maloney murine leukemia virus reverse transcriptase, random hexamer primers and dNTPs. cDNA templates were amplified by PCR with specific primers in the presence of dNTPs, [α -³²P] dCTP and Taq polymerase applying 19 cycles of 2 min at 94°C, 1 min at 93°C, 1 min at 60°C, 2.5 min at 72°C, and ended with 1 cycle for 10 min at 72°C.

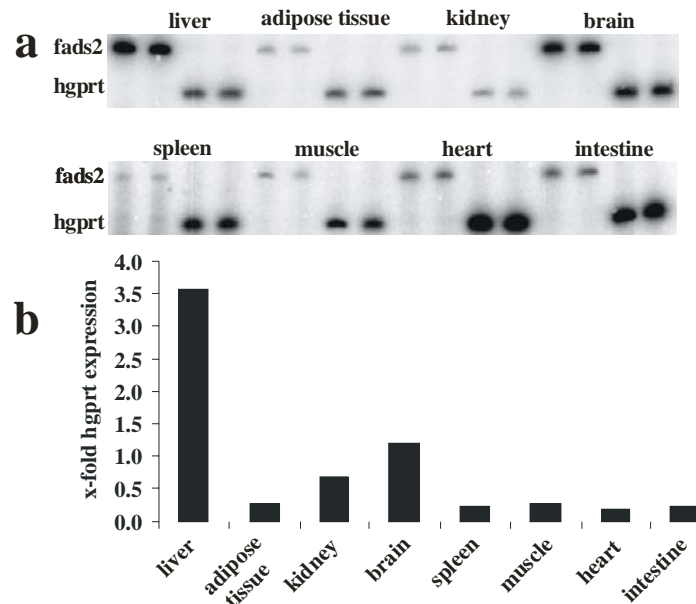


Figure SI.

Steady state levels of *fads2*/*hgprt* in organs of wt littermates. (a) Semiquantitative RT-PCR of multi-tissue RNA (liver, white adipose tissue, kidney, brain, spleen, muscle, heart and intestine) was carried out using primers d6s 5'-TATGGCAAGAAGAAGCTGAAATACCTGCCC-3' and d6as 5'-TGCTTACCTCCATAAATGAAGCTGCCGTC, which yielded a 420bp fragment. *hgprt* was used as internal marker. No signals were obtained with RNAs of organs of *fads2*^{-/-} mice (not shown).

(b) Densitometric analysis of steady state levels showing the *fads2*/*hgprt* ratios in arbitrary units.

Western blot of liver proteins of wt and *fads2*^{-/-} mice

Liver microsomes (100.000xg sediment) were isolated by cell fractionation. Microsomal proteins were separated by gradient (4-12% bis-Tris gel) polyacrylamide-SDS electrophoresis (SDS-PAGE) using the Invitrogen NuPAGE system (Invitrogen life technologies, Karlsruhe, Germany) and transferred by capillary electroblotting to a nitrocellulose membrane for immuno-detection. Western blot analysis was carried out using affinity purified rabbit anti-FADS2 antibodies. The antigenic peptide of the C-terminal sequence his329 to lys444 of mouse FADS2 were obtained as recombinant polypeptides, C-

terminally fused to GST. HPLC-purified peptide was used as antigen to raise affinity purified rabbit anti-FADS2 antibodies.

Glucose tolerance test with *wt* and *fads2*^{-/-} mice

We studied the role of the disrupted metabolism of the essential FAs and the absence of long chain PUFAs on carbohydrate and lipid metabolism in the *fads2 null* mouse. Blood glucose and glucose tolerance test of *fads2* mutants were in the range of control mice as shown in Figure S2.

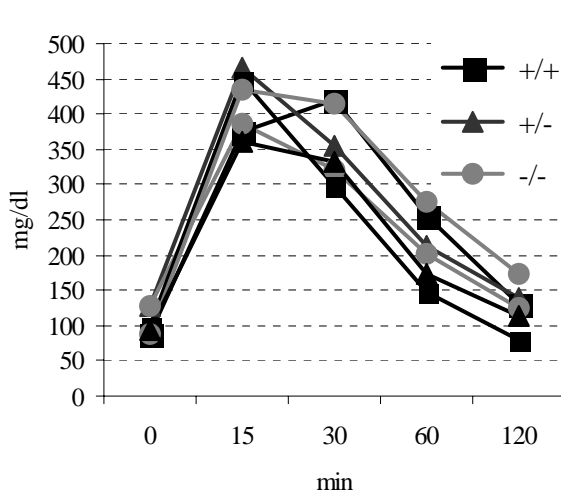


Figure S2. Glucose tolerance test of *wt* +/+, heterozygous +/- and homozygous -/- *fads2* mice (each n=10)

Serum lipoprotein analysis

Lipoproteins from 100-200 μ l serum were separated by fractionation on a Superose-6 FPLC column (HR 10/30) in phosphate buffer, pH 7.5 (0.01M Na₂HPO₄, 0.1M EDTA and 0.15M NaCl) at a flow rate of 0.5 ml/min/fractions. Fractions were analyzed for cholesterol and apolipoprotein A1 by Western blot analysis. Proteins of fractions were separated by gradient (4-12% bis-Tris gel) polyacrylamide-SDS electrophoresis (SDS-PAGE) using the Invitrogen NuPAGE system (Invitrogen life technologies, Karlsruhe, Germany).

