SUPPLEMENTARY INFORMATION

Pharmacological correction of a defect in PPARγ signaling ameliorates disease severity in *Cftr*-deficient mice

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SUPPLEMENTARY METHODS

Intestinal epithelial cell isolation

Colonic epithelial cells were harvested from sibling female wild-type or *Cftr*$^{-/-}$ and *Pparg*$^{f/f}$ or *Pparg*$^{IEC-/-}$ mice using the method of Rogler et al[35]. Briefly, intestinal tissue was dissected, washed in phosphate buffered saline (PBS) and incubated in 2 mM EDTA and 1 mM EGTA in Hanks' balanced salt solution without calcium or magnesium for 12 min at 37°C. The supernatant was discarded and the remaining mucosa vortexed in ice cold PBS. The suspension was centrifuged (75 x g for 5 min at 4°C) to isolate intact crypts, the supernatant discarded, and the pellet retained at -80°C for RNA and protein analysis.

Transcriptome Analysis

Two Sentrix Mouse-Ref8 v1.1 Expression Beadchips (Illumina) were used to identify differentially expressed genes from 16,075 probes between littermate wild-type and *Cftr*$^{-/-}$ mice treated with or without rosiglitazone as previously described[1]. Briefly, 1 μg RNA isolated as described above from each sample was amplified to cDNA, transcribed to cRNA, biotin labeled, hybridized and detected with streptavidin-Cy3 following the manufacturer's protocol. BeadChips were scanned with Illumina BeadArray Reader and analyzed as previously described[2]. Data were normalized and analyzed with GENESPRING (Silicon Genetics). GeneOntology (GO) Analysis was performed using the DAVID bioinformatics resource as previously described[3]. Genes down- or up-regulated in knock-out mice were identified by using a false discovery rate of 0.05 and a maximal p value of 0.10 (unpaired, two-tailed t-test) and connected to biological process annotations provided by the GO Consortium[4].

Cell culture

The human colonic epithelial cell line, T84, was cultured in Dulbecco's modified Eagle's medium/Hams' F-12 medium (1:1) supplemented with 5% newborn calf serum (HyClone). COS-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone). For Ussing chamber studies, T84 cells were seeded on 12-mm Millicell-HA inserts (Millipore) and cultured for 21 days to achieve transepithelial resistance of >1200 $\Omega \cdot cm^2$ as measured by an epithelial voltohmmeter (EVOM, World Precision Instruments) prior to experiments.

Colonic epithelial ion transport

Segments of mid-distal colon were stripped of muscle layers and mounted on Ussing chamber inserts with a window area of 0.07 cm^2 as described previously[5]. Tissues were bathed in oxygenated Ringer's solution at 37°C containing (in mM) 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 0.8 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 $H_2PO_4^-$, 0.4 HPO_4^{2-}, and 10 glucose. The tissues were voltage-clamped to zero potential difference and short-circuit current recorded using a DVC-1000 Dual voltage-clamp (World Precision Instruments). Results were normalized and expressed as ΔIsc ($\mu A/cm^2$). Chloride-free Ringer's solution contained (in mM) 145 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 2.4 SO_4^{2-}, 2.8 PO_4^{3-}, 25 HCO_3^-, 120.1 isethionate and 10 glucose. Apical chloride-free solution was substituted to increase the driving force for chloride transport and provide a measurable ΔIsc in *Cftr*$^{-/-}$ colon following the addition of carbachol. Short-circuit current was recorded in response to application of forskolin (10 μM) at the mucosal and serosal surface or carbachol (100 μM) at the serosal surface.

T84 cells were grown as monolayers on Millicell-HA supports and mounted in Ussing chambers with a window of 0.6 cm^2 as described[6]. For CFTR inhibitor studies, the basolateral membrane was permeabilized with 100 μM amphotericin B (Sigma) followed by treatment with 10 μM $CFTR_{inh}$-172 (Sigma) at the basolateral surface. The

buffer was changed to standard Ringer's on the basolateral side and chloride-free Ringer's on the apical side, and the response to forskolin or carbachol recorded.

