Supplemental Material

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FXR activation prevents the development of vascular calcification in ApoE$^{-/-}$ mice with chronic kidney disease

1. **Extended Materials and Methods**

Animal Studies: 5/6 nephrectomized and sham-operated ApoE$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Details of 5/6 nephrectomy are described in the Supplemental Methods. At 8 weeks of age, animals were fed either a high-fat/high-cholesterol diet (Western diet) (Harlan Teklad, 000750, containing 21% fat, 0.15% cholesterol [w/w]) with or without INT-747 for 12 weeks. For Figure 2 and S1, animals were fed western diet for 24 weeks. Where indicated, diets were supplemented with INT-747 [EC_{50} 85 nM on FXR], at a level sufficient to provide 10 or 20 milligrams per kilogram body weight (mpk) dose on consumption of a 3-g diet by a 25-g mouse per day.[1, 2] *Animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Colorado at Denver.*

Cell culture studies: Bovine calcifying vascular cells (CVC) were cultured in DMEM containing 15% FBS with either 1.2 mM phosphate (normal-phosphate concentration) or 2.0 mM phosphate (high-phosphate concentration). CVC were treated with several concentrations of 6α-ethyl-chenodeoxycholic acid (INT-747, Intercept Pharmaceuticals Inc., New York) and the medium were changed every 2-3 days.[1] At 14 days of the confluence, cells were stained with either Alizarin red which identifies calcium deposit or Oil red O which identifies neutral lipid deposit.

Calcium content in cultured cells and aorta: Calcium deposition in the plates was quantified as previously described.[3] Cells were decalcified using 0.6M HCl solution. After collecting the supernatant, the cells were washed with PBS and solubilized with 0.1N NaOH/0.1% SDS solution for protein quantification. The aortas were removed and frozen at -20°C until analysis. The dried aorta was defatted with chloroform and methanol (2:1) for 48 hours and dehydrated by acetone for three hours. The dried samples were incinerated to ashes at

600°C for 24 hours using an electric muffle furnace (Thermo Scientific), then extracted with HCl and diluted with distilled water. The levels of calcium in the aorta were determined and represented as the weight of calcium per dry weight of aorta.[4] The calcium content was quantified calorimetrically using the o-cresolphtalein method. The protein content was measured using a BCA protein assay kit.

Histology and lesion analysis: For *en face* analysis, mice were euthanized and the aorta dissected out, opened longitudinally from heart to the iliac arteries, and stained with Sudan IV to determine lesion area. Images were captured by use of a Zeiss Axiocam-CCD video camera and analyzed by a single technician who was blinded to the study protocol and used AxioVision image analysis software. The extent of lesion formation is expressed as the percentage of the total aortic surface area covered by lesions. Atherosclerotic lesions at the aortic valve were analyzed as described. The upper portion of the heart and proximal aorta were obtained, embedded in OCT compound, and stored at −80°C. 10-μm sections were analyzed for a distance of 800 μm. Sections were stained with Von Kossa and Oil Red O. The lipid-staining and calcified areas on 25 sections were determined in a blinded fashion by light microscopy. The mean value of lesion area of aortic wall per section was then calculated. [5]

RNA analysis: Total RNA was isolated by using Tri reagent coupled with RNAeasy kit. Real-time quantitative PCR assays were performed by using an Applied Biosystems StepOne qPCR instrument. In brief, 1 μg of total RNA was reverse transcribed with random hexamers by using High Quality Reverse Transcription Reagents Kit (Applied Biosystems). Each amplification mixture (10 μl) contained 25 ng cDNA, 900 nM forward primer, 900 nM reverse primer and 5 μl of Universal fast PCR Master Mix. Samples were analyzed for 18S rRNA expression in parallel in the same run. Quantitative expression values were calculated from absolute standard curve method using the plasmid template for each target gene. Primer sequences are available upon request.

Lentiviral and adenoviral transduction for CVC: Lentiviral CMV vectors (Lenti-X, Clontech) expressing mouse FXR dominant negative (FXRDN) with a FLAG tag were generated as previously described[6]. The dominant negative FXRα, lacking the C-terminal 10 amino acids and the AF-2 subdomain was prepared by a mutagenesis kit (Stratagene). Lentiviral stocks were obtained by transfection of 5×10^6 293T cells with Lenti-X HT Packing system (Clontech). The

titers were analyzed by Lenti-X qRT-PCR Titration kit (Clontech). Cells were infected with recombinant lentiviruses and adenoviruses at a multiplicity of infection (MOI) of 10 and 40, respectively. Adenovirus expressing Msx2 with a c-myc tag, osterix with a FLAG tag and VP16FXRα (VP16 domain was inserted into the N-terminus of FXRα) were generated in the Vector Core in the Diabetes Endocrinology Research Center (DERC), University of Colorado-Denver or by RAPAd.I system (Cell Biolabs). The adenoviral cosmid vector contains IRES-GFP to check the transfection efficiency. CVC was infected with either lentivirus or adenovirus in DMEM 15% FBS containing 5 μg/ml Polybrene® (Sigma). After 24 or 48 h, the infected cells were treated with media containing 2mM phosphate.

