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Supplementary Information for

Orphan Nuclear Receptor ERRgamma Regulates Hepatic FGF23 Production in Acute Kidney Injury.

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Materials and Methods

Cell culture, transient transfection and Luciferase assay

AML12 (mouse immortalized hepatocytes), HepG2 (human hepatoma cells), Huh7 (hepatocellular carcinoma cells) and 293T (human embryonic kidney cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained as described previously (1). Transient transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Luciferase assay carried out as described previously (1). Briefly, cells were transfected with indicated reporter plasmids together with expression vectors encoding ERRγ or treated with IL-6. Total cDNA used for each transfection was adjusted to 1 mg/well by adding an appropriate amount of empty vector and pCMV–β-gal plasmid was used as an internal control. The luciferase activity was normalized to b-galactosidase activity.

Isolation of primary hepatocytes

Primary hepatocytes were isolated from C57BL/6J mice by collagenase perfusion (2) and seeded with Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics in a humidified atmosphere containing 5% CO2 at 37°C. After attachment, cells are infected with adenovirus (Ad-GFP, Ad-ERRγ or Ad-shERRγ) or treated with IL-6 for the indicated time period.

Chemical and Antibodies

Recombinant human IL-6 is obtained from Prospec Protein Specialists (Cat # cyt-213), and GSK5182 was synthesized as previously described (3-6). Antibodies used in this study were as follows: mouse monoclonal anti-ERRγ (Perseus Proteomics, Clone # H6812, Cat # PP-H6812-00), rabbit polyclonal anti-alpha tubulin (AbFRONTIER, Cat # LF-PA0146) and rat monoclonal anti-FGF23 (R&D Systems, Clone # 283511, Cat # MAB2629) for western blot. For immunohistochemical staining of endogenous ERRγ, rabbit anti-ERRγ serum was generated

using a peptide (404-AGQHMEDPRRAGKMLM-419) from mouse ERRγ helix 9 (7) (AbFrontier/Young in Frontier, Seoul, Korea). Antibody was affinity purified using the same peptide and tested by Western blotting. No cross reactivity to ERRα or ERRβ was noted (8). Rat monoclonal anti-FGF23 (R&D systems, Clone # 283507, Cat # MAB26291) was used for immunohistochemical staining of FGF23. Rat monoclonal anti-IL6 antibody (BioXCell, Clone # MP5-20F3, Cat # BE0046) was used for IL-6 neutralization in mice, and rat IgG1 antibody as isotype control.

Clinical Trial of Patients Undergoing Cardiac Surgery

Details of the study cohort have been described previously (9). The study included patients undergoing elective cardiac surgery at the Saarland University Medical Centre (Homburg, Germany) recruited between 2010 and 2011. Patients younger than 18 years, patients with hemodynamic instability, emergency cardiac surgery or those who were unable to give written consent were excluded. AKI was defined according to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines as an increase in serum creatinine of at least 0.3 mg/dL within 48 h or an increase in serum creatinine at least 1.5 times higher than baseline within 7 days, or a urine volume of less than 0.5 ml/kg/h for 6 h (10). Patients with chronic kidney disease (CKD) and requiring renal therapy were excluded. Blood samples for the measurement of IL-6 and FGF23 were collected before surgery. Creatinine measurements were calibrated to the gold standard, that is isotope dilution mass spectrometry. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-Epidemiology Collaboration equation. C-terminal FGF23 levels were measured as described previously from plasma samples by enzyme-linked immunosorbent assay (lowest cutoff value of 3 relative units (RU)/ml, highest cutoff value of 2,000 RU/ml; Immunotopics International, San Clemente, CA, USA) (11). The results are reported as relative units, whereby 1 RU/ml equates to 2 pg/ml according to the manufacturer's guidelines.

IL-6 measurement

The interleukin-6 ECLIA assay (elecsys IL-6 05109442, Roche Diagnostics, Mannheim,

Germany) was performed on human plasma samples (n = 709) using the Cobas e411 analyzer (Roche Diagnostics) according to the manufacturer's instructions. IL-6 concentrations were calculated according to a stored calibration curve of optical densities with a measurement range of 1.5 - 5000 pg/ml and results were reported as pg/ml. The assay is highly specific for IL-6 with no relevant cross-reactivity for IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-8 and TNF-alpha. The coefficient of variance for imprecision of this assay is <6% for concentrations within the reference interval (<7 pg/ml) and <2.6% for samples for concentrations above the reference interval.

Statistical Analysis

In the clinical study, continuous variables are presented as means ± SD when normally distributed or as medians (IQR) for variables with skewed distribution. Categorical data are presented as percentages. Statistical differences between continuous or categorical variables were established using One-way ANOVA, Kruskal-Wallis test, or $\chi 2$ test where appropriate. For subsequent analyses, IL-6 was divided in tertiles. To visualize the association between IL-6 and FGF23 a violin plot was used. Median and quartiles are marked with lines. Correlation between IL-6, hsCRP, and FGF23 was assessed by calculating the Spearman-Rho correlation coefficient. To assess the association between tertiles of IL-6 and risk of AKI logistic regression analyses were performed including multivariate adjusted models for age, sex, body mass index, systolic blood pressure, eGFR, diabetes, smoking, CRP. To assess the interaction between IL-6 and FGF23 an interaction term between both parameters was included. A two-sided p-value <0.05 was considered statistically significant. Analyses were performed using SPSS version 21.0.