Transient Transfection and Reporter Studies

COS-7 cells were transfected with 0.1 μg of the luciferase reporter plasmid and 0.1 μg of the PPARγ expression plasmid or empty vector with FuGENE 6 Transfection Reagent (Roche). A β-galactosidase expression vector was co-transfected as an internal control. The reporter construct was pTAL (Clonetech). Enhancers containing 250-400 bp sequence were cloned from C57Bl/6 genomic DNA to the reporter construct using Phusion High-Fidelty DNA Polymerase (Finnzymes). Cells were treated with Ro (1 μM) as indicated. Luciferase reporter gene activity was measure after 24 hr Ro treatment and performed as previously described[7].

Lipidomics analysis

The sample preparation, liquid chromatography mass spectrometry, and gas chromatography mass spectrometry were conducted as previously described[38-40]. Adaptations to the LC/MS/MS method include using the scheduled MRM mode (Analyst 1.5 software, Applied Biosystems) to increase the total MRM transitions monitored to 174. Multiquant 1.1 software (Applied Biosystems) was used in the quantitation of each metabolite. Briefly, right colon epithelial cells from six wild-type and five *Cftr*$^{-/-}$ were harvested via scrapping, weighed and immediately frozen. Cells were suspended in solution containing 800 μl of H_2O, 200 μl of a deuterated mix of internal fatty acid and eicosanoid standards in methanol. Cells were lysed using a sonicator probe. 80 ul was removed for liquid liquid extraction and fatty acid analysis by gas chromatography mass spectrometry (GC/MS). 920 μl was subjected to solid phase extraction. All extracted samples were stored at -80° C until analyzed via mass spectrometry. Extracted eicosanoid samples were dried under vacuum centrifugation before resuspension in 100 μl of solvent A (H_2O-acetonitrile-acetic acid (70:30:0.02; v/v/v)). An aliquot of 40 μl was

injected onto a reverse-phase HPLC system linked in series with an Applied Biosystems Q-Trap 4000. Fatty acids were analyzed using an Agilent Technologies GC 6890N linked with a 5975 iMSD.

SUPPLEMENTARY REFERENCES

1. Ogawa, S.*, et al.* Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* **122**, 707-721 (2005).
2. Sasik, R., Calvo, E. & Corbeil, J. Statistical analysis of high-density oligonucleotide arrays: a multiplicative noise model. *Bioinformatics* **18**, 1633-1640 (2002).
3. Huang da, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57 (2009).
4. Creating the gene ontology resource: design and implementation. *Genome Res* **11**, 1425-1433 (2001).
5. McCole, D.F., Rogler, G., Varki, N. & Barrett, K.E. Epidermal growth factor partially restores colonic ion transport responses in mouse models of chronic colitis. *Gastroenterology* **129**, 591-608 (2005).
6. Chappell, A.E.*, et al.* Hydrogen peroxide inhibits Ca2+-dependent chloride secretion across colonic epithelial cells via distinct kinase signaling pathways and ion transport proteins. *FASEB J* **22**, 2023-2036 (2008).
7. Pascual, G.*, et al.* A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-763 (2005).

Supplementary Figure Legends

Supplementary Figure 1. Effect of PPARγ activation on gene expression in mice. (a) GeneOntology (GO) terms significantly enriched in genes down-regulated and up-regulated in *Cftr*$^{-/-}$ colonic epithelial cells, indicating p values and Benjamini correction for multiple hypothesis testing. (b) Effect of Ro treatment on expression of 388 genes down-regulated and 328 genes up-regulated in *Cftr*$^{-/-}$ colonic epithelial cells compared to control cells.

Supplementary Figure 2. Additional PPARγ-dependent target genes in *Cftr*- and *Pparg*-deficient mice. (a) Q-PCR analysis of *Aqp8* and *Adfp* mRNAs in colonic epithelial cells derived from wild-type and *Cftr*$^{-/-}$ mice treated with Ro (20 mg/kg/d for 5 days) or maintained on a control diet (n=10 mice per group). mRNA levels are normalized to GAPDH and expressed relative to untreated wild-type cells. (b) Western blot of PPARγ using colonic epithelial cell lysates derived from *Pparg*$^{f/f}$ and *Pparg*$^{IEC-/-}$ mice. (c) Q-PCR analysis of mRNAs shown in panel a in *Pparg*$^{f/f}$ and *Pparg*$^{IEC-/-}$ colonic epithelial cells (n=6 mice per group). Values are means ± s.e.m. For mice treated with Ro, *P<0.01 and **P<0.05 versus untreated mice of the same genotype. For untreated knock-out mice (*Cftr*$^{-/-}$ or *Pparg*$^{IEC-/-}$), +P<0.01 and ++P<0.05 versus wild-type or *Pparg*$^{f/f}$ controls.