Western blotting: The cells were washed four times with ice-cold PBS and the nuclear extracts were isolated using a nuclear extract kit (Active Motif). These samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with a FXR monoclonal antibody (R&D systems), FLAG monoclonal antibody (Sigma), GFP antibody (Santa Cruz), myc monoclonal antibody (Covance), phospho-AKT antibody (Cell Signaling), phospho-JNK antibody (Cell Signaling), phsopho-p38 MAPK antibody (Cell Signaling), phospho-ERK antibody (Cell Signaling), total JNK antibody (Cell Signaling) or total ERK antibody (Cell Signaling) and visualized with horseradish peroxidase coupled to anti-mouse secondary antibody with enhancement by ECL detection kits.

5/6 nephrectomy: Under anesthesia with ketamine/Xylazine, a two-step procedure was performed. In the first step of surgery the left kidney was decapsulated and 1/3rd of each kidney pole ligated with a silk suture. The kidney tissue from each pole was then excised with scissors. The muscle and skin layers of the incision are then closed with sterile surgical staples. The animals recovered for one week before the second surgery. In the second surgery, the entire right kidney was decapsulated and surgically removed by ligating with silk suture at the bottom of the renal artery and then excising the tissue with scissors to complete the 5/6 nephrectomy.

Tissue lipid composition: Hepatic and CVC cholesterol and triglycerides were analyzed using gas chromatography as previously described. [7, 8]

Blood chemistry: Total serum cholesterol, triglycerides, calcium and phosphate, blood urea nitrogen, were determined enzymatically in the serum or plasma of mice.

Statistical analysis: Statistical analyses for multiple groups were performed with 1-way ANOVA with Tukey post hoc test (GraphPad Prism, CA). Student t test was used when 2 groups were compared. Data are presented as mean ± SEM.

Supplemental References

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2. Online Figure Legends

Online Figure I. Chronic kidney disease (CKD) accelerates atherosclerosis and vascular calcification. A) *En face* analysis of atherosclerosis in ApoE$^{-/-}$ mice with CKD. 8 week-old male ApoE$^{-/-}$ mice with or without 5/6 nephrectomy were obtained from The Jackson Laboratory and fed ad libitum either control or a western diet for 24 weeks. B) Quantification of aortic atherosclerosis in control diet and western diet fed-mice. The aorta including ascending arch, thoracic and abdominal segments was dissected and stained with Sudan IV. The surface lesion was quantified with commercially available software (Image Quant, Zeiss). **C)** Aortic calcium levels in ApoE$^{-/-}$ mice with CKD.

Online Figure II. INT-747 reduced gene expression of osteogenic transcription factors, osteogenic markers and lipogenic enzyme in CVC treated with high-phosphate. Cells were treated with INT-747 in the presence of 2 mM phosphate for 14 days. Black bar; vehicle, gray bar; 0.3 μM INT-747, white bar; 3.0 μM INT-747. Osteogenic markers: A) ALP, B) COL1A1 and C) MGP. Osteogenic transcription factors: D) Msx2, E) Runx2 and F) Osterix. Lipogenic genes: G) SREBP-1, H) SREBP-2, I) FAS, and J) ACC1. FXR target genes: K) SHP and L) AT2R.

Online Figure III. INT-747 treatment induces SHP expression in CVC. Cells were treated with INT-747 in the presence of 2 mM phosphate for 24 hours. Black bar; vehicle, gray bar; 0.3 μM INT-747, white bar; 3.0 μM INT-747.