Supplementary Figures

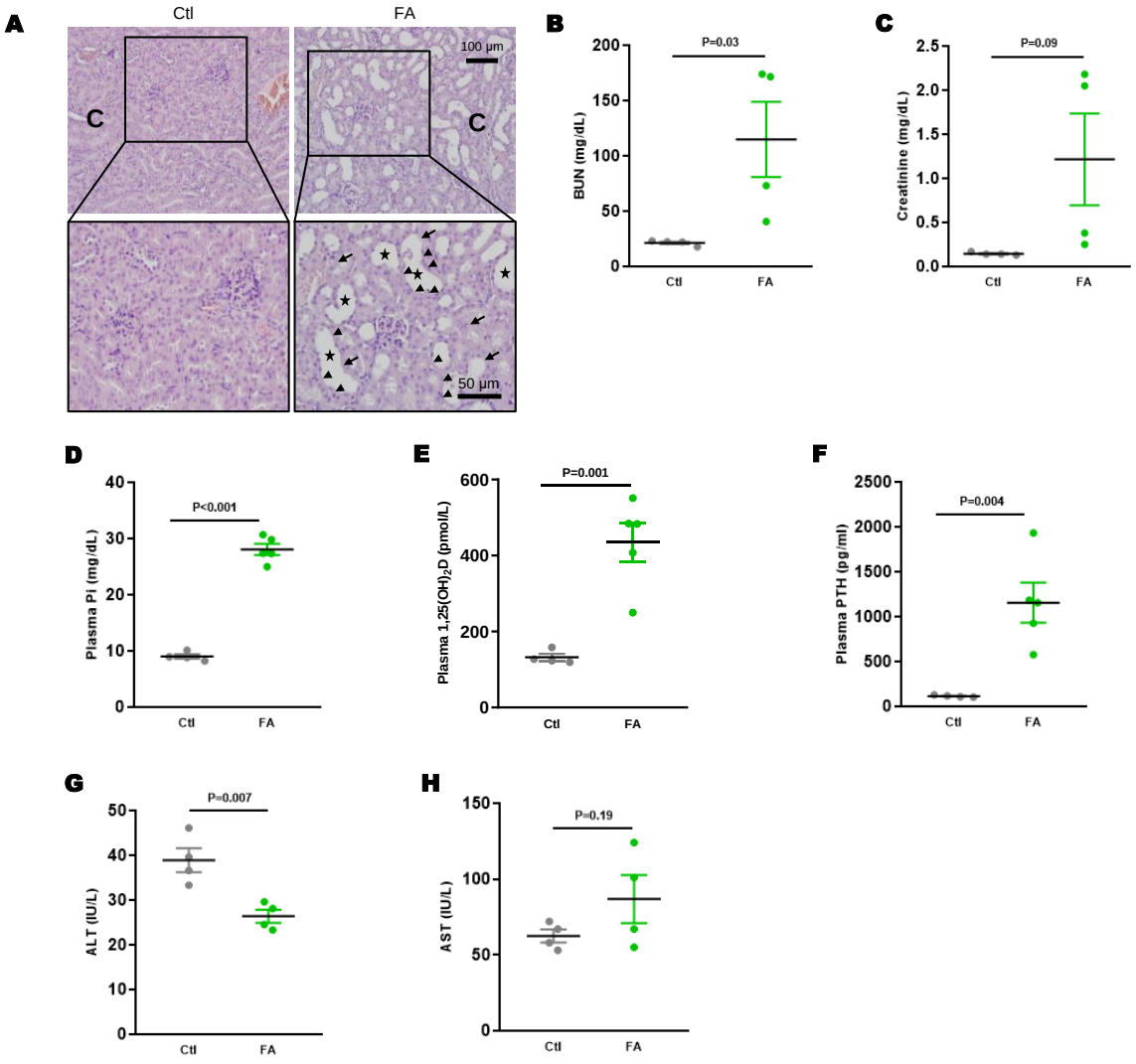

Fig. S1. Kidney H&E staining and plasma biochemistry of FA-AKI mice. C57BL/6J mice were intraperitoneally injected with a high dose of FA (240 mg/kg dissolved in vehicle: 0.15 M of NaHCO3 with pH 7.4) and sacrificed after 24 h (control n = 4 and FA n=5 mice). (A) Representative images of hematoxylin and eosin (H&E) staining in control and FA treated kidney sections with zoomed-in images. C: cortex, asterisk (tubular dilation), arrow head (flattening of tubule), and arrow (loss of tubular cells). Plasma levels of (B) blood urea nitrogen (BUN), (C) creatinine, (D) phosphate, (E) 1,25$(OH)_2$D (F) PTH and (G, H) liver injury markers in control and FA treated mice.