Supplementary Figure 3. Genes up-regulated in *Cftr*- and *Pparg*-deficient mice. (a) Q-PCR analysis of *Pap*, *Reg3g*, *Cxcl1*, and *Cxcl2* mRNAs in colonic epithelial cells derived from wild-type and *Cftr*$^{-/-}$ mice treated with Ro (20 mg/kg/d for 5 days) or maintained on a control diet (n=10 mice per group). mRNA levels are normalized to GAPDH and expressed relative to untreated wild-type cells. (b) Q-PCR analysis of mRNAs shown in panel a in *Pparg*$^{f/f}$ and *Pparg*$^{IEC-/-}$ colonic epithelial cells (n=6 mice per group). Values are means ± s.e.m. For mice treated with Ro, *P<0.01 and **P<0.05 versus untreated mice of the same genotype. For untreated knock-out mice (*Cftr*$^{-/-}$ or *Pparg*$^{IEC-/-}$), +P<0.01 and ++P<0.05 versus wild-type or *Pparg*$^{f/f}$ controls.

Supplementary Figure 4. Deletion of PPARγ exacerbates the phenotype of *Cftr*$^{-/-}$ mice. (a) Weight (grams) of male and female wild-type and *Cftr*$^{-/-}$ mice with combined *Pparg*$^{f/f}$ or *Pparg*$^{IEC-/-}$ genotypes demonstrated significantly reduced weight (age 30 days, 10 mice per group) by the combined deletion (*Cftr*/*Pparg*^{DKO}) compared to control mice (*Cftr*$^{-/-}$/*Pparg*$^{f/f}$). There was no difference in the weight of *Pparg*$^{IEC-/-}$ compared to control *Pparg*$^{f/f}$ mice. Values are means ± s.e.m. *P<0.001 and **P<0.01. (b) Representative

histology demonstrated alcian blue-positive mucin accumulation at the point of colonic obstruction in *Cftr*/*Pparg*^{DKO} mice.

Supplementary Figure 5. Ro effect on PPARγ target genes in IB3-1 and S9 cells. (a) *Angptl4* and *Adfp* mRNAs are reduced in CFTR mutant bronchial epithelial cell line IB3-1 and expression is restored by treatment with Ro. Values are means ± s.d. For cells treated with Ro, *P<0.01 versus untreated cells and +P<0.01 IB3-1 versus S9 control cells.

Supplementary Figure 6. PPARγ agonist rosiglitazone (Ro) does not affect calcium-dependent chloride secretion. (a) Ussing chamber experiments from wild-type or sibling *Cftr*$^{-/-}$ mice treated with or without Ro demonstrated reduced responses to carbachol (100 μM) in *Cftr*$^{-/-}$ colon and no difference in the response in *Cftr*$^{-/-}$ pre-treated for 5 days with Ro. (b) Pre-treatment with Ro for 48 hrs did not change the response to carbachol in T84 cells mounted in mucosal and serosal Ringer's solution. (c) Left panel; Pre-treatment with *CFTR*$_{inh}$-172 in amphotericin permeabilized T84 cells significantly reduced the peak current response to forskolin (10 μM) consistent with CFTR inhibition. Right panel; Ro did not affect the carbachol-responsive current in CFTR inhibited cells (p=0.12). Values are means ± s.e.m. *P<0.01 and **P<0.001. Results are representative of n=7 mice per group or n=6 T84 inserts per group.

Supplementary Figure 7. Quantitative expression for candidate ion transporter genes in *Pparg*$^{f/f}$ and *Pparg*$^{IEC-/-}$ mice treated with or without rosiglitazone (Ro). The results demonstrated increased expression of the basolaterally localized *Slc4a2* (AE2, chloride-bicarbonate exchanger 2, P<0.05) by rosiglitazone in *Pparg*$^{f/f}$ mice and increased expression for *Slc26a6* (PAT1, chloride-bicarbonate exchanger) in *Pparg*$^{IEC-/-}$ mice (p<0.05). The expression of other candidate genes was unchanged. *Slc4a1* (AE1) and *Slc12a1* (NKCC2) were not present in colonic epithelial cells. mRNA levels are normalized to GAPDH. Expression for *Slc26a6* was 200x less than *Slc26a3* (DRA1, chloride-bicarbonate exchanger) suggesting that DRA1 is the major apical chloride-bicarbonate exchanger in the colon.