Online Figure IV. FXRDN blocks the alterations of gene expressions by INT-747 treatment. A) SHP, B) ALP, C) COL1A1, D) MGP, E) Msx2 and F) osterix (Osx). Cells were treated with lentivirus expressing mock or FXRDN at 10 MOI. 24 hour after infection, cells were treated with 3μM INT-747 in the presence of 2 mM phosphate for 24 hours. $^{**}p<0.001$ vs. vehicle

Online Figure V. VP16FXR reduces Msx2, osterix, ALP and COL1A1 mRNA levels. CVCs were treated with adenovirus expressing VP16FXR at 40 MOI. 24 hour after infection, cells were treated with high-phosphate for 7 days. $^{**}p<0.001$ vs. Mock

Online Figure VI. **Msx2 and osterix (Osx) overexpression augments mineralization of CVC:** A) western blot analysis, B) Alizarin staining C) Calcium content, and D) ALP and COL1A1 mRNA. CVC were treated with adenovirus Mock (empty), Msx2 and Osx at 40 MOI in the presence of high (2.0 mM) phosphate for 7 days. Msx2 and Osx proteins were analyzed by immunoblot analysis with anti-GFP, anti-myc and anti-FLAG antibodies, respectively. GFP was used to determine the transfection efficiency. Data are representative of combined from three independent experiments. $^{**}p<0.001$ vs. Mock

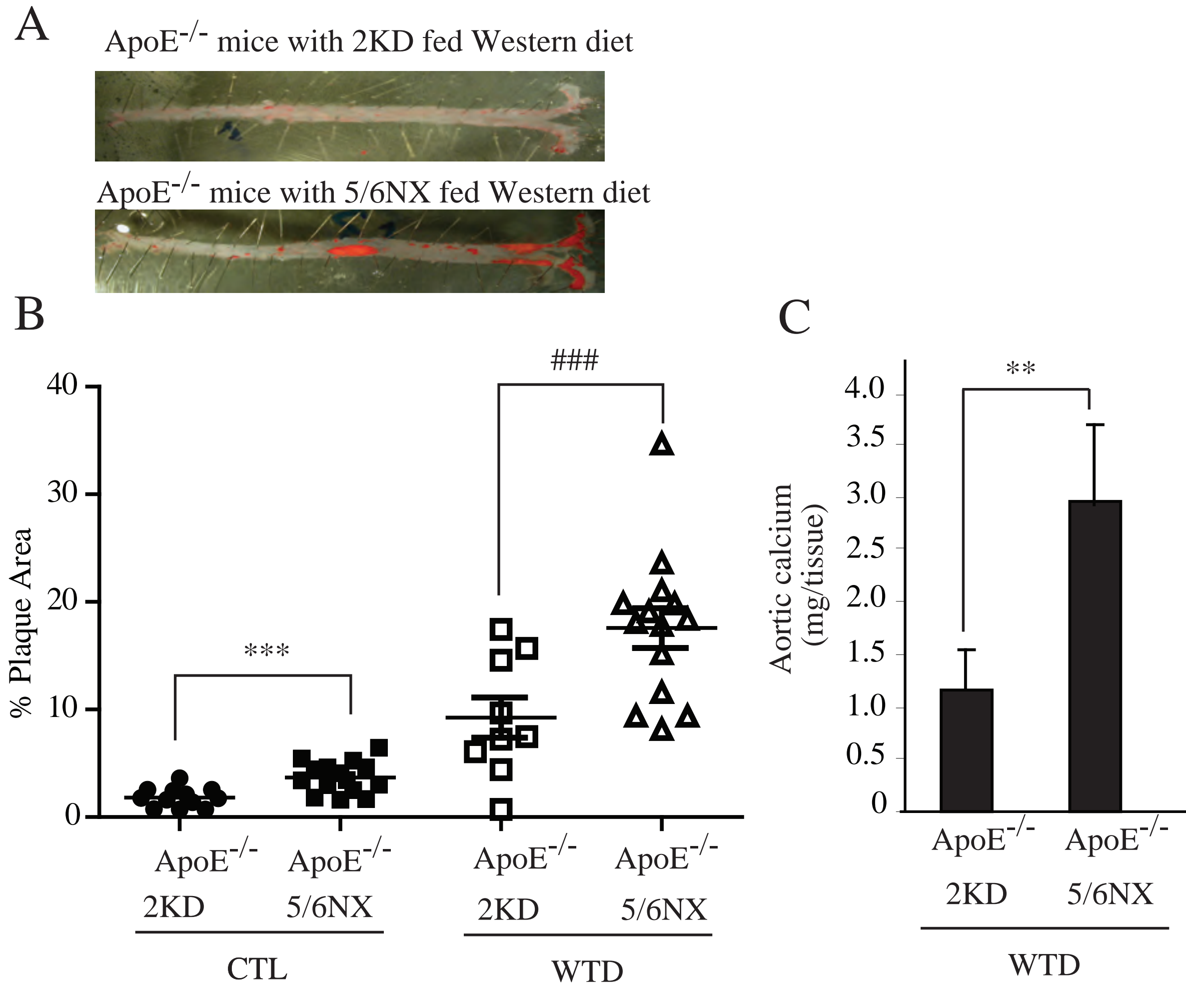
Online Figure VII. INT-747 did not alter gene expression of and macrophage makers (CD68 and F4/80) and pro-inflammatory cytokines (TNFα and IL-1β) in the aorta of ApoE$^{-/-}$ mice with CKD. Male mice were fed a western diet for 12 weeks. A) Macrophage markers: CD68 and F4/80. B) Pro-inflammatory cytokines: TNFα and IL-1β.