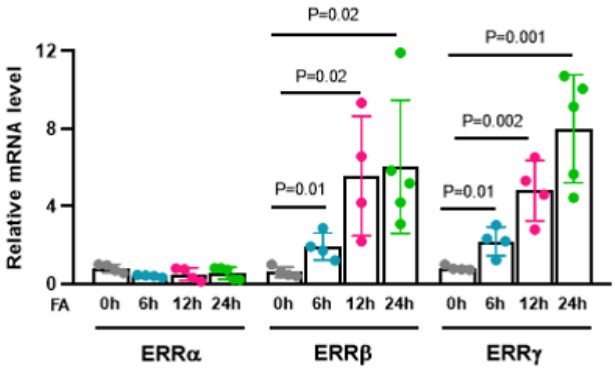

Fig. S2. Liver mRNA analysis of FA-AKI mice. C57BL/6J mice were intraperitoneally injected with a high dose of FA (240 mg/kg dissolved in vehicle - 0.15 M of NaHCO3 with pH 7.4) and sacrificed at the indicated time points (0 h, 6 h, 12 h n = 4 and 24 h n = 5 per group). Q-PCR analysis of total RNA from control and FA treated mouse livers. Data represent mean ± SEM. All data were analysed by two-tailed Student's t test.

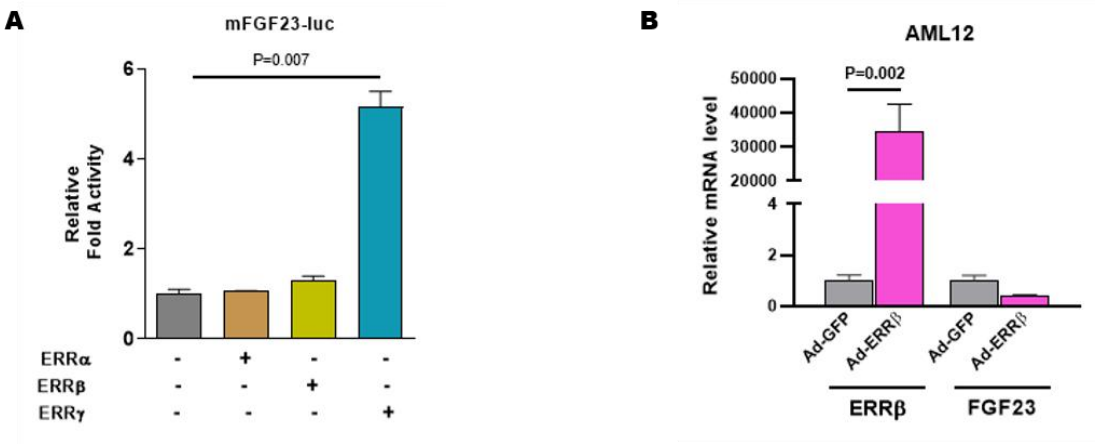

Fig. S3. ERRγ but not ERRα or ERRβ induces FGF23 promoter activation and effect of ERRβ on induction of FGF23 mRNA level. (A) Activation of the FGF23 gene promoter by ERRs. 293T cells were transfected with the wild type FGF23 promoter, along with ERRα, ERRβ or ERRγ expression vectors. Luciferase activity is presented. (B) Q-PCR analysis of total RNA from AML12 cells infected with Ad-GFP or Ad-ERRβ. All cell culture experiments were performed as three independent replicates. Data represent mean $\pm$ SEM. All data were analysed by two-tailed Student's t test.