Lipid analysis

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 HPTLC using a C/M/2N NH₄OH 65:25:4 (v/v/v) solvent system and identified by Zinzadze reagent for phospholipids, anthrone reagent for carbohydrate-containing sphingolipids, and by charring with 50% sulfuric acid/H₂O for all lipid classes. For fatty acid analysis, lipid bands were visualized with 2,7-

dichlorofluorescein (5 mM) in ethanol, collected on small-fitted glass filters and eluted with C/M 2:1(v/v). Ester lipids were saponified in 0.5 N KOH in methanol for 2 h at 40°C. Unsaponifiable lipids were extracted with dichloromethane, the aqueous phase was acidified with 2 N HCl, fatty acids were extracted with hexane/ether 1:1 (v/v), concentrated, and then esterified with 5% HCl-methanol at 80°C for 2 h under reflux. One volume of water was added and the fatty acid methylesters extracted with hexane for GC-MS and GC analysis. Esters were separated on 0.25 mm silica columns on a Carlo Erba Instrument Model GC8000 using a 15 m HP-5MS fused silica column applying a temperature gradient between 160°C to 250°C at 3°C per min.

Steady state mRNA concentrations of genes involved in the regulation of lipid metabolism were examined by semiquantitative RT-PCR. Primer pairs used have been described previously (Binczek et al., 2007). Figure S3 indicates that FADS2 deficiency has no impact on the expression of the regulatory transcription factors or enzymes of lipid metabolism listed below.

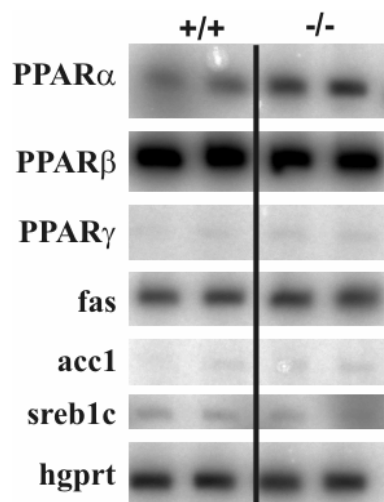


Figure S3.

Steady state mRNA concentrations of genes involved in the regulation of lipid metabolism

Fatty acid analysis

Fatty acid methylesters were separated, identified and quantified by combined gas-liquid-chromatography - electrospray ionization tandem mass spectrometry (ESI-MS/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 μ m) or on a Carlo Erba Instrument Model GC8000. Samples of TMS-derivatized fatty acid methylesters and long chain bases (2 μ l) were injected with a split ratio of 10:1 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. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed on an Applied Biosystems (Darmstadt, Germany) QTrap analyzer. Analytes were dissolved in methanol containing 5mM ammonium acetate and injected into the ion source by means of a Hamilton syringe pump at 3 μ L/min. The ESI source was operated at room temperature using a spray voltage of 5500 V. 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 50 V in precursor as well as product ion scan experiments. A mass range of 200-750 u and 50-700 u was recorded, respectively.

Histology and immunohistochemistry

Two-month-old *wt* (+/+), (+/-) and (-/-) *fads 2* 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 anti-mouse FADS2-specific antibody, anti-connexin 43, anti-ZO1, anti-JAM-A, anti- β -catenin and anti G-actin antibodies. F-actin was stained with phalloidin. Cy3 conjugated anti-rabbit IgG secondary antibodies were used for fluorescence microscopy 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 for excitation of GFP and the 568nm krypton ion laser line for excitation of the Cy3-fluorophor.

Electron Microscopy

Mice were anesthetized with Ketanest (100mg/kg) (Parke-Davis; Berlin, Germany) and 10 μ l Rompun (Bayer, Leverkusen Germany) in 250 μ l sterile 0.9% NaCl, and perfused transcardially with 2% paraformaldehyde, 2% glutaraldehyde and 0.2% picric acid in 0.1 M cacodylate buffer (pH 7.35, 22°C). The perfusion-fixed tissue (testes and ovaries) were cut into small pieces (1 mm³) and fixed for another 2 h at room temperature. Tissue specimens were post-fixed in 1% OsO₄ solution for 1 h, and stained in 1% uranyl acetate for 1 h at room temperature. After dehydration, specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultra-thin sections (about 70 nm) were stained with uranyl acetate and lead citrate and were examined by electron microscopy (Zeiss 902 A, Zeiss, Oberkochen, Germany). The semi-thin sections (1 μ m) were stained with methylene blue for light microscopy.

Quantification of eicosanoids

Thromboxane B₂, prostaglandin E₂ and leukotriene B₄ were quantified by ELISA using the Enzyme Immuno assay kit Correlate EIA Thromboxane (TXB₂), Leukotriene B₄ (LTB₄) and ProstaglandinE₂ (PGE₂) (Assay Designs, Inc. Ann Arbor, Mi, USA).