Online Table I. Serum parameters in 5/6 nephrectomezed (5/6NX) ApoE$^{-/-}$ mice maintained for 12 weeks on a western diet in the presence or absence of INT-747

Strain	C57Bl/6	ApoE$^{-/-}$	ApoE$^{-/-}$	ApoE$^{-/-}$	ApoE$^{-/-}$
Kidney	2	2	5/6NX	5/6NX	5/6NX
Diet	WTD	WTD	WTD	WTD	WTD
Treatment	Vehicle	Vehicle	Vehicle	INT-747 10 mpk	INT-747 20 mpk
Total serum CHOL (mg/dl)	330.0 ± 21.2	1556.7 ± 161.9	1447.5 ± 205.6	1423.6 ± 84.2	1492.5 ± 95.8
Triglycerides (mg/dl)	67.4 ± 8.8	184.0 ± 31.0	168.6 ± 3.2#	90.5 ± 0.9*	93.1 ± 0.7*
Glucose (mg/dl)	189.8 ± 19.9	190.5 ± 22.6	163.2 ± 25.9	148.0 ± 6.7	144.3 ± 9.6
Calcium (mg/dl)	8.5 ± 0.4	9.4 ± 0.6	10.0 ± 0.3	9.5 ± 0.2	9.4 ± 0.2
Phosphate (mg/dl)	9.0 ± 0.7	11.4 ± 0.3	14.7 ± 1.2#	13.7 ± 0.6#	14.0 ± 0.4#
BUN (mg/dl)	13.6 ± 3.7	16.4 ± 2.4	34.7 ± 2.7#	31.7 ± 1.8#	35.4 ± 3.4#
Total liver CHOL (mg/g)	8.0 ± 0.5	8.4 ± 1.6	9.2 ± 1.9	8.58 ± 1.4	7.10 ± 0.6**
Liver triglycerides (mg/g)	14.2 ± 2.6	18.3 ± 4.0	20.7 ± 3.5	13.20 ± 1.8**	10.72 ± 1.3**

Data expressed as mean ± SEM. *$p<0.05$; **$p<0.01$ vs. ApoE$^{-/-}$ mice with 5/6NX fed WTD without INT-747
#$p<0.05$ vs. ApoE$^{-/-}$ with 2 kidneys fed WTD without INT-747