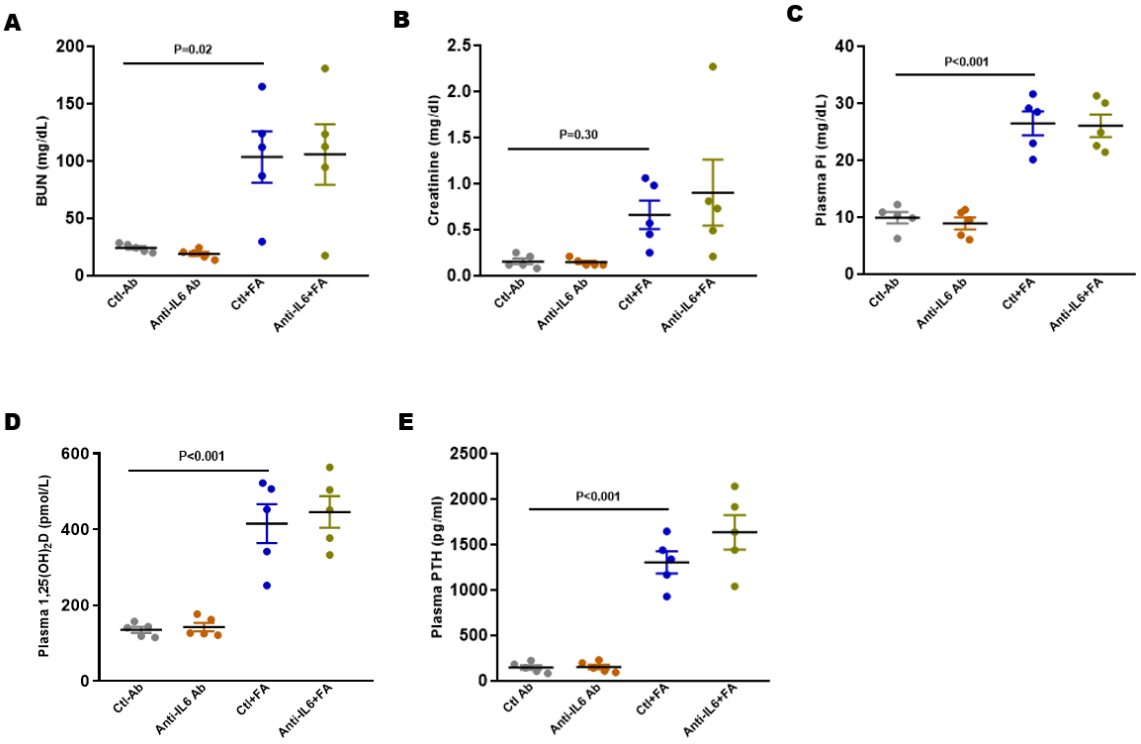

Fig. S4. Plasma biochemistry of IL-6 neutralized mice treated with FA. Plasma levels of (A) blood urea nitrogen (BUN), (B) creatinine, (C) phosphate (D) $1,25(OH)_2D$ and (E) PTH in C57BL/6J mice intraperitoneally injected with control antibody (rat IGg1) or IL-6 neutralizing (anti-IL-6) antibody (200µg/200µl/mouse) in presence or absence of FA treatment for 24h (n = 5 per group). Data represent mean $\pm$ SEM. All data were analysed by one-way ANOVA with Tukey's multiple comparison test.

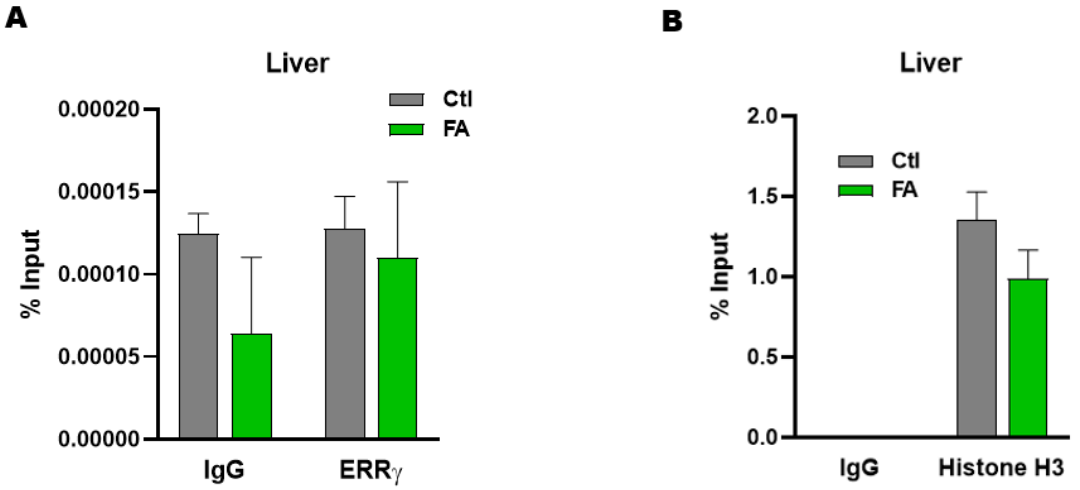

Fig. S5. ChIP analysis of FA treated mouse liver. (A, B) Soluble chromatin from FA-treated mouse livers were immunoprecipitated with (A) mouse IgG or ERRγ antibody and (B) rabbit IgG or Histone H3 (D2B12) antibody. Q-PCR analysis of purified DNA with primers corresponding to (A) ERRE non-binding region of mouse FGF23 gene promoter and (B) intron 2 of mouse RPL30 gene. All cell culture experiments were performed as three independent replicates. Data represent mean ± SEM. All data were analysed by two-tailed Student's t test.