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 Biosystems QTrap 2000 mass spectrometer (Darmstadt, Germany). The liquid chromatograph was equipped with a Macherey-

Nagel (Düren, 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 – 105, 348 – 91, 348 – 81, and 348 – 77, which were employed to unambiguously identify anandamide.

Steady state levels of RNA of genes expressed at differential stages of testicular germ cell (table SI) and follicle development (table SII) were quantified using the genes and primers listed below. Results of semiquantitative RT-PCR of testis RNA are presented in figure 4.

Table SI. Primers used for semiquantitative RT-PCR of testis RNA

<i>Hlf3 s</i>	TCTTGACCATGTTCGGAAACGGCTCCTGCGG
<i>Hlf3 as</i>	GCTGCCCTGGCTTTGGCGGGGCTTTTACGT
<i>Sprm-1 s</i>	CTATGTTTGGGAAGGTTCTCAGCCAGACGA
<i>Sprm-1 as</i>	AGTTCTCTGACTTAGTGAGGGTGGGAGTGG
<i>Ccna1 s</i>	GCTGTGGTTACTAGGCAATGCATCGCCAG
<i>Ccna1 as</i>	GCTGCTTCCTCATGTAGTGAGCCTTGGGTC
<i>Cdc25c s</i>	GTAACTGGCAAGGATTTTCACCAGGACCT
<i>Cdc25c as</i>	TCTCGGAGGAGAATTCACAGAGGAACACAA
<i>Prm1 s</i>	GACCCCTGCTCACAGGTTGGCTGGCTCGA
<i>Prm1 as</i>	ATTGGCAGGTGGCATTGTTCCTTAGCAGGC

Table SII. Primers used for semiquantitative RT-PCR of ovary RNA

<i>SF1 s</i>	GGCTGCAGGGCTGCAGGGGTTTATAAGGGC
<i>SF1 as</i>	GAAAGTGTGTGAGAGAGAGTGGGCAGGAG
<i>DAZL as</i>	TCTCTGGTCTGTTTAACCCTGAGAACAGAC
<i>Podocalycin s</i>	GCAAATGGTTCTCCCCTAGCTGCAAACCCC
<i>Podocalycin as</i>	ACATTCTTGTTTCAGAAGTGGGGAGGGAGGC
<i>Selectin s</i>	TGAACGAGAAGTGTGCCATTGGCGTCACTC
<i>Selectin as</i>	TGTTCTGGGTGATCTGGCGCTTGGTTATCA
<i>Folistatin1 s</i>	CTCGGAGCCTGGTGATAAGCGACGCTCCTA
<i>Folistatin1 as</i>	GCTCATCGCTGTTAGCTTGATAGCAGACAA

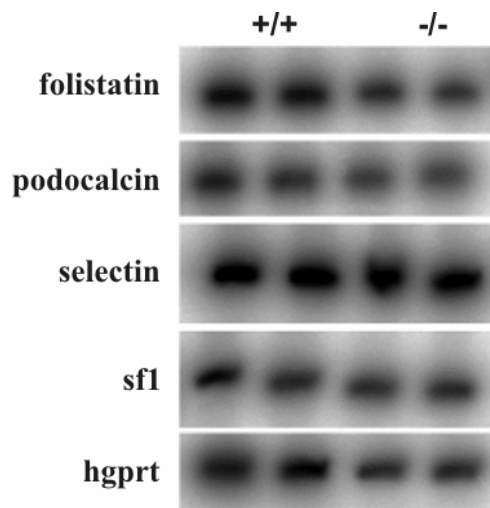


Figure S4. Steady state levels of RNA of genes expressed at differential stages of follicle development

Bleeding time and platelet aggregation assay

Bleeding time was measured by trans-section of the mouse tail of anesthetized *wt* and *fads2*^{-/-} mice at about 2mm diameter, and the bleeding time determined with the tail inserted in a glass beaker filled with saline at 37°C. The mouse was placed on a 30°C heat pad. The bleeding time was recorded for 30min.

Induced arterial thrombosis

Arterial thrombosis was induced using 5% FeCl₃ following the procedure as described previously (Farrehi et al., 1998). Thrombosis was documented in cross-sections of the common carotid artery of *wt* and *fads2*^{-/-} mutant, stained with hematoxylin-eosin.

Peritoneal macrophage stimulation assay and measurement of leukotriene synthesis in unstimulated and stimulated macrophages. Mice were injected with 0.5ml of a 3% thioglycolate in PBS intraperitoneally and sacrificed after 48h. The peritoneum cavity was opened and two peritoneum washes with 5 ml of ice-cold DMEM-FCS (10%, 10mM Hepes) were collected and plated on Petri dishes. Cultures were stimulated with 1µl of a 10µg/ml stock solution lipopolysaccharide (LPS)(SIGMA). The washes were filtered through a nylon filter into a 15 ml Falcon tube, centrifuged at 1200 rpm, at 4°C, for 5 min. Cells were resuspended in DMEM medium and the concentration of counted cells adjusted to 20x10⁶/ml. They were stimulated with 1µl of a stock of 10µg/ml lipopolysaccharide (LPS) (Sigma) for 4, 8 and 24 h and the supernatant was taken for leukotriene determination.