Online Figure I



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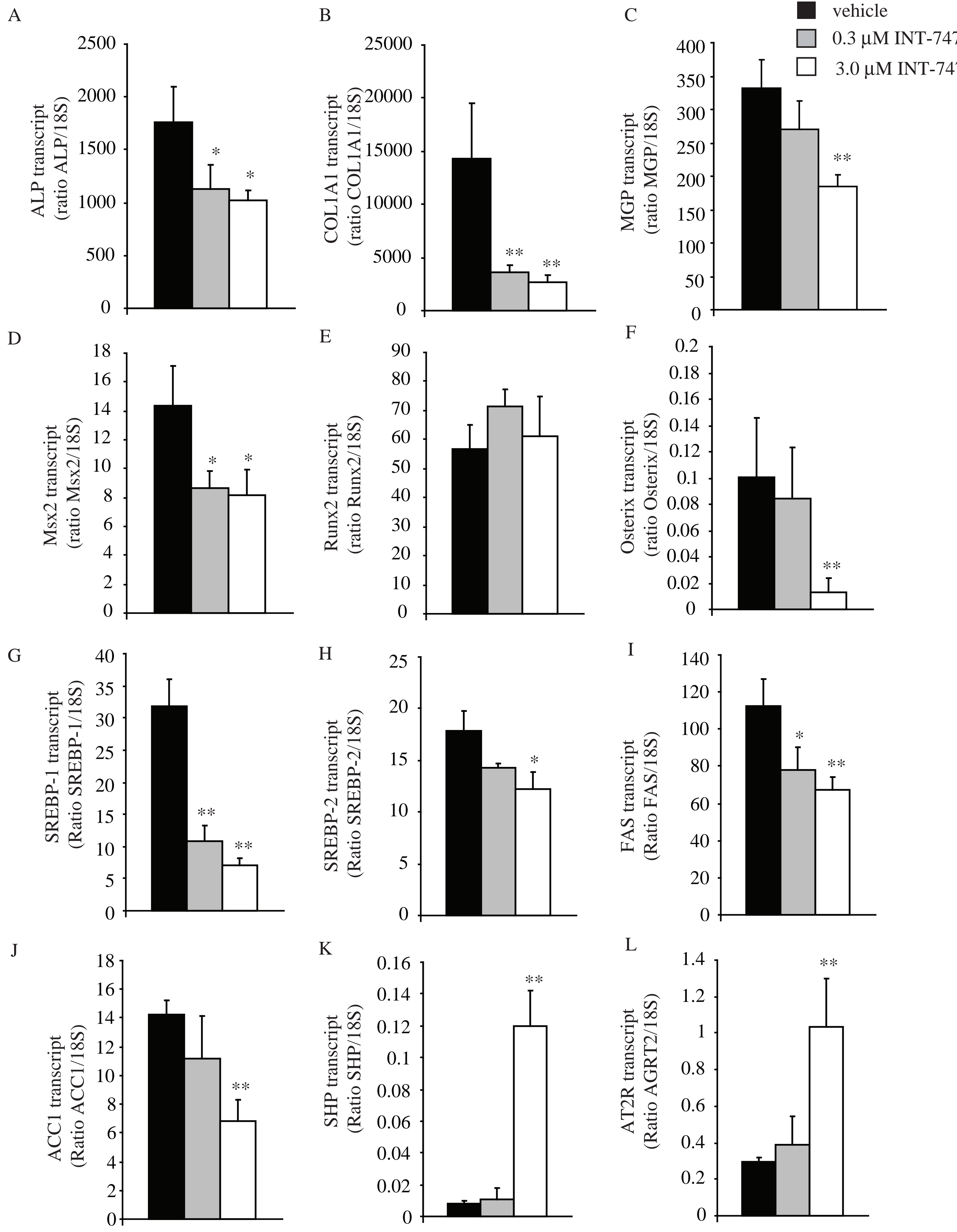

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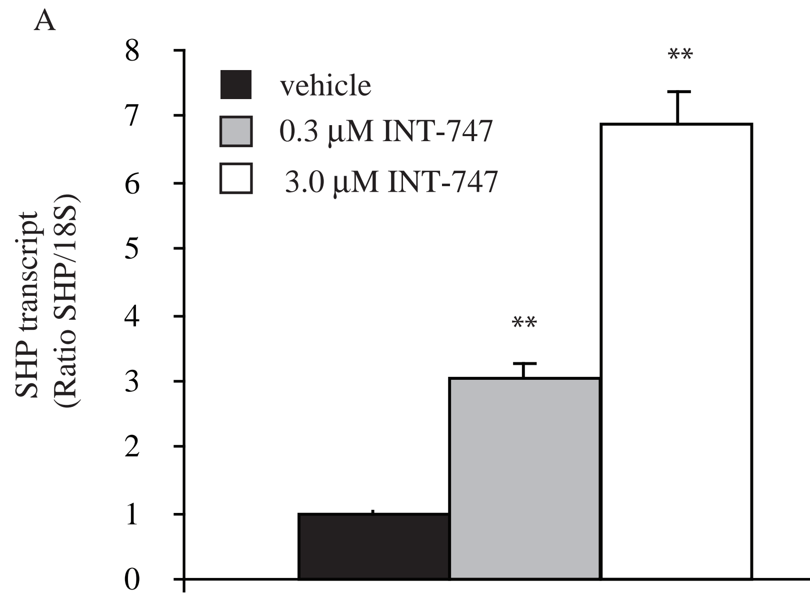

