Supplementary Material

Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways

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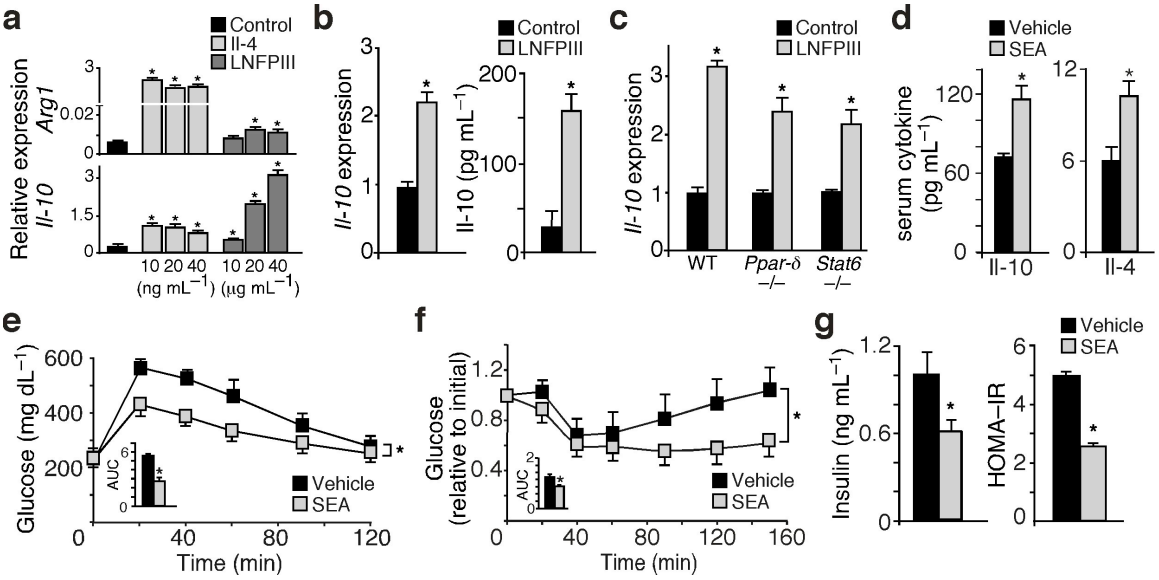
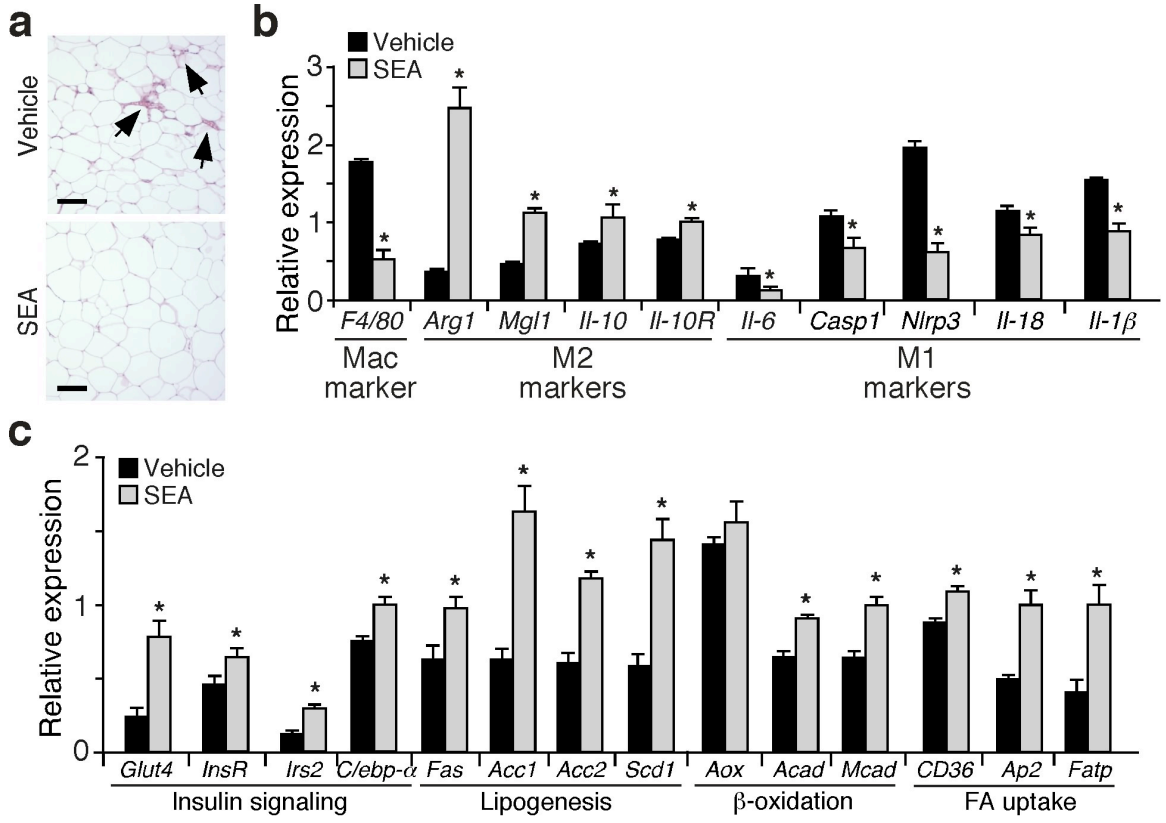
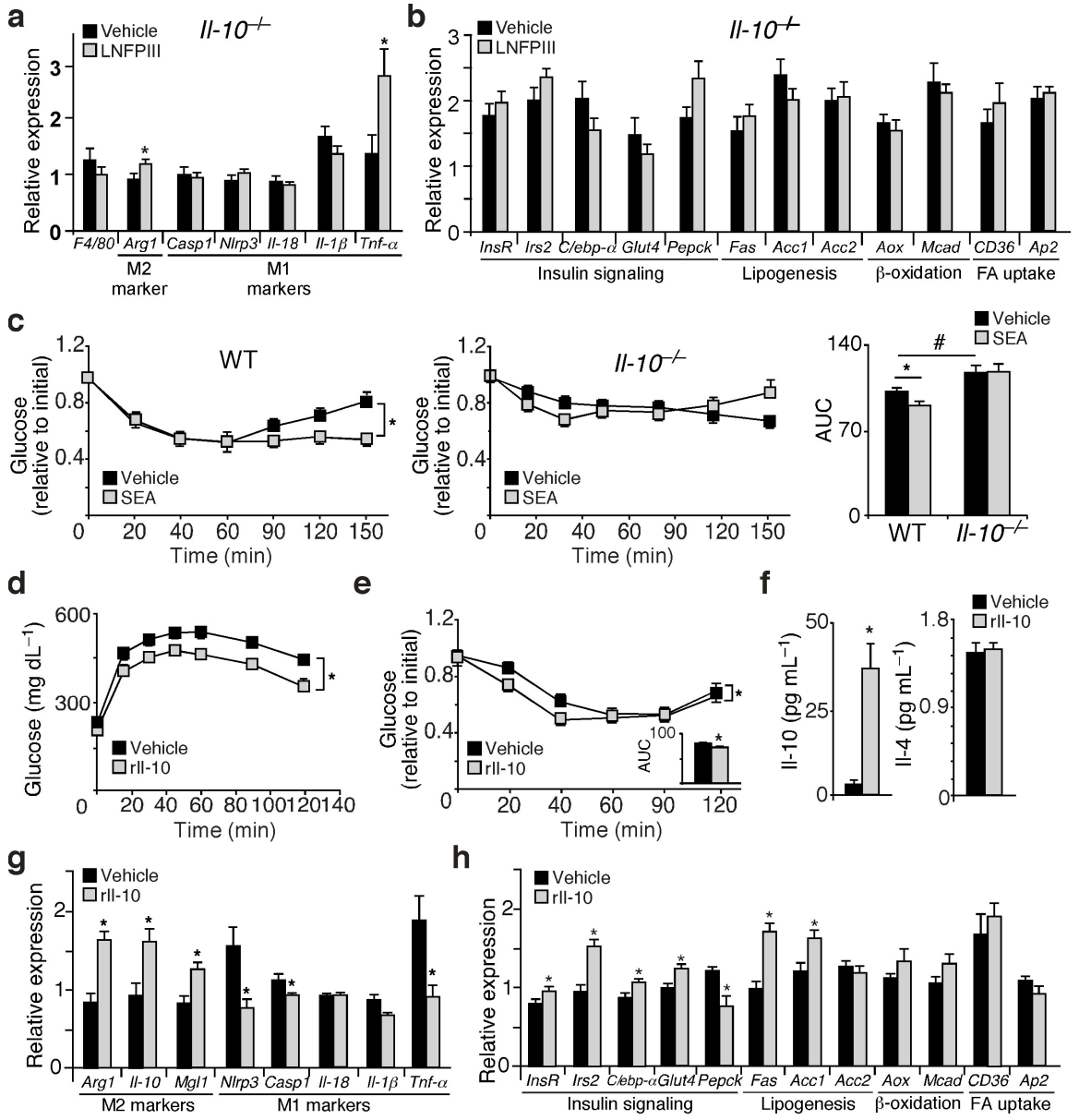


