Supplemental Material

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Supplementary Materials

	Alcoholic Patients
N	10
Age, years	51 (39 - 66)
Sex, Male/Total	9/10
Race	White (10/10)
MELD	31 (22 - 40)
Prothrombin Time (INR)	2.49 (1.50 - 3.24)
Total Bilirubin (mg/dl)	5.15 (1.50 - 20.60)
Creatinine	2.13 (1.01 - 4.04)
AST, IU/L	41.50 (30.00 - 1425.00)
ALP, IU/L	163.50 (84.00 - 326.00)
Albumin, g/dL	3.30 (2.70 - 4.70)
Years since abstinence	0.67 (0.083 - 2)
Months since last drink	8.00 (1 - 24)
Collection type	Transplant (10/10)
Primary diagnosis	Alcoholic cirrhosis (10/10)
Hepatitis A, IgG+ /Total available	4/10
Hepatitis B core, IgG+ /Total available	0/10
Hepatitis B surface, IgG+ /Total available	2/10
Hepatitis C, IgG+ /Total available	0/10
HIV 1 and 2, IgG+ /Total available	1/10
CMV, IgG+ /Total available	5/10
EBV, IgG+ /Total available	10/10
Pathology report: Evidence of Dysplasia	0/10

Supplementary Table 1.

Demographic and patient data from human samples.

	Primers used in this study	
Gene	**Forward Primer – 5'-3'**	**Reverse primer – 5'-3'**
mTNFα	CACCACCATCAAGGACTCAA	AGGCAACCTGACCACTCTCC
mMCP-1	CAGGTCCCTGTCATGCTTCT	CAGGTCCCTGTCATGCTTCT
mActa2	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
mTGFβ	ATTCCTGGCGTTACCTTG	CTGTATTCCGTCTCCTTGGTT
mCollagen1a1	GCTCCTCTTAGGGGCCAT	CCACGTCTCACCATTGGG
mHIF1α	CAAGATCTCGGC GAAGCA A	GGTGAGCCTCATAACAGAAGCTTT
mHNF4	AGCTCGAGGCTCCGTAGTGTTT	GAAAATGTGCAGGTGTTGACCA
mF4/80	TGCATCTAGCAATGGACAGC	GCCTTCTGGATCCATTTGAA
mCD68	CCCACAGGGCAGCACAGTGGAC	TCCACAGCAGAAGCTTTGGCCC
mIL-1B	CTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG
mHNF6	TTCCAGCGCATGTCGGCGCTC	GGTACTAGTCCGTGGTTCTTC
mPPAR-γ	GGAAGACCACTCGCATTCCTT	TCGCACTTTGGTATTCTTGGAG
m/h18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
hGRHL2	GAAAGTCCAGTTTCACCAGAGG	GGCACTAAGGCCACTAGTCTTTT

Supplementary Table 2. Primers used, all are listed in 5' to 3'.

Supplementary Fig. 1: (A) Serum ALT from week 2, of a Lieber DeCarli chronic alcohol feeding model. (B) Serum Gaussia Luciferase (Gluc) activity measured weekly from (n=3-5) alcohol-fed mice given $6x10^{11}$ viral particles by tail vein injection. (C) Schematic representation of scAAV8-MIR122-OX treatment model. (D) Hepatocyte pri-MIR122 expression following in MIR122-OX treated mice following 5-weeks of Et or PF diet. (E) 8-week Lieber DeCarli and CCl4 alcoholic cirrhosis model utilizing 0.05 ul/kg CCl4

every 3 days and treatment with $6x10^{11}$ viral particles of AAV8 containing either Scr or MIR122-OX construct on week 4 (n=5-10/group). Percentages represent % EtOH vol/vol. (F) Serum GLuc activity measured weekly beginning 2 weeks following AAV8 MIR122-OX administration in murine model alcoholic cirrhosis (n=6-7group). (E) Total liver MIR122 expression following 8 weeks of alcoholic cirrhosis model. **P* < 0.05, ***P*<0.005, ****P*<0.0005 by Student's *t* test or two-way ANOVA.

Supplementary Figure 2: (a) Schematic representation of MIR122 promoter, transcription start site (TSS), Grainyhead 2 (GRHL2) dimer binding site and location of pre-MIR122 stem-loop. (B) Schematic representation of GRHL2 full length and spliced protein products, denoting the N-terminal transactivation domain in the FL variants, the central DNA binding domain, and the C-terminal dimerization domain. Percentages indicate sequence similarity between indicated domains of the FL and splice variant.

Supplementary Figure 3: (A) 5-week Lieber DeCarli chronic alcohol feeding model utilizing pretreatment with either AAV8-MIR122-TuD or Scrambled vector. Hepatic expression of (B) MIR122, and (C) pri-MIR122 in scrambled or MIR122-TuD treated WT or HIF-1α$^{hep-/-}$ mice after 5 weeks of control (PF) or alcohol (Et) diet. $*P < 0.05$, $**P<0.005$, $***P<0.0005$ by two-way ANOVA (n=6-14).

Supplementary Figure 4: Schematic representation of GRHL2-MIR122-HIF-1α axis in alcoholic liver disease.