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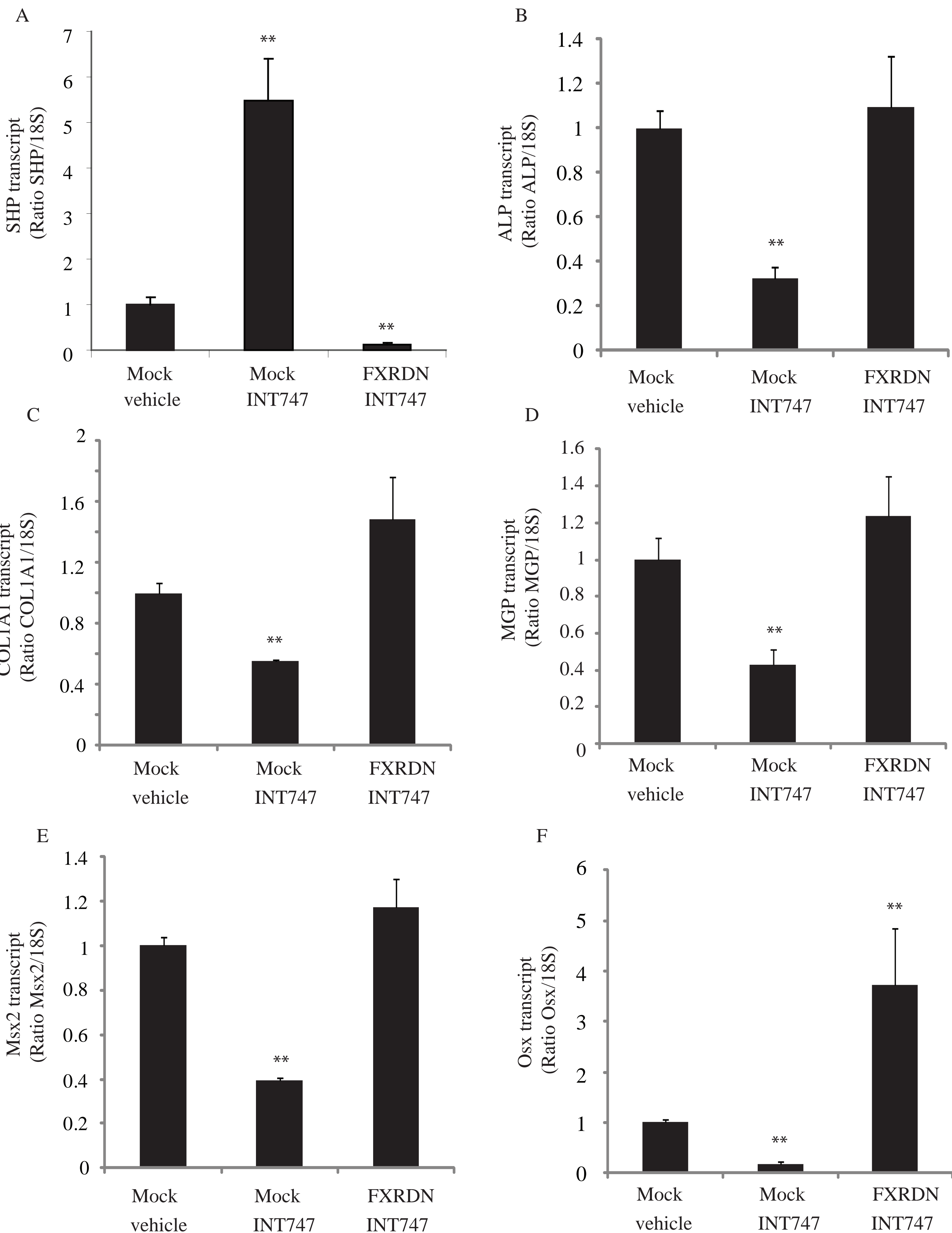

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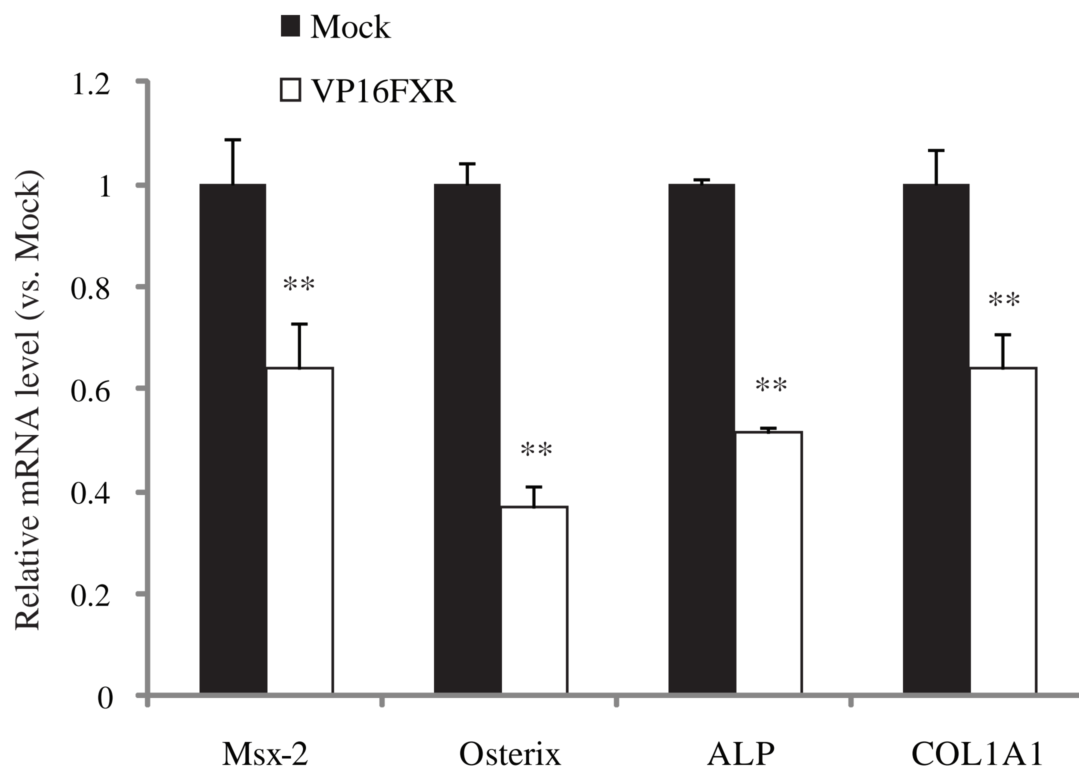