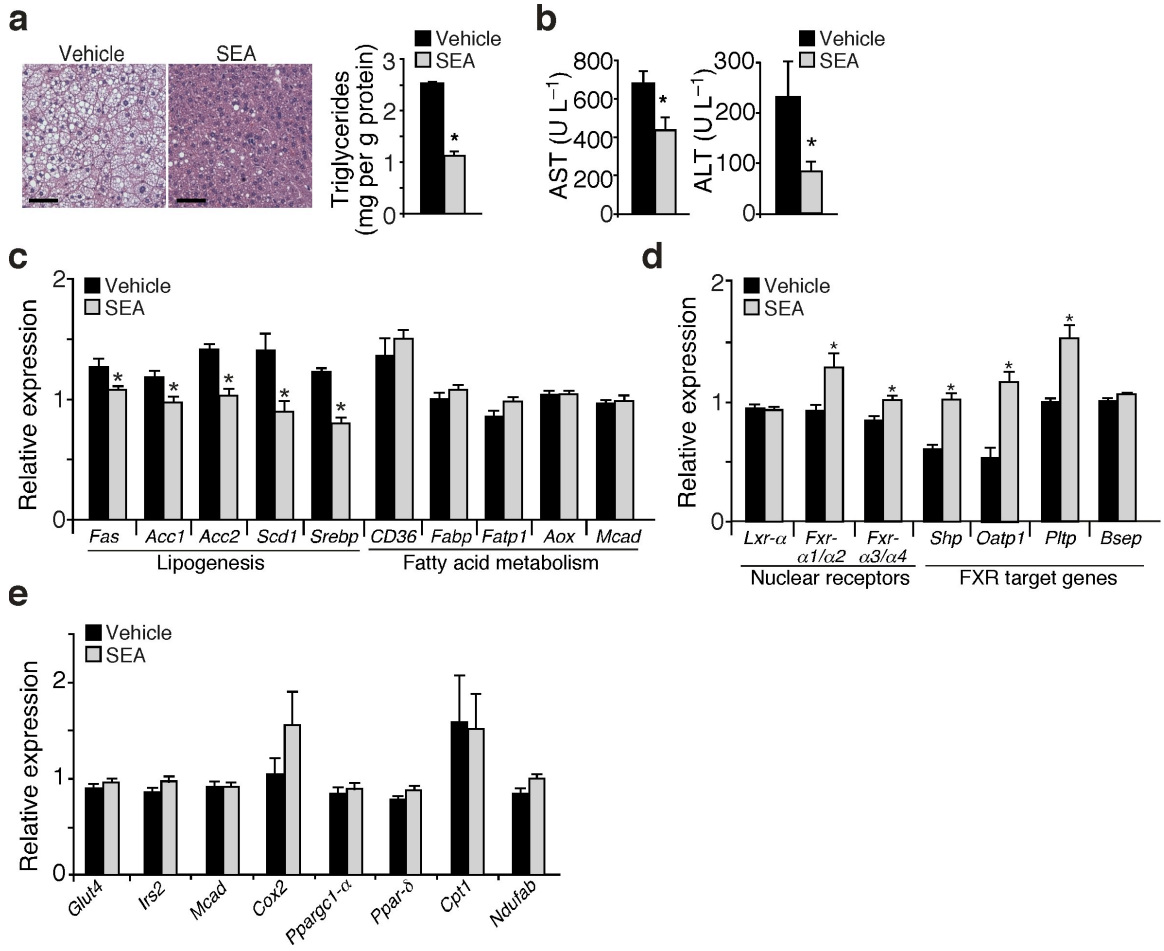
Supplementary Figure 1 Induction of Il-10 by LNFPIII is independent of Th2 cytokines. (**a**) Real-time q-PCR analyses examining the expression of *Il-10* and *Arg1* in bone marrow derived macrophages treated with 10, 20 or 40 ng mL^{-1} of Il-4 or 10, 20 or 40 μg mL^{-1} of LNFPIII. (**b**) The expression (left panel) and production (right panel) of Il-10 by LNFPIII (20 μg mL^{-1}) in dendritic cells. (**c**) *Il-10* expression determined by real-time q-PCR in wild-type (WT), *Ppar-δ*$^{-/-}$ and *Stat6*$^{-/-}$ macrophages given 20 μg mL^{-1} LNFPIII overnight. (**d**) Serum Il-10 and Il-4 concentrations in vehicle or SEA treated mice (n = 4–6 per treatment). (**e**) Glucose tolerance test and (**f**) insulin tolerance test in high fat fed mice (n = 4–6) treated with vehicle or SEA (25 μg, twice a week). Inset: area under the curve (AUC). (**g**) Fasting insulin concentrations and HOMA-IR to assess insulin sensitivity in vehicle and SEA treated mice. Values are expressed as means ± SEM. For *in vitro* assays, the mean and SEM were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. *In vivo* studies were reproduced in three mouse cohorts (n = 4–6 per treatment). *P < 0.05 (SEA versus vehicle control).