Feeding experiments

The following feeding regiments were carried out with cohorts (n=5) of *wt* control and *fads2*^{-/-} littermates (n=5). Mice had free access to regular laboratory chow (Altromin 1314, R/M Zuchtdiät (Ratte/Maus), which contained 10394 mg/1000 g 18:1; 23702 mg/1000 g 18:2, 3253 mg/1000 g 18:3, 546 mg/1000 g 20:4, 243 mg/1000 g 20:5. Special fat-free diet, Altromin C1056 (Altromin Gesellschaft für Tierernährung mbH, D-32791-Lage, Germany) was supplemented by daily oral application with soy phosphatidylcholine fatty acid ethylesters or cod liver oil fatty acids ethylesters. Soy phosphatidylcholine fatty acid ethylesters contained 237 mg/10 g 18:2 and 33 mg/10 g α -18:3. Cohorts of 5 male and female *fads2*^{-/-} mice each were fed a fat-free diet supplemented with 10 mg/d cod liver oil fatty acid ethylester containing 0.5 mg α -18:3, 5 mg/d 20:5 (EPA) and 5 mg/d 22:6 (DHA), another cohort (n=5) with supplemented with AA: 20:4 (AA) (10 mg/d). These diets were orally administered to pregnant mice during pregnancy and the lactation period. Feeding of *fads2*^{-/-} siblings was continued up to p50.

Table SIII. Fatty acid composition (% of total) in regular, EFA (soy fatty acids) and 20:5/22:6 supplemented diet. Polyunsaturated fatty acids are marked in bold.

FA	regular	EFA	20:5, 22:6
16:0	0.2	-	18.2
16:1	12.9	-	9.2
18:0	3.7	-	-
18:1	22.3	28.0	20.0
18:2	50.9	58.0	2.0
α -18:3	7.0	7.0	1.0
20:0	0.4	-	-
20:1	0.9	-	-
20:4	1.2	-	-
20:5	0.5	-	11.4
22:1	-	-	11.2
22:6	-	-	12.0

Table SIV. Fatty acid composition (% of total) of lipid extracts of testis (*wt*, *fads2*^{-/-}, *fads2*^{-/-} 20:4 supplemented diet) and liver (*fads2*^{-/-} 20:4). Polyunsaturated fatty acids are marked in bold.

FA	testis <i>wt</i>	testis <i>-/-</i> 20:4	liver <i>-/-</i>	liver <i>-/-</i> 20:4
14:0	1.7	3.6	4.8	2.9
16:0	28.8	31.1	23.5	27.8
18:2	24.9	20.4	39.0	5.2
18:1	-	21.9	25.2	14.2
18:0	9.7	7.9	3.9	22.8
20:4	14.5	12.9	<1	20.9
20:5	1.1	1.9	<1	5.9
22:6	14.4	0.4	<1	0.3

	testis -/- 20:5/22:6	liver -/- 20:5/22:6
14:0	1.6	0.8
16:1	6.3	6.7
16:0	35.0	28.4
18:2	7.0	3.3
18:1	31.9	27.9
18:0	6.4	8.8
20:5	1.1	8.4
22:6	8.2	13.2
22:5	2.6	2.6

Table SV. Fatty acid composition of testis lipids of *fads2*^{-/-} mice (2 mo) fed with a cod liver oil (20:5/22:6) supplemented diet (fatty acid composition see table SIII)

A

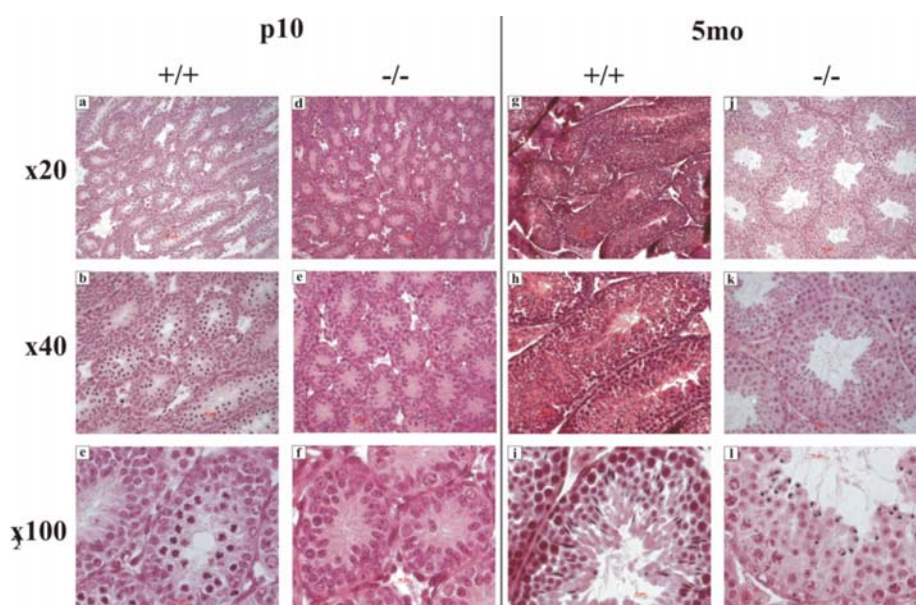
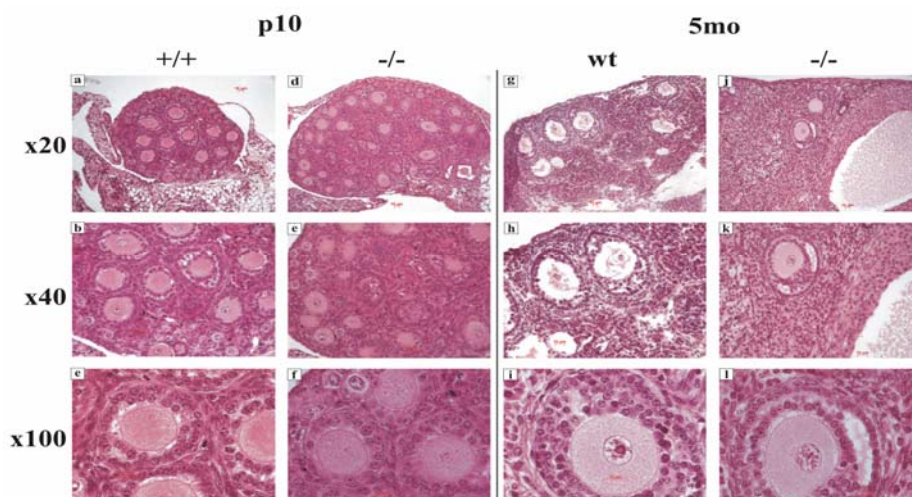