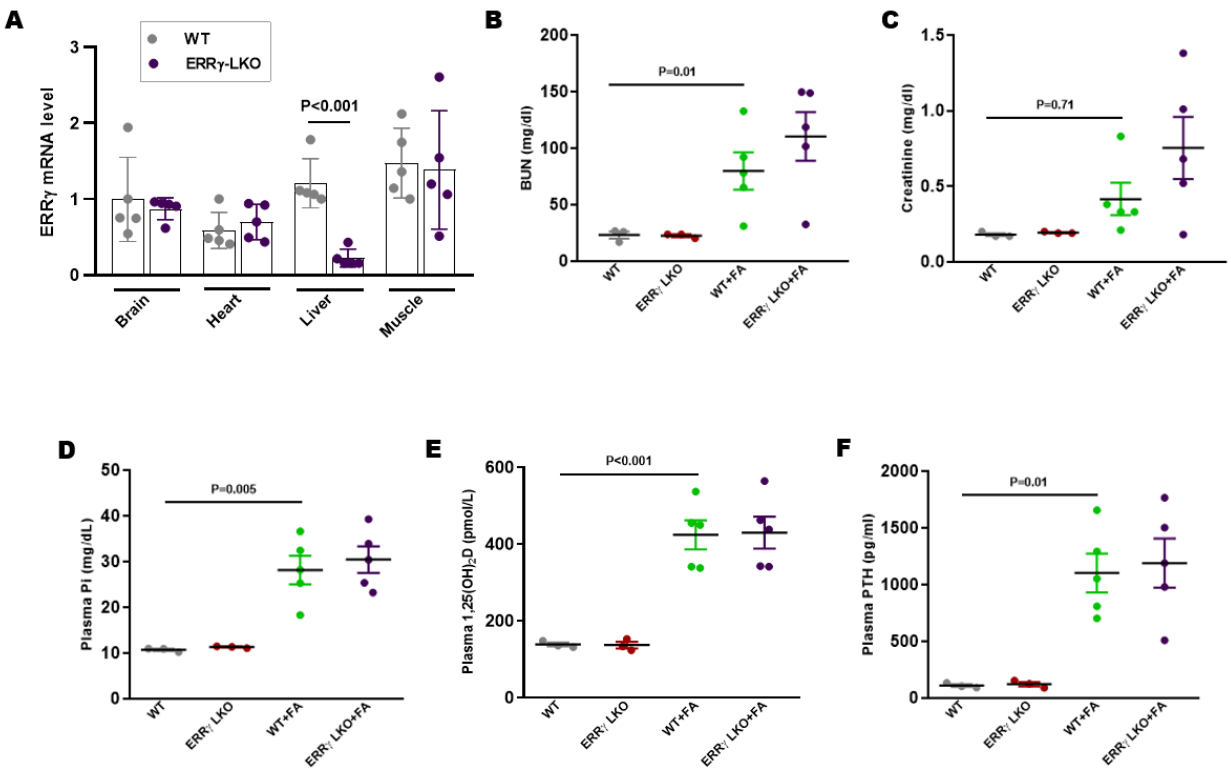

Fig. S6. Liver specific knock-out of ERRγ gene expression and plasma biochemistry of WT or ERRγ-LKO mice treated with FA. (A) Q-PCR analysis of total RNAs isolated from different tissues of wild type or ERRγ-LKO mice (n = 5 per group). Plasma levels of (B) blood urea nitrogen (BUN), (C) creatinine, (D) phosphate, (E) $1,25(OH)_2D$ and (F) PTH in WT or ERRγ-LKO mice treated with FA (240 mg/kg) (WT and ERRγ-LKO n = 3 per group ; WT+FA and ERRγ-LKO+FA n = 5 per group). Data represent mean ± SEM. Data in A was analysed by two-tailed Student's t test. Data in B-F were analysed by one-way ANOVA with Tukey's multiple comparison test.