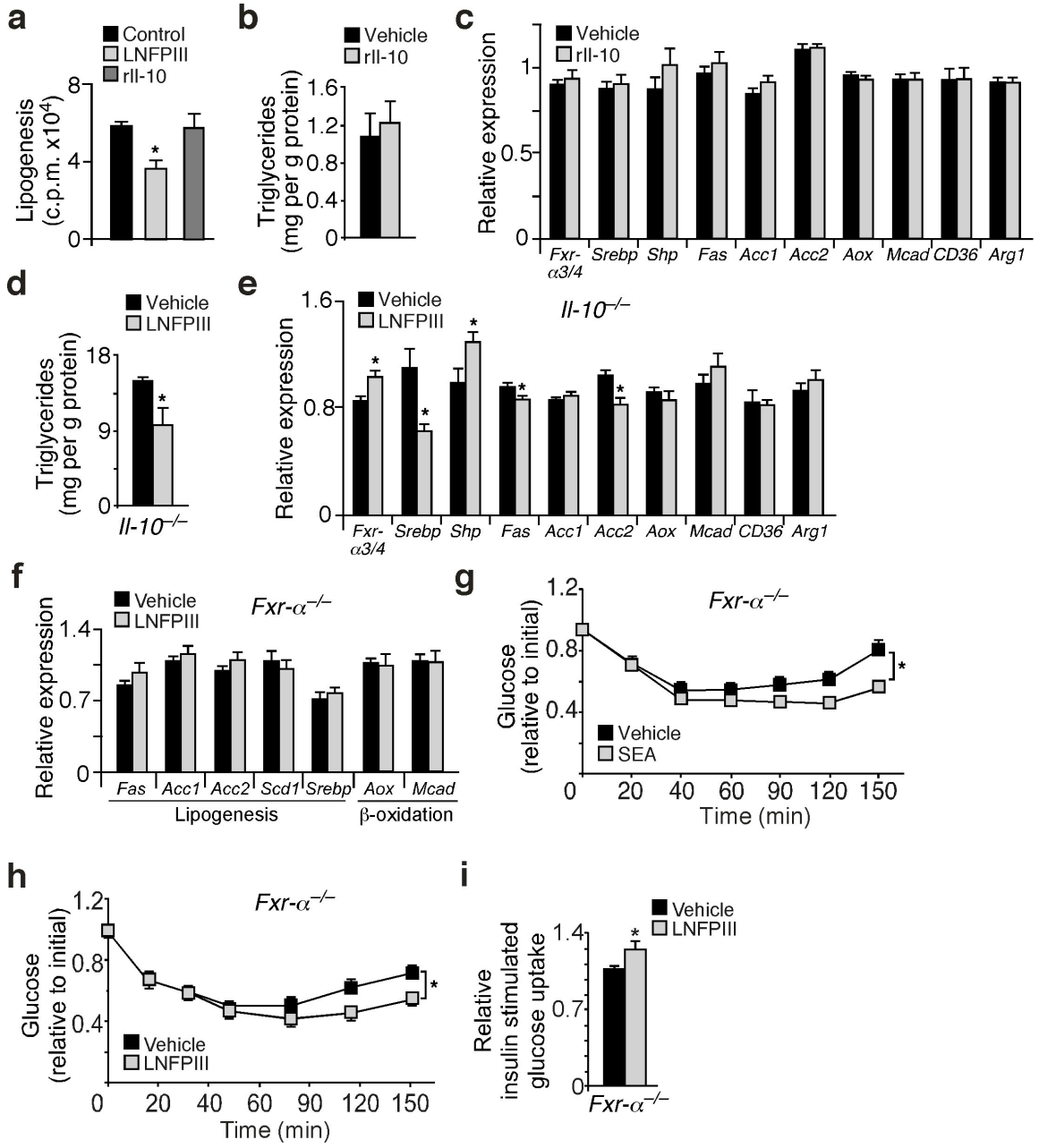
Supplementary Figure 2 SEA reduces inflammation and improves metabolic homeostasis in WAT. (**a**) WAT histology showing crown-like structures (indicated with arrows). Scale bar = 200 μm. (**b**) Real-time q-PCR analyses examining the expression of M1 and M2 genes in WAT from vehicle or SEA treated mice (n = 5 per treatment). (**c**) WAT metabolic gene expression. Values are expressed as means ± SEM. Metabolic studies were reproduced in three mouse cohorts (n = 4–6 per treatment). Histology and expression analyses were examined in one and two of the three cohorts, respectively. *P < 0.05 (SEA versus vehicle control).