Figure S5.

Light-microscopy of sections of A) testis and B) ovary at p10 and 5 mo displayed at low (x20), medium (x40) and high magnification (x100).

B



Sertoli cell development in mature *fads2*^{-/-} mice is normal.

To assess the developmental state of SCs in adult (*fads2*^{-/-}) males we studied the expression of three stable markers of mature Sertoli cells, Sox9, the androgen receptor (AR) and the transcription factor WT-1 (Wilms tumor-1). SOX9 plays a central role in the differentiation of Sertoli cells. SOX9 is translocated during male sexual differentiation from the cytosol to its nuclear localization in adult testis. Androgen receptor (AR) is expressed in mature SCs. AR and is a sensitive indicator of the maturation state of SC, (Morais da Silva et al., 1996; Vidal et al., 2001) for review see (Sharpe et al., 2003). Transcription factor WT-1 (Wilms tumor-1) is expressed in early fetal life and then continuously in mature SCs. WT-1 is a stable SC marker. Wnt-1 is a negative regulator of β -catenin signalling in testicular cord maintenance. Sox9 is an essential sex differentiation factor which is expressed in the cytoplasm of somatic cells of both sexes. It is translocated during male sexual differentiation into the nucleus.

The similar nuclear location of AR and WT1 in Sertoli cells in adult wt and *fads2* null indicates that they have reached the mature state.