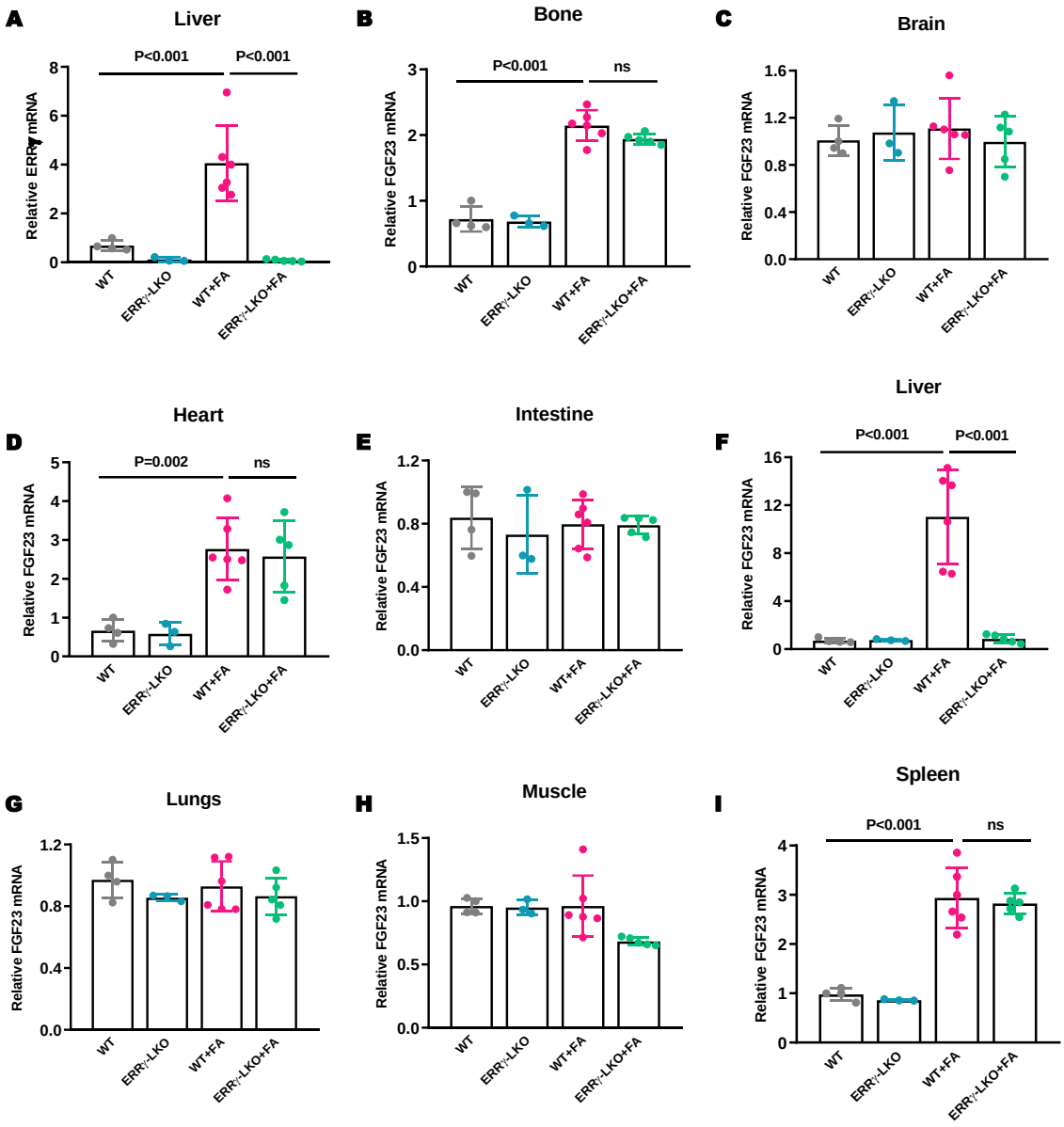

Fig. S7. Q-PCR analysis of total RNAs isolated from different tissues of FA treated WT and ERRγ-LKO mice. (A) ERRγ and (B-I) FGF23 mRNA expressions were measured from different tissues (bone, brain, heart, intestine, liver, lungs, muscle and spleen) of WT and ERRγ-LKO mice intraperitoneally injected with a high dose of FA and sacrificed after 24 h (WT n=4; ERRγ-LKO n=3; WT+FA n=6 per group and ERRγ-LKO+FA n=5 mice). Data represent mean + SEM. All data were analysed by one-way ANOVA with Tukey's multiple comparison test.

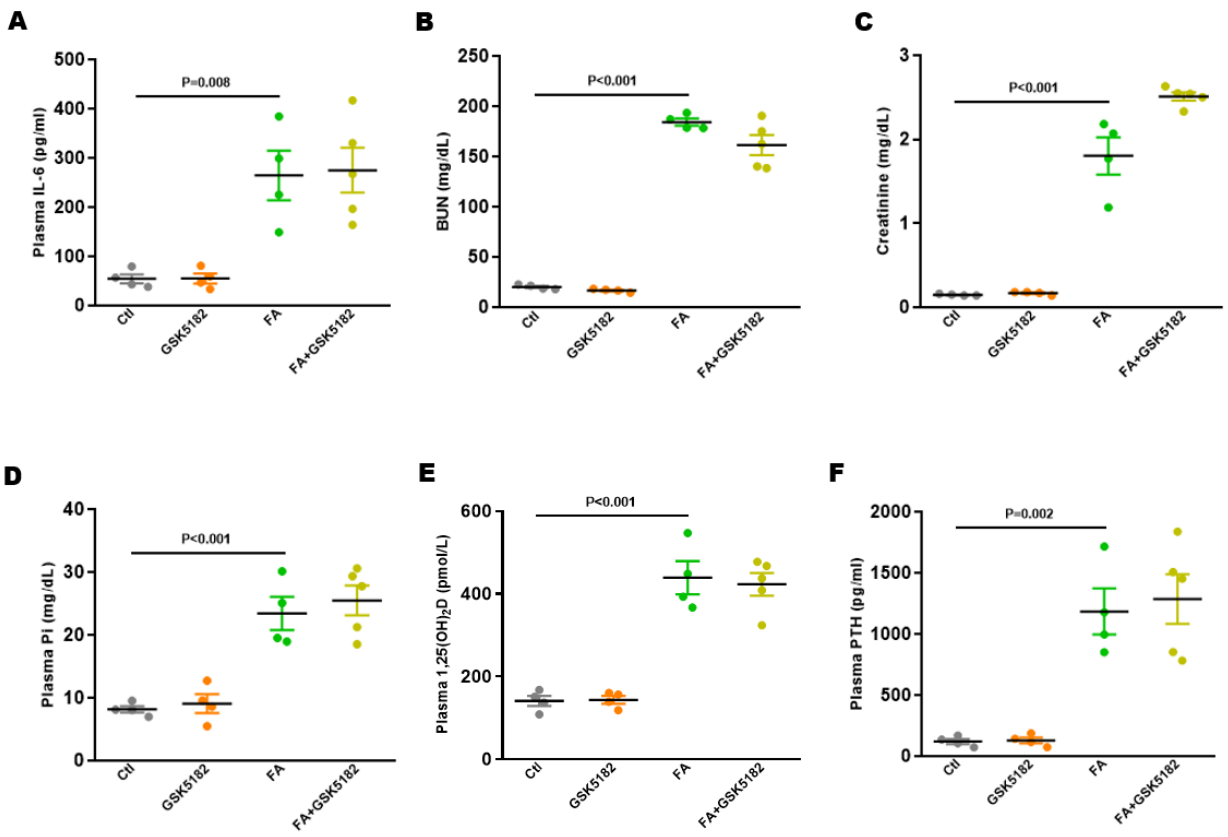

Fig. S8. Plasma biochemistry of mice treated with FA in presence or absence of GSK5182. Plasma levels of (A) IL-6, (B) blood urea nitrogen (BUN), (C) creatinine, (D) phosphate, (E) $1,25(OH)_2D$ and (F) PTH in C57BL/6J mice intraperitoneally injected with FA (240 mg/kg) in presence or absence of GSK5182 (40 mg/kg, dissolved in 30% of PEG400) (control, GSK5182 and FA n = 4 per group; FA+GSK5182 n = 5). Data represent mean $\pm$ SEM. All data were analysed by one-way ANOVA with Tukey's multiple comparison test.