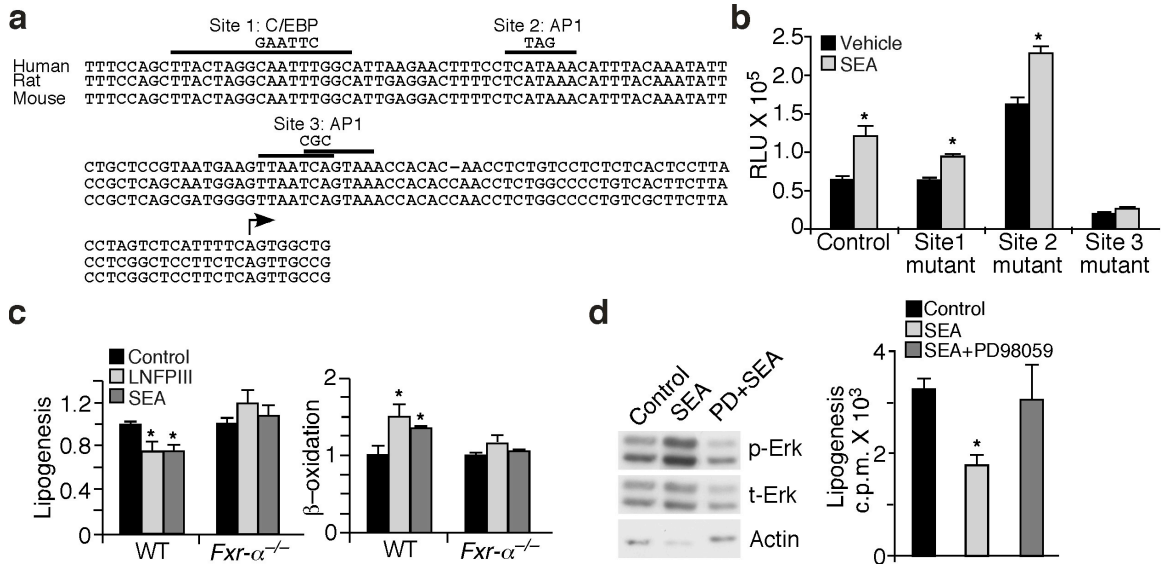
Supplementary Figure 3 The insulin sensitizing activity of LNFPIII and SEA is mediated by Il-10. (**a** and **b**) The expression of inflammatory and metabolic genes in WAT of *Il-10*$^{-/-}$ mice treated with vehicle or LNFPIII (n = 6 per treatment). (**c**) Insulin tolerance test in wild-type (WT, left panel) and *Il-10*$^{-/-}$ mice (middle panel) treated with vehicle or SEA (n = 6 per treatment). Right panel: area under the curve of ITT. (**d**) Glucose tolerance and (**e**) insulin tolerance test in high fat fed male C57BL/6J mice given PBS (vehicle) or rIl-10 (1 μg, every other day, 3 doses, n = 6 per treatment). (**f**) Serum Il-10 and Il-4 concentrations in vehicle or rIl-10 treated mice. (**g** and **h**) The expression of inflammatory and metabolic genes in WAT of mice treated with vehicle or rIl-10 (n = 6 per treatment). Values are expressed as means ± SEM. Studies using *Il-10*$^{-/-}$ and control mice (n = 6) and in rIl-10 treatment (n = 6) were conducted in 1 cohort. *$P < 0.05$ (treatment versus vehicle control); #$P < 0.05$ (*Il-10*$^{-/-}$ versus wt).