Supplementary Figure 8. PPARγ binding sites function to enhance promoter activity. COS-7 cells were transiently transfected with control vector DNA (empty) or vector containing the previously identified PPARγ binding site for *Angptl4* and the novel sites

for *Acaa1b*, *Mgll*, and *Car4* upstream of a minimal thymidine kinase TATA like promoter (pTAL) directing firefly luciferase expression. Values are means ± s.d. of n=3 technical replicates (*P<0.01). Results are representative of 3 independent biological replicates.

a

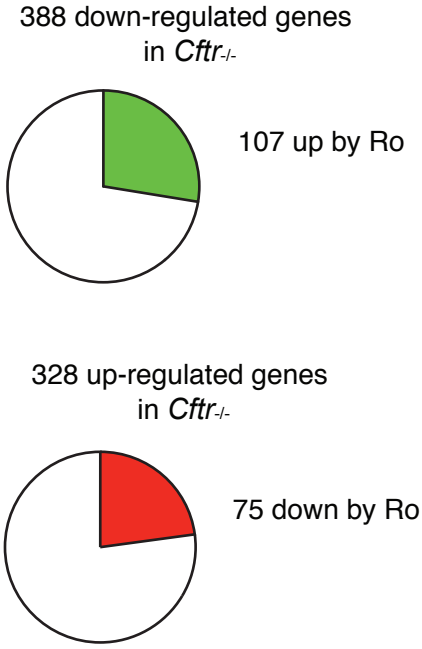
Down-regulated genes

GO Term	p value	Benjamini
Biological Process		
Lipid metabolism	5.0e-8	2.6e-4
Carboxylic acid metabolism	2.3e-9	3.0e-6
Molecular Function		
Oxidoreductase activity	9.3e-10	1.3e-6