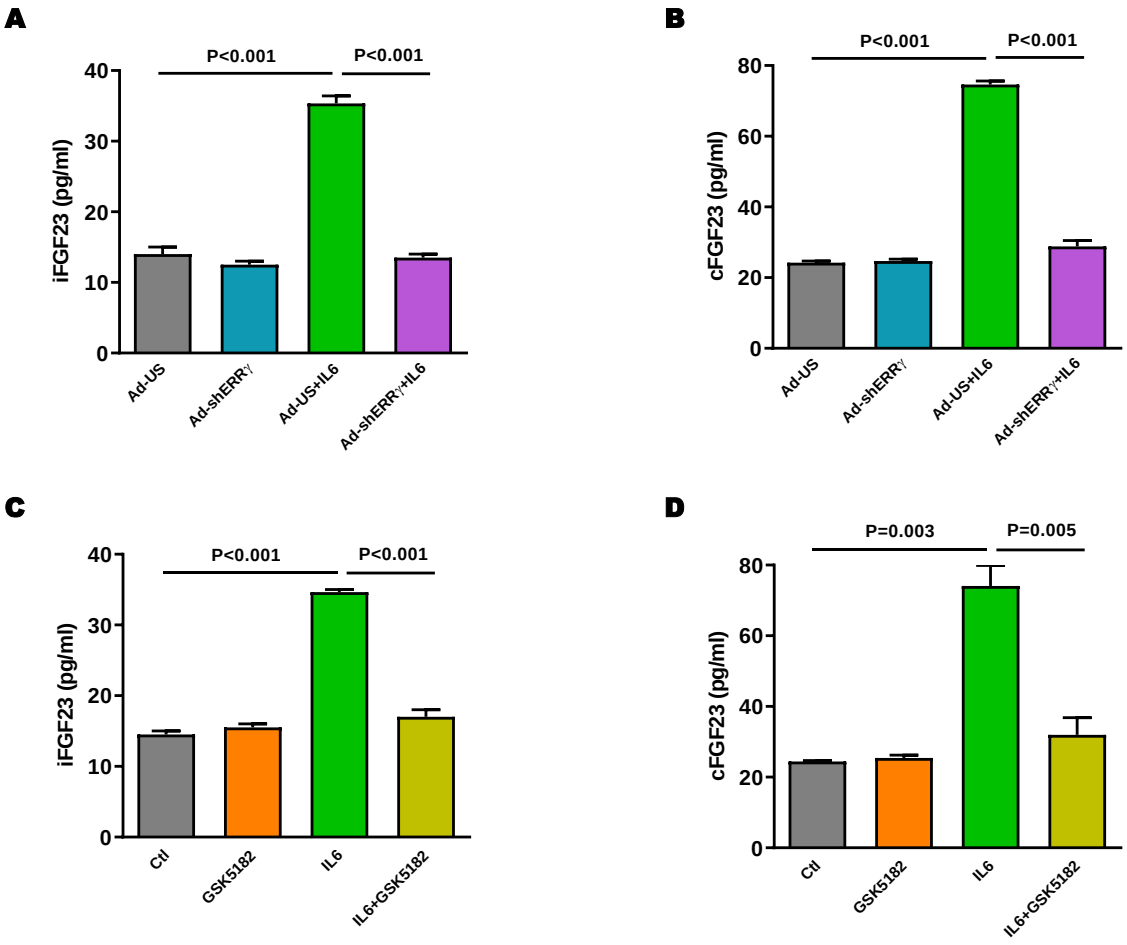

Fig. S9. Secreted FGF23 levels were measured in MPH culture medium. (A and C) Intact FGF23 and (B and D) c-terminal FGF23 levels were measured in culture medium of MPH treated with IL-6 in the presence of (A and B) adeno-viral sh ERRγ (Ad-sh ERRγ) or (C and D) GSK5182. All experiments were performed as three independent replicates. Data represent mean ± SEM. All data were analysed by one-way ANOVA with Tukey's multiple comparison test.