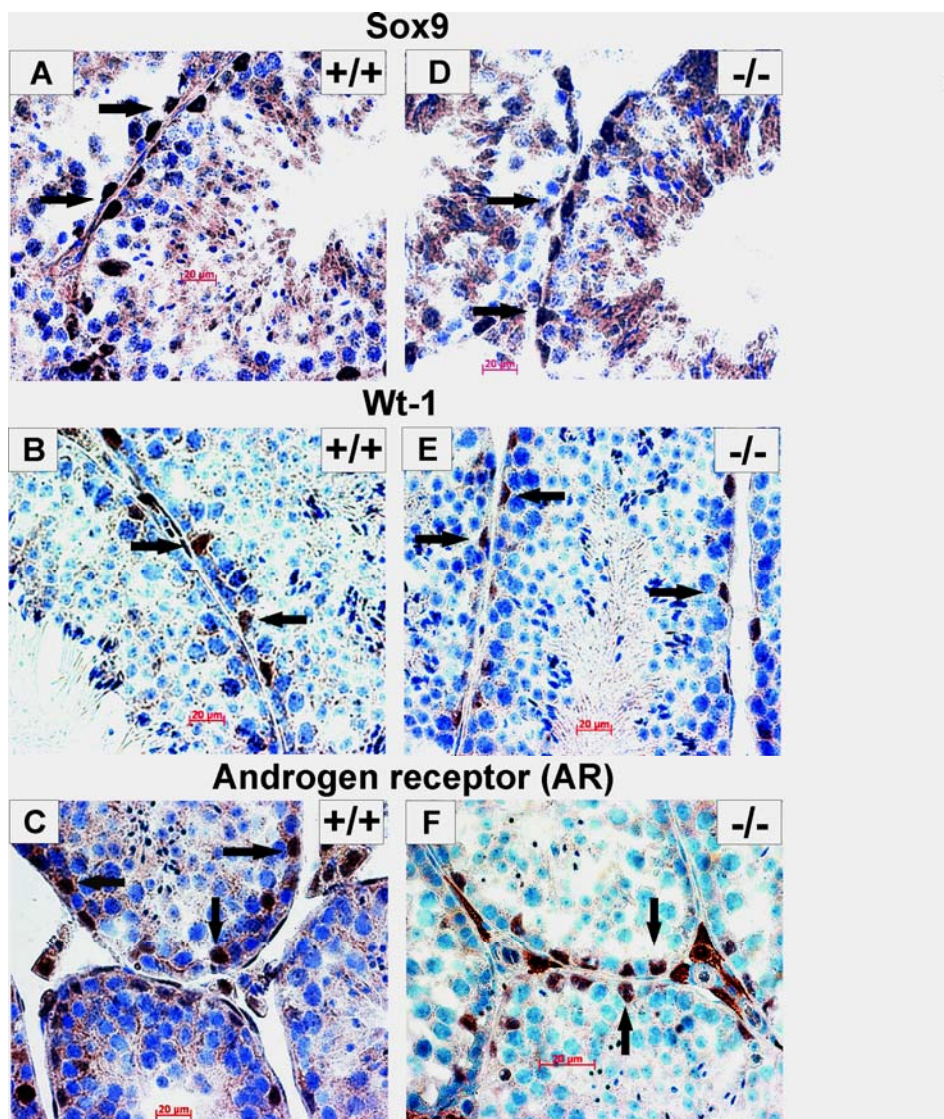


Figure S6. Sox9-, AR and Wt-1 expression in mature (age 4months) wt (A, C and E) and *fads2*^{-/-} Sertoli cells (B, D and F). Cryo sections were counterstained with heme alaun. Arrows highlight the nuclear staining of Sertoli cells with anti Sox9-, AR- and anti Wt1- antibodies.

Cell polarity of enterocytes and of the epithelial cells of the lining of trachea

Enterocytes were double immunofluorescently stained with anti occludin (Fig. S7A,B) and claudin 11 (Fig. S7C, D) antibodies. The merged images (Fig. S7E, F) indicate the colocalization of these tight junction specific marker proteins in the plasma membrane of the highly polarized enterocytes of *wt* (Fig. S7G) and *fads2 null* mice (Fig. S7H) (2months of age). Podocin antibodies visualize the ciliated apical domain of epithelial cells of tracheae of *wt* and *fads2*^{-/-} mice.

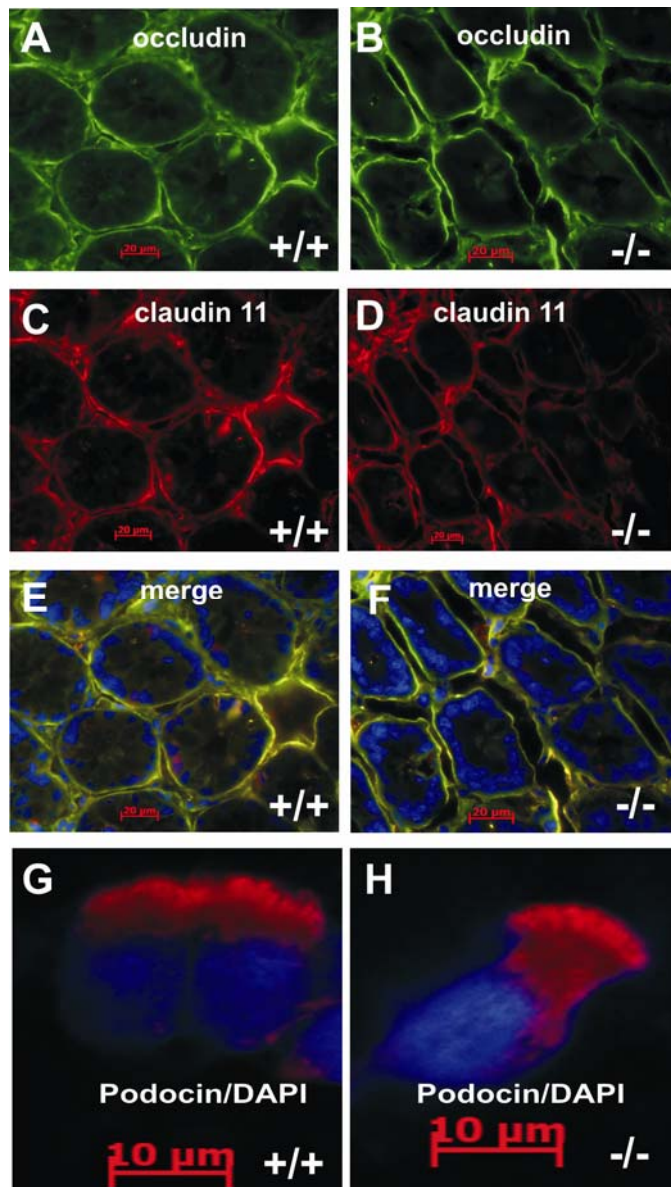


Figure S7. Confocal images of *wt* +/+ (A, C, E) and *fads2*^{-/-} (B, D, F) enterocytes of jejunum, stained with TL specific anti occludin, anti claudin 11 and merged images. Sections 5 μm thickness. Isolated epithelium cells of *wt* (G) and *fads2*^{-/-} (H) trachea stained with anti podocin (red) using a Cy2 conjugated second antibody.