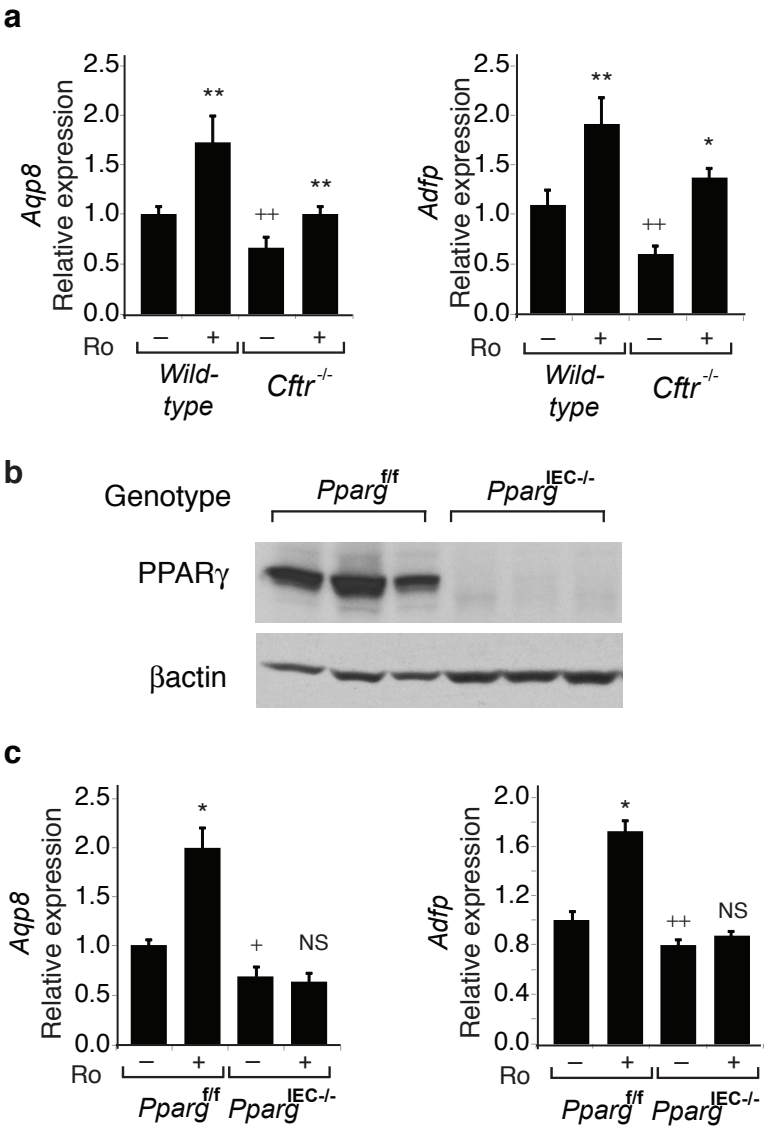
Up-regulated genes

GO Term	p value	Benjamini
Biological Process		
Inflammatory responses	1.5e-5	8.6e-3
Response to wounding	4.6e-5	2.3e-2
Molecular Function		
Actin binding	2.6e-8	3.5e-5