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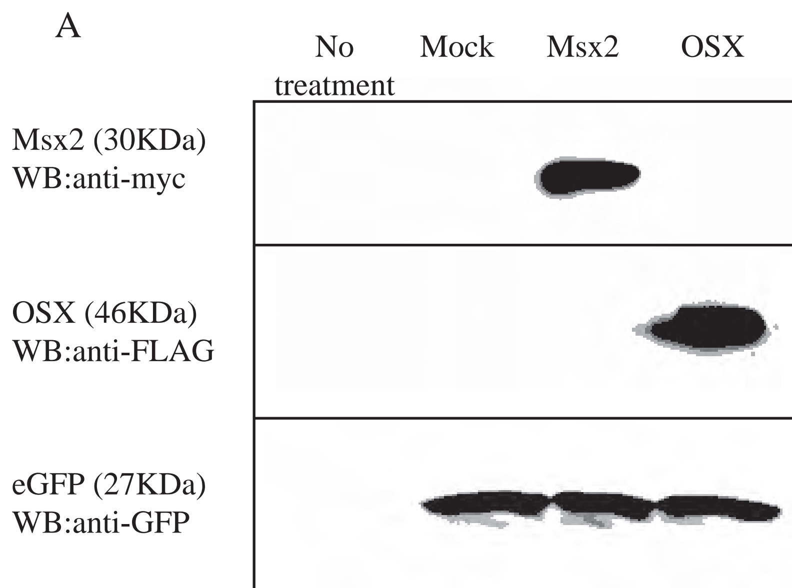