Supplementary Figure 5: Full immunoblots of for GRHL2 in Human and Murine Livers.

Supplementary Methods

Isolation and culture of primary mouse hepatocytes and LMNCs

Anesthetized animals were perfused by way of portal vein with saline solution, followed by enzymatic digestion, as previously described [14]. The hepatocytes were separated by centrifugation, and LMNCs were purified by centrifugation in Percoll gradient followed by microbead selection using MACS LS columns (MACS). Primary hepatocytes were cultured in low-glucose DMEM supplemented with 10% fetal bovine serum, 1% Anti-Anti, and 1% insulin, transferrin, selenium solution. Primary hepatocytes were seeded in 6-well collagen-coated plates (Biocoat, Becton Dickinson). Before starting stimulation experiments, hepatocytes were rested for 4 hours.

Biochemical assays and cytokines

Serum alanine aminotransferase (ALT) levels were determined using a commercially available reagent (Advanced Diagnostics, Inc.) as described [1]. Liver triglycerides were extracted using a 5% NP-40 lysis solution buffer. Triglycerides were quantified using a commercially available kit (Wako Chemicals) followed normalization to protein amount by BCA protein assay (Pierce).

ELISA

Cytokine levels were monitored in liver whole cell lysates diluted in assay diluent following the manufacturer's instructions. MCP-1 and TNFα were measured using a specific anti-mouse ELISA (BioLegend). IL-1β was measured using a specific anti-mouse ELISA (R&D Systems) that recognizes pro-IL-1β and cleaved IL-1β. For normalization, the total protein loaded per sample was determined using BCA protein

assay (Pierce) using an aliquot of the whole cell lysate.

Confocal microscopy – immunofluorescence

Confocal images were processed as previously described [4]. Primary hepatocytes were fixed, permeabilized, and stained O/N with either anti-GRHL2 (Atlas antibodies, HPA004820), or normal rabbit IgG sc-2027 (Santa Cruz Biotechnology). Actin was stained using ActinGreen 488 anti-ReadyProbes Reagent (Cat. No. #R37110, Molecular Probes). Secondary antibody used was anti-rabbit Alexa Fluor 594 (Cat. No. #A-21207, Molecular Probes). Images were acquired using a Leica TCS SP5 II Laser Scanning Confocal Microscope.

RNA extraction and real-time PCR

Total RNA was extracted using the Qiagen miRNeasy kit (Qiagen) according to the manufacturer's instructions. Briefly, tissue samples were lysed in QIAzol Lysis reagent (Qiagen), homogenized with stainless steel beads in TissueLyser II (Qiagen) followed by miRNA isolation following the manufacturer's instructions and DNase I digest. RNA was quantified using Nanodrop 2000 (Thermo Scientific). Complementary DNA (cDNA) synthesis was performed by reverse transcription of 1 μg total RNA using the iScript Reverse Transcription Supermix (BIO-RAD). Real-time quantitative PCR was performed using Bio-Rad iTaq Universal SYBR Green Supermix and a CFX96 real-time detection system (Bio-Rad Laboratories). Primers were synthesized by IDT, Inc. The primer sequences are listed in Table S2 below. Relative gene expression was calculated by the comparative cycle threshold (Ct) method. The expression level of target genes was normalized to the house-keeping gene, 18S rRNA, in each sample and the fold-change

in the target gene expression between experimental groups was expressed as a ratio. Melt-curve analysis was used to confirm the authenticity of the PCR products.

Detection of pri-MIR122

Total liver cDNA was generated as stated above. Taqman Primer and probes specific for mmu-pri-MIR122-FAM and hsa-pri-MIR122-FAM were purchased from Applied Biosystems. Human-GAPDH-Hex (Applied Biosystems) or mouse-GAPDH-Hex (Bio-Rad), were multiplexed with target genes for normalization.

Western blot analysis

Whole cell lysates, nuclear and cytoplasmic extracts were prepared from mouse livers as described previously [4]. All westerns were performed under reducing conditions using Tris-glycine buffer system and blotted to nitrocellulose membranes. Proteins of interest were detected by immunoblotting with specific primary antibodies against GRHL2 (Atlas antibodies, HPA004820) and beta-actin-HRP (ab9482; Abcam). Respective horseradish peroxidase–labeled secondary antibodies were from Santa Cruz Biotechnology. The specific immunoreactive bands of interest were detected by chemiluminescence (Bio-Rad) The immunoreactive bands were quantified by densitometric analysis using an UVP System (Bio-Rad Laboratories, Hercules, CA).

EMSA

The DNA binding activity of HIF-1α was assessed by electrophoretic mobility shift assay as described previously [14]. Briefly, nuclear protein extract from liver (5 μg) was incubated with 50,000 cpm γ^{32}P-labeled HIF-1α consensus oligonucleotide at room temperature for 30 min. All reactions were run on a 4% polyacrylamide gel, and the

dried gel was exposed to an X-ray film at -80°C for different times. For the cold competition reaction, a 20-fold excess of the same, unlabeled, double-stranded oligonucleotide was added to the reaction mixture before adding the labeled consensus oligonucleotide.

HIF-1α Activity Assay

The DNA binding activity of HIF-1α was