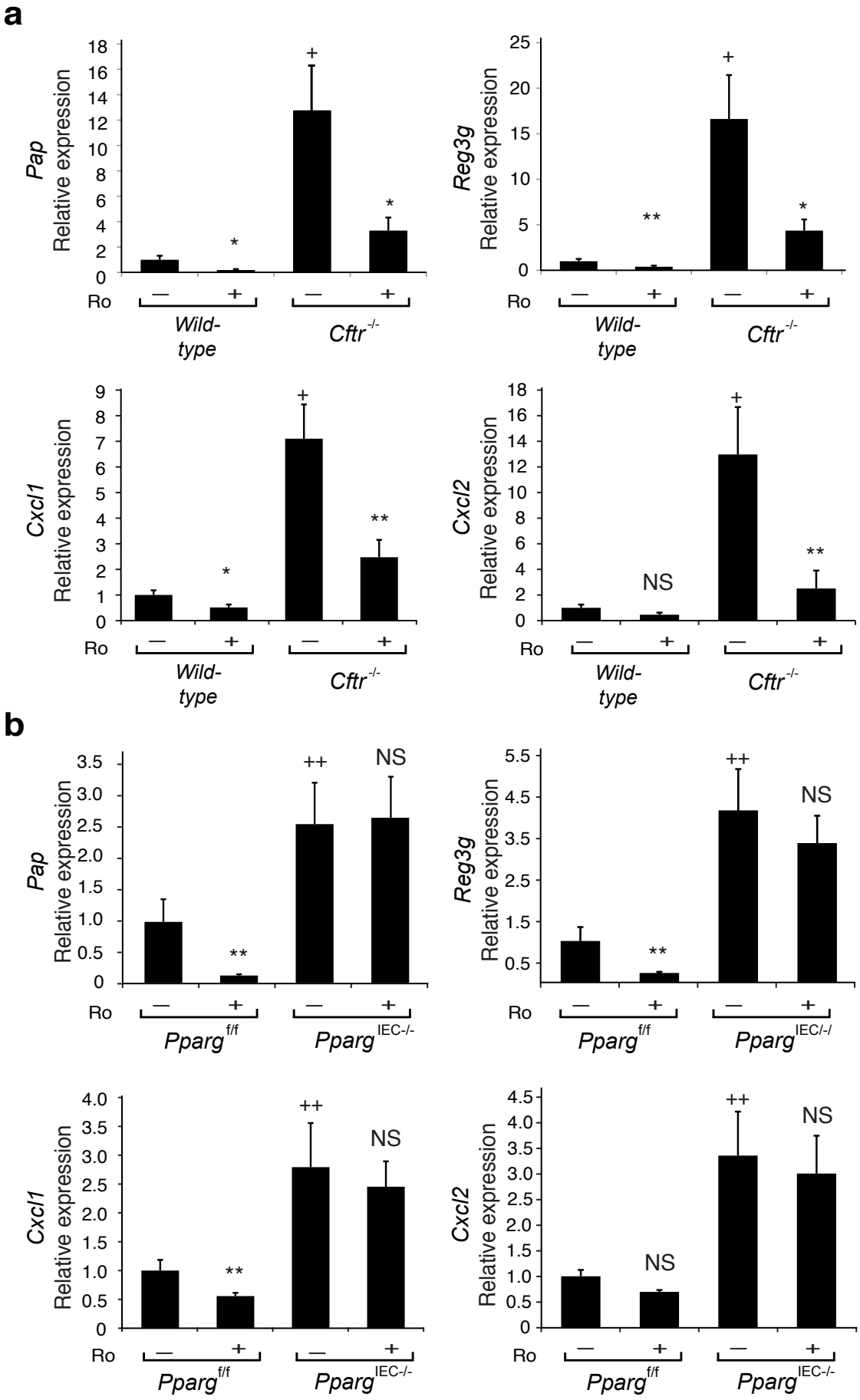
b



Harmon, et al., Supplementary Fig. 1

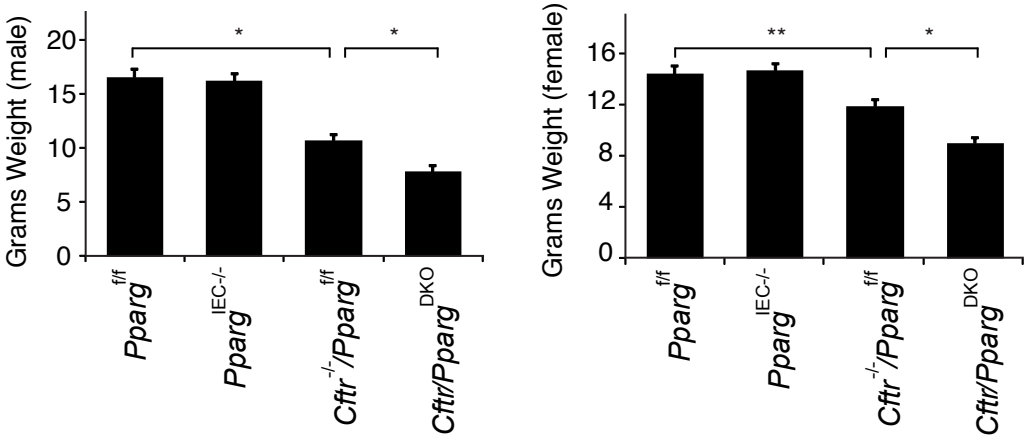


Harmon, et al., Supplementary Fig. 2



Harmon, et al., Supplementary Fig. 3