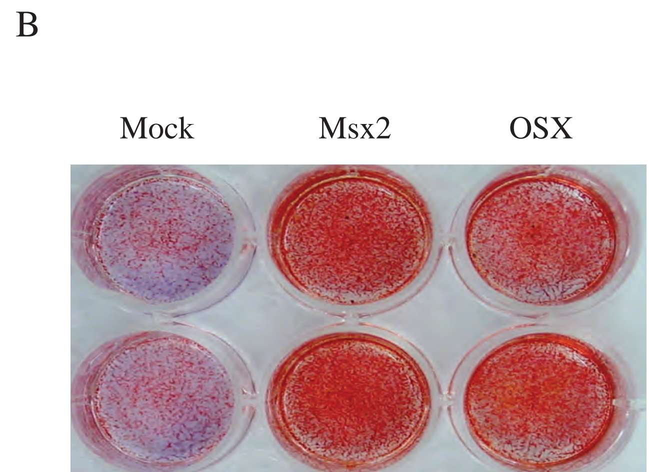

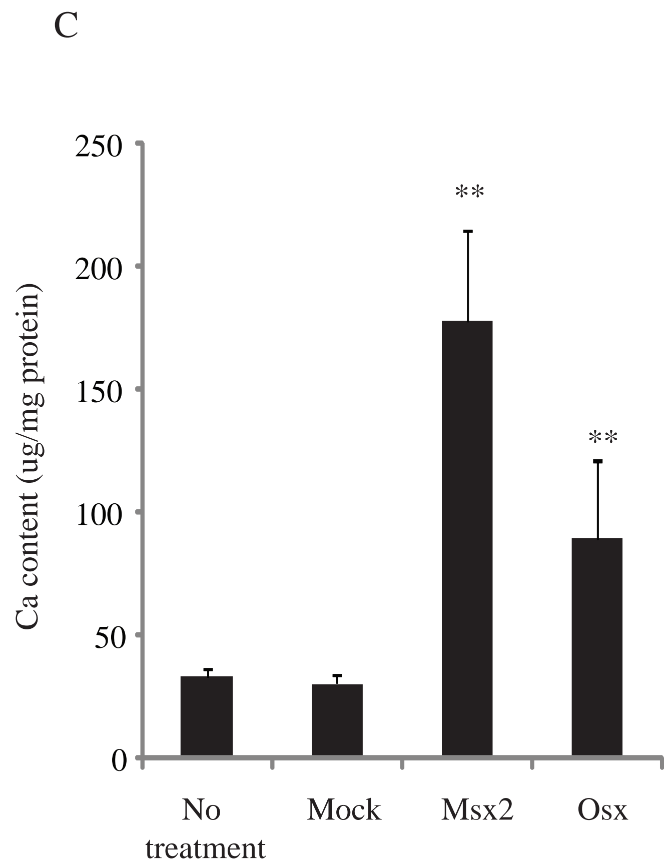

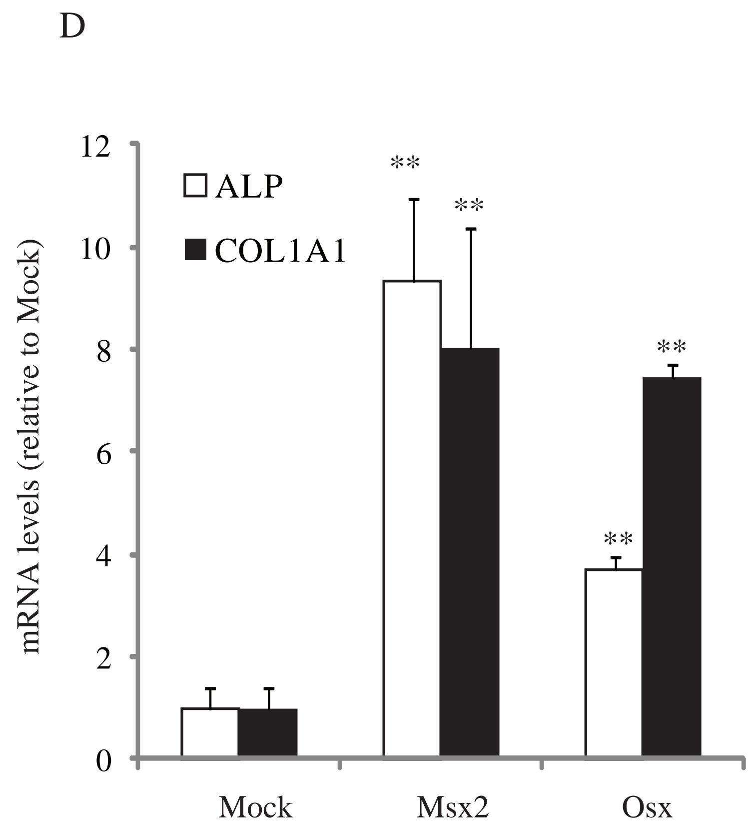

Online Figure VII

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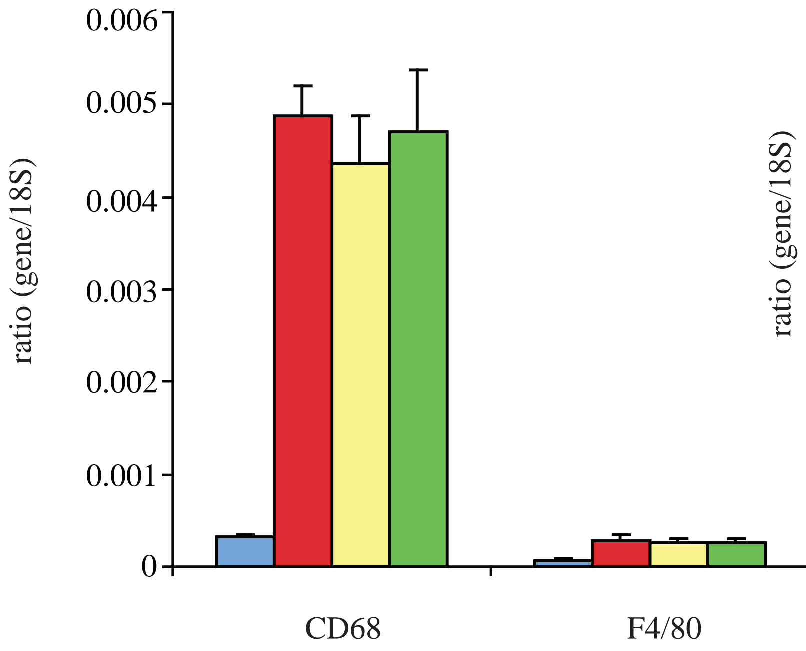

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