Supplementary Figure 4 SEA protects against hepatic steatosis and suppresses lipogenic gene expression. (**a**) Liver histology and triglyceride content analyses to determine hepatic fat accumulation in vehicle and SEA treated mice. Scale bar = 100 μm. (**b**) Circulating AST and ALT concentrations to assess liver function. (**c** and **d**) Expression analyses of metabolic genes and nuclear receptor signaling pathways known to regulate lipogenesis in livers from vehicle or SEA treated mice (n = 5) by real-time q-PCR. (**e**) Gene expression in muscle from vehicle or SEA treated mice determined by real-time q-PCR. Values are expressed as means ± SEM. Metabolic studies were reproduced in 3 mouse cohorts (n = 4–6 per treatment). Histology was examined in one and lipid and expression analyses were examined in two of the three cohorts. *$P < 0.05$ (SEA versus vehicle control).



Supplementary Figure 5 LNFPIII, but not Il-10, suppresses de novo lipogenesis in the liver. (**a**) *Ex vivo* lipogenesis assays in primary hepatocytes treated with vehicle, LNFPIII (20 μg ml^{-1}) or rIl-10 (10 ng ml^{-1}) for 24 hr. (**b** and **c**) Triglyceride content and lipogenic gene expression in the liver from vehicle or rIl-10 treated mice (n = 6). (**d** and **e**) Triglyceride content and lipogenic gene expression in the liver of *Il-10*$^{-/-}$ mice treated with vehicle or LNFPIII (n = 6) (**f**) Gene expression analyses in livers of *Fxr-α*$^{-/-}$ mice treated with vehicle or LNFPIII (n = 6) by real-time q-PCR. (**g** and **h**) Insulin tolerance tests in *Fxr-α*$^{-/-}$ mice (n = 6) treated with vehicle, SEA or LNFPIII. (**i**) *Ex vivo* insulin stimulated glucose uptake in adipose tissue slices. Values are expressed as means ± SEM. For *in vitro* assays, the mean and SEM were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. Studies using rIl-10 (n = 6), *Il-10*$^{-/-}$ (n = 6), *Fxr-α*$^{-/-}$ (n = 6) and corresponding controls were performed in 1 mouse cohort. *P < 0.05 (treatment versus control).



Supplementary Figure 6 *Fxr-α* is a molecular target of LNFPIII and SEA. (**a**) Sequence comparison of the 5' proximal regulatory region of human, rat and mouse *FXR-α* downstream promoter (promoter 2). The transcriptional start site is indicated with an arrow. Putative binding sites for C/EBP (site 1) and AP1 (site 2 and site 3) are highlighted. Sequences mutated for the reporter assays are indicated above the highlighted binding sites. (**b**) Reporter assays showing that the induction of human *FXR-α* promoter 2 is mediated by AP1 binding sequences in site 3. Similar results were obtained with LNFPIII treatment (data not shown). (**c**) Lipogenic and fatty acid oxidation assays conducted in hepatocytes isolated from wild type (WT) or *Fxr-α*$^{-/-}$ mice treated with vehicle, LNFPIII (20 μg ml^{-1}) or SEA (2 μg ml^{-1}). (**d**) Left panel: Western blot analyses showing Erk phosphorylation in hepatocytes ± SEA ± PD98059. p-Erk: phospho-Erk; t-Erk: total Erk. Actin was included as a loading control. Right panel: *de novo* lipogenesis assays in hepatocytes. Values are expressed as means ± SEM. Mean and SEM were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. *P < 0.05 (SEA or LNFPIII versus vehicle control).