a



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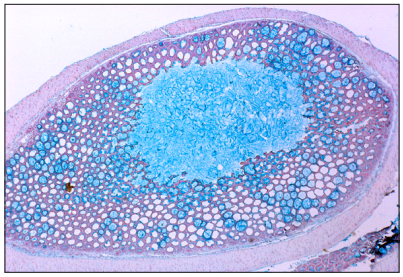
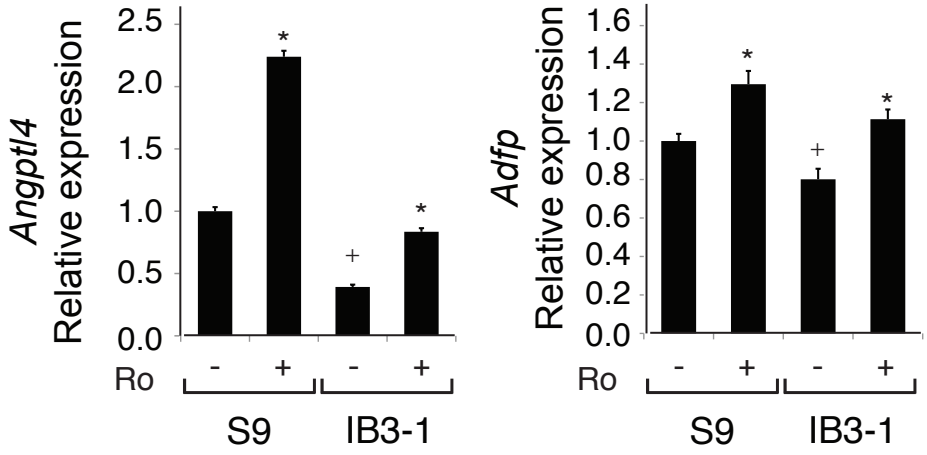
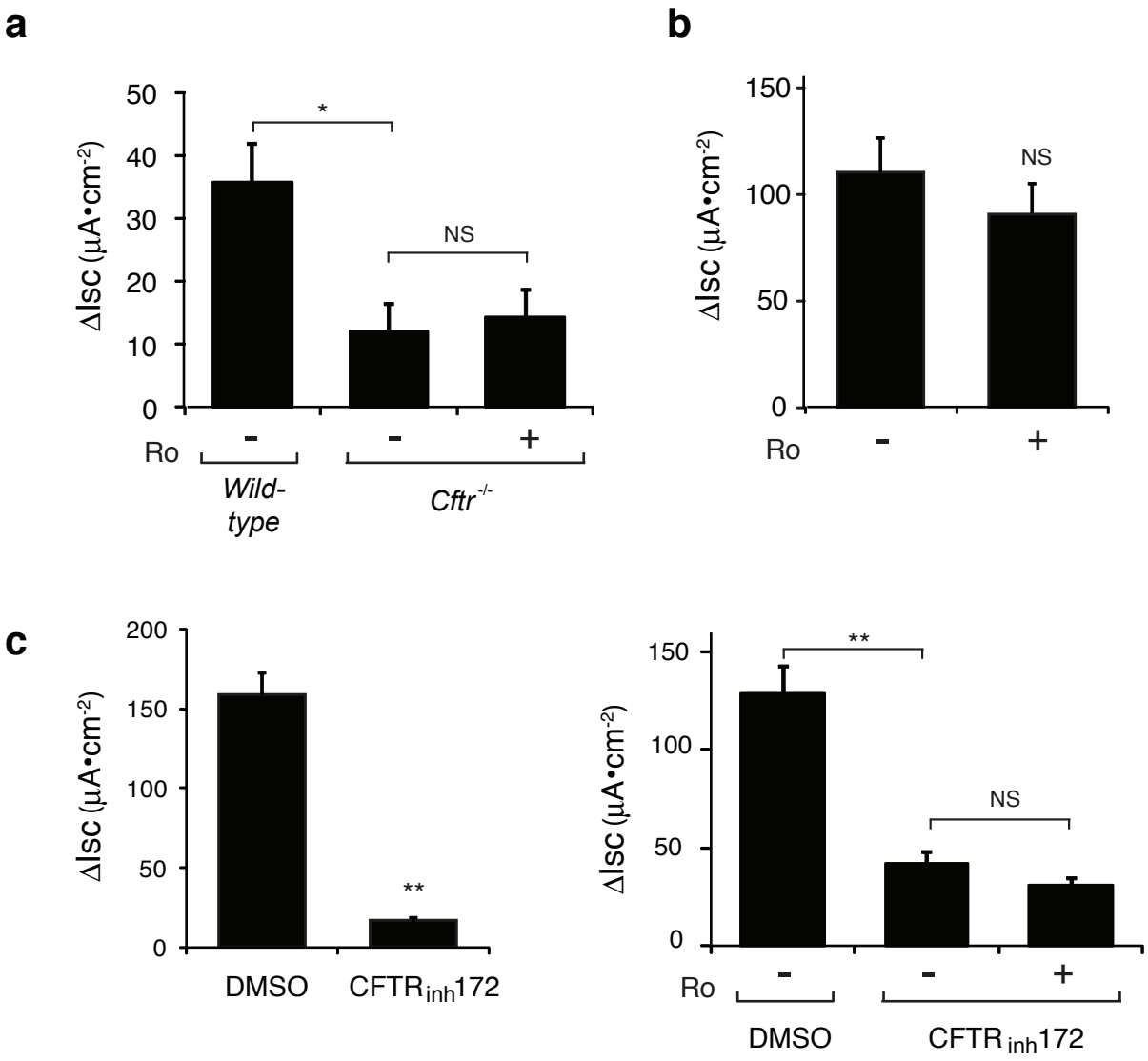