Lipid polarity in Sertoli cell membrane is disturbed

The disruption of SC polarity in *fads2* null testis is further supported by fluorescence studies using filipin, a high affinity ligand of cholesterol in cell membranes. In polarized cells such as SCs, cholesterol, sphingomyelin and glycosphingolipids segregate into the apical cell membrane. When

cryosection of wt and *fads2*^{-/-} testes were treated with filipin, the adluminal domain of wt seminiferous tubuli became strongly fluorescently labelled, (Figure S8A), whereas in *fads2*^{-/-} testis, the filipin-cholesterol complex was distributed throughout the basolateral and apical compartment of SCs, (Figure S8B).

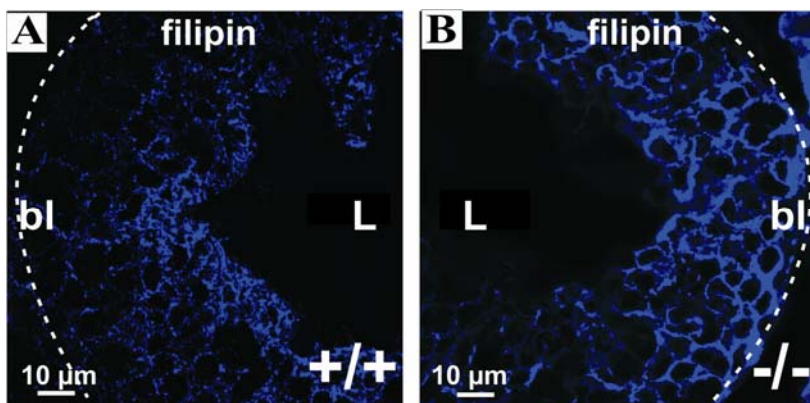


Figure S8. Cholesterol localization in the plasma membrane of *wt* (A) and *fads2*^{-/-} (B) SCs, visualized by the fluorescent ligand filipin:cholesterol rich apical domains of SCs in *wt* seminiferous tubuli, redistribution of cholesterol into the basolateral and apical compartment of SCs of *fads2*^{-/-} males.

The *fads2* null mutant is an auxotrophic mutant

We asked whether the *fads2* null mouse is an auxotrophic mutant, in which the genetic defect with its complex phenotype can be overcome by the dietary supply of AA and/or EPA and DHA. The regular diet of *wt* and *fads2* null mice provided the daily requirement of EFAs linoleic, α -linolenic acid, AA and EPA (Table SIII). However, this diet was not sufficient to prevent or reverse the *fads2* null phenotype. We therefore orally fed cohorts of pregnant heterozygous *fads2*^{+/-} females (n=10 each) previously mated with *fads2*^{+/-} either ω 6-20:4 (AA) or ω 3-20:5/22:6 (cod liver fatty acids: EPA/DHA) daily and their homozygous F1 male and female offspring until adulthood.

Crossing these homozygous males and females with fertile *fads2*^{+/-} males and females yielded *fads2*^{-/-} and *fads2*^{+/-} offsprings with a Mendelian distribution as shown by genotyping of tail DNA by PCR analysis. Figure S9A presents paradigmatically the genotyping results of two mating experiments with *fads2*^{-/-} mice fed on a ω 3-20:5/22:6-rich diet. The supply of ω 6-20:4 as well as ω 3-20:5/22:6 *ab ovo* rescued a normal spermatogenesis in homozygous *fads2* males and a regular follicle development in the complete rescue of male and female fertility and fecundity, although litters from 10 crossings of homozygous males and females, maintained on the 20:4-supplemented diet, were smaller (3-6 littermates). Light microscopy of hematoxylin-eosin stained cross-sections of wt testes (Fig. S9B, C) and of PUFA-fed *fads2* null mice (Figure S9D, E) showed normal spermatogenesis. Like wt

epididymis (Figure S9F, G) also the epididymal tubular system of homozygous *fads2* males (Fig. S9H, I) revealed mature spermatozoa in the lumen of the seminiferous tubuli.

Normal development of ovarian follicles was rescued in homozygous *fads2*^{-/-} females by the PUFDA substituted diet, Figure S9 J-M.

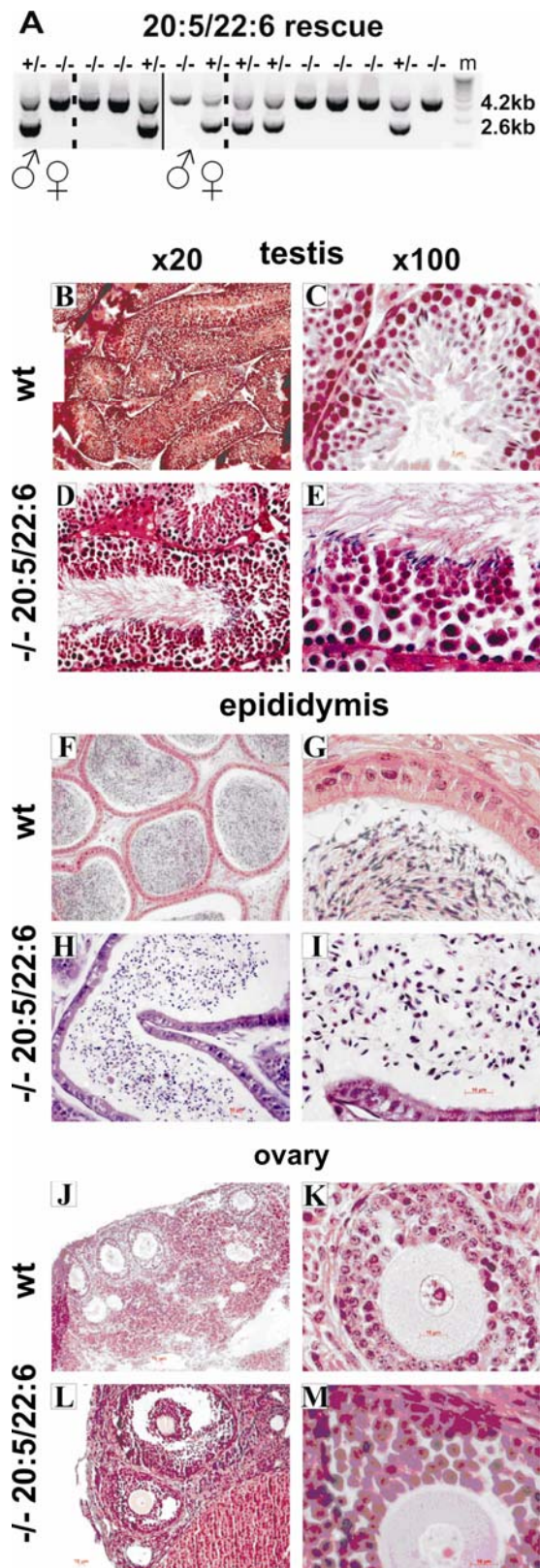


Figure S9. *Fads2* homozygosity is an auxotrophic mutation. *Fads*^{+/-} females and *fads*^{+/-} males (n=10 each) were crossed and administered orally a daily diet supplemented with ω6-20:4 (AA) or ω3-20:5/22:6 (cod liver oil: EPA/DHA) during pregnancy, lactation period and after weaning. Homozygous F1-female offsprings were fed the supplemented diet to fertility. These females were crossed with fertile *fads*^{+/-} males and showed rescue of fertility. (A) Genotyping of offsprings of crossing of *fads2*^{-/-} females and males, fed a 20:5/22:6-supplemented diet, with their heterozygous counterpart by PCR of tail genomic DNA. Histology of paraffin-embedded sections of (B, C) *wt* and (D, E) rescued *fads2 null* testis. Histology of paraffin-embedded sections of epididymis. (F, G) *wt* and (H, I) rescued *fads2 null* epididymis. Rescue of normal folliculogenesis in *fads2*^{-/-} female mice. Histology of paraffin-embedded sections of (J, K) *wt* ovary and (L, M) a *fads2 null* rescued foster mother. Magnification x20 and x100.

20:4 supplemented diet rescued platelet aggregation and FeCl₃ induced thromboembolism in carotid artery in *fads2*^{-/-} mice.

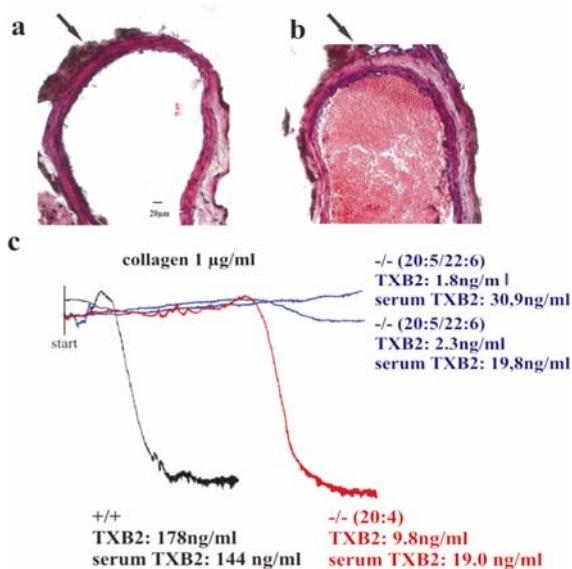


Figure S10. Induction of thromboembolism by FeCl₃ in carotid artery of (a) *fads2*^{-/-} and (b) *fads2*^{-/-} (supplemented 20:4) (c) Platelet aggregation assay with plasma of wt (+/+) (black), 20:4 (red) and 20:5/22:6 (blue) supplemented *fads2*^{-/-} mice.

	%
14:0	2.0
16:1	6.6
16:0	19.0
18:2	1.7
18:1	28.6
18:0	7.3
20:5	5.6
20:3	2.7
20:1	3.3
22:6	8.9
22:5	3.7
22:4	4.3
24:5	6.4

Table SVI. Fatty acid composition of testis lipids of *fads2*^{-/-} mice (2 mo) fed with a cod liver oil (20:5/22:6) supplemented diet (fatty acid composition see table SIII).

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