Supplementary Table 1. Metabolic parameters of mouse cohorts used in the study. Serum samples were collected after 6 h fasting. *$P < 0.05$.

a. WT mice treated with SEA and LNFPIII

Treatment	Vehicle	SEA	Vehicle	LNFPIII
Body weight (g)	43.63 ± 1.74	41.62 ± 1.36	45.26 ± 0.94	46.86 ± 1.09
WAT/body weight	0.044 ± 0.0044	0.068 ± 0.002*	0.031 ± 0.015	0.039 ± 0.001
Liver/body weight	0.042 ± 0.004	0.041 ± 0.003	0.038 ± 0.002	0.043 ± 0.002
Triglyceride (mg dL^{-1})	102.38 ± 3.89	130.78 ± 7.52	67.17 ± 2.01	69.79 ± 1.18
Free fatty acid (mmol L^{-1})	1.06 ± 0.05	1.14 ± 0.04	1.08 ± 0.08	1.02 ± 0.03
Cholesterol (mg dL^{-1})	165.90 ± 8.59	166.78 ± 12.35	154.72 ± 14.06	144.24 ± 15.99
Glucose (mg dL^{-1})	221.67 ± 15.17	221.17 ± 8.53	236.2 ± 18.45	217 ± 12.01
Insulin (ng mL^{-1})	1.002 ± 0.156	0.615 ± 0.081*	1.545 ± 0.065	1.288 ± 0.059*
Adiponectin (μg mL^{-1})	5.92 ± 0.16	6.35 ± 0.13	5.59 ± 0.32	5.84 ± 0.10

b. *Il-10*$^{-/-}$ mice treated with LNFPIII

Treatment	Vehicle	LNFPIII
Body weight (g)	33.67 ± 3.84	37.03 ± 4.14
WAT/body weight	0.019 ± 0.004	0.028 ± 0.005
Liver/body weight	0.038 ± 0.002	0.032 ± 0.003
Triglyceride (mg dL^{-1})	29.11 ± 1.11	36.14 ± 3.61
Free fatty acid (mmol L^{-1})	0.49 ± 0.05	0.54 ± 0.08
Cholesterol (mg dL^{-1})	136.36 ± 10.87	153.89 ± 19.95
Glucose (mg dL^{-1})	132.17 ± 17.36	142.5 ± 16.42
Insulin (ng mL^{-1})	0.260 ± 0.033	0.259 ± 0.048

c. WT mice treated with rIl-10

Treatment	Vehicle	rIl-10
Body weight (g)	31.93 ± 1.75	32.67 ± 1.03
WAT/body weight	0.025 ± 0.001	0.032 ± 0.001*
Liver/body weight	0.036 ± 0.001	0.035 ± 0.001
Triglyceride (mg dL^{-1})	76.69 ± 5.04	84.83 ± 4.2
Free fatty acid (mmol L^{-1})	0.57 ± 0.04	0.61 ± 0.06
Cholesterol (mg dL^{-1})	106 ± 3.80	104.21 ± 4.31
Glucose (mg dL^{-1})	226.5 ± 11.93	228 ± 8.47

d. *Fxr-*$\alpha^{-/-}$ mice treated with LNFPIII

Treatment	Vehicle	LNFPIII
Body weight (g)	40.73 ± 3.23	36.33 ± 3.54
WAT/body weight	0.029 ± 0.003	0.041 ± 0.003
Liver/body weight	0.046 ± 0.004	0.053 ± 0.011
Triglyceride (mg dL^{-1})	46.12 ± 5.22	44.11 ± 4.90
Free fatty acid (mmol L^{-1})	0.40 ± 0.02	0.49 ± 0.03
Cholesterol (mg dL^{-1})	303.18 ± 27.10	315.47 ± 8.16
Glucose (mg dL^{-1})	175 ± 4.38	150 ± 18.78
Insulin (ng mL^{-1})	0.450 ± 0.025	0.198 ± 0.026*