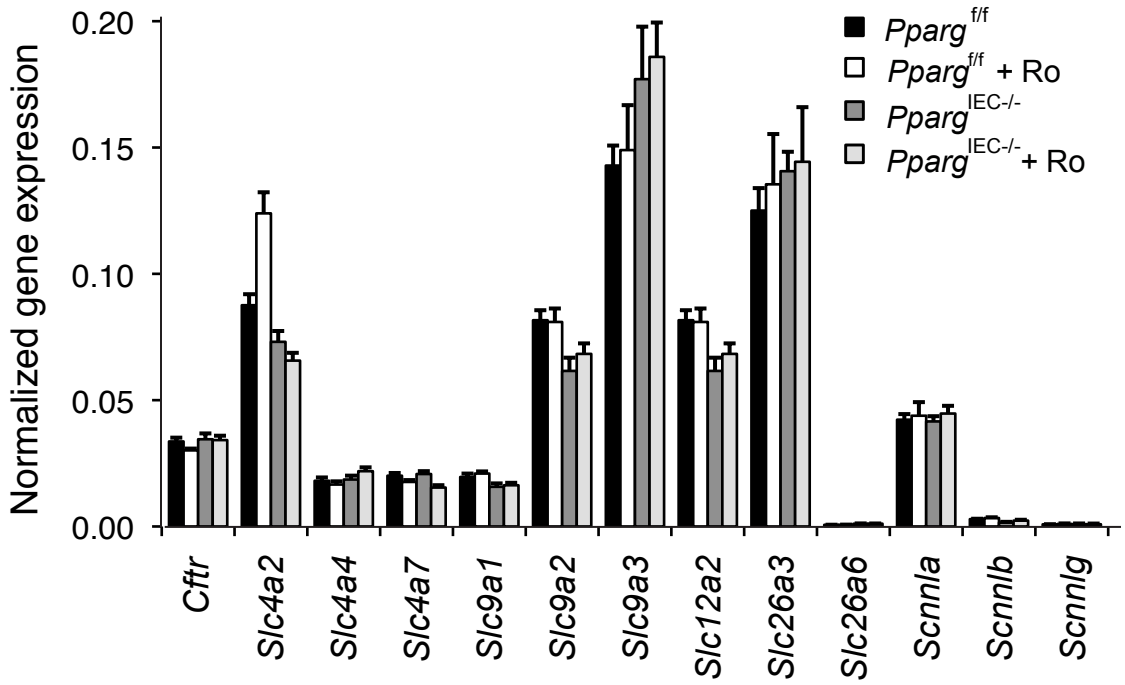
Harmon, et al., Supplementary Fig. 4



Harmon, et al., Supplementary Fig. 5



Harmon, et al., Supplementary Fig. 6



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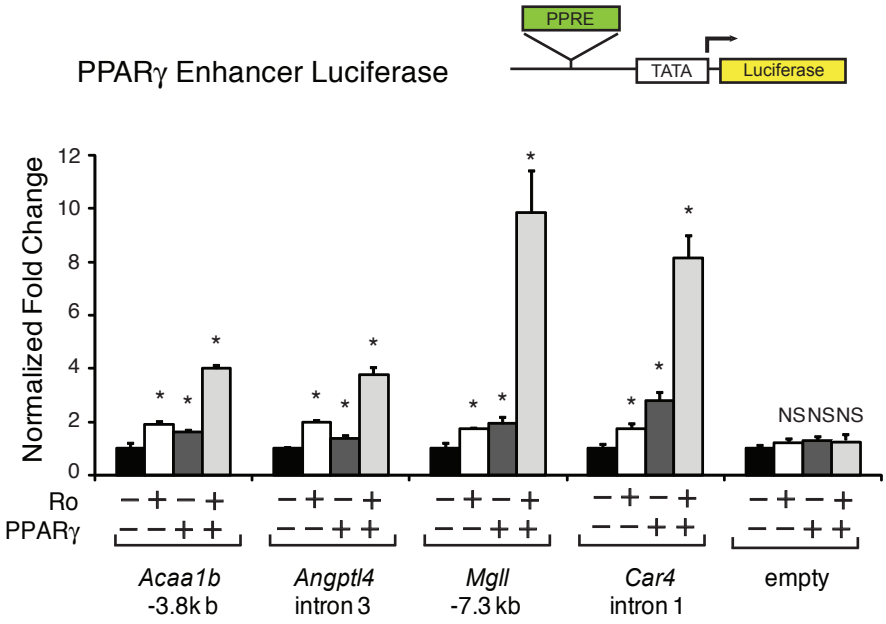

Harmon, et al., Supplemental Fig. 8