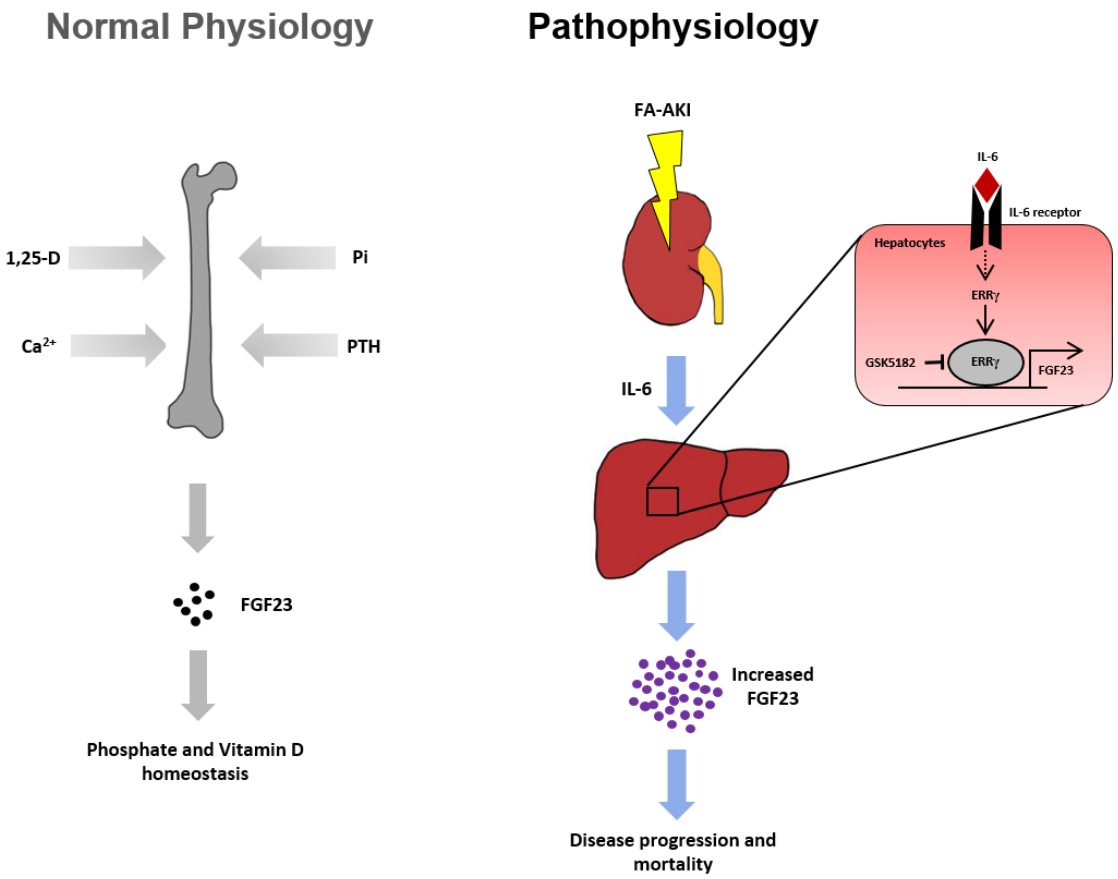

Fig. S10. A schematic model of ERRγ regulated hepatic FGF23 production in FA mediated AKI. In normal physiology 1,25-D, phosphate, calcium and PTH induces bone production of FGF23 to control phosphate and vitamin D homeostasis. In pathophysiological condition, FA-AKI injury produces IL-6 in kidney which in turn regulates ERRγ mediated FGF23 production in liver. GSK5182 inhibits ERRγ transactivation and there by controls ERRγ mediated hepatic FGF23 production.

Supplementary Tables

Table S1. Baseline characteristics of patients undergoing cardiac surgery according to tertiles of IL-6. CABG, coronary artery bypass grafting. eGFR, estimated glomerular filtration rate.

	Total (N=709)	IL-6 ≤2.6 pg/mL (N=233)	IL-6 2.7-6.3 pg/mL (N=241)	IL-6 >6.3 pg/mL (N=235)	P
Age (years)	63.6±14.5	56.0±15.4	65.4±13.3	68.7±11.5	<0.001
Body mass index (kg/m^2)	27.8±4.6	26.9±3.8	28.3±5.0	28.0±4.8	0.004
Systolic blood pressure (mmHg)	125±36	130±28	127±33	119±44	0.097
Diastolic blood pressure (mmHg)	72±22	75±17	73±20	67±26	0.007
Arterial hypertension (%)	87.6	85.4	89.7	87.7	0.370
Smoking (%)	12.3	9.9	15.3	11.5	0.180
Diabetes (%)	18.5	9.3	18.1	28.0	<0.001
Valve surgery (%)	70.3	76.8	70.7	63.4	0.006
CABG (%)	37.7	27.0	43.0	43.0	<0.001

Combined surgery (%)	33.7	31.3	35.1	34.5	0.648
Serum creatinine (mg/dL)	1.0 (0.3)	1.0 (0.2)	1.0 (0.3)	1.2 (0.5)	<0.001
eGFR (ml/min/1.73m^2)	90.7±19.5	100.6±17.9	90.9±17.4	81.4±19.1	<0.001
FGF-23 (RU/mL)	64.1 (67.0)	50.3 (26.0)	62.3 (53.0)	99.8 (217.0)	<0.001

Table S2. Correlation between IL-6, hsCRP, and FGF-23.

hsCRP, high sensitivity C-reactive protein.

	Spearman-Rho correlation coefficient for the correlation with FGF-23	P
IL-6	0.448	<0.0001
hsCRP	0.033	0.368

Table S3. Logistic regression analysis on the association between tertiles of IL-6 and risk of postoperative acute kidney injury (AKI).

Adjusted 1: age and sex

Adjusted 2: age, sex, body mass index, systolic blood pressure, eGFR, diabetes, smoking

Adjusted 3: age, sex, body mass index, systolic blood pressure, eGFR, diabetes, smoking, CRP

Interaction term for IL-6 and FGF-23, P<0.0001.

Model	Tertile of IL-6	OR for AKI	95% CI	P
Crude	1	Reference		
	2	3.05	1.81-5.12	<0.0001
	3	7.24	4.38-11.95	<0.0001
Adjusted 1	1	Reference		
	2	2.08	1.21-3.57	0.008
	3	4.60	2.72-7.78	<0.0001
Adjusted 2	1	Reference		
	2	1.89	1.05-3.39	0.033
	3	3.03	1.70-5.39	0.0002
Adjusted 3	1	Reference		
	2	1.90	1.06-3.40	0.032
	3	2.96	1.65-5.29	0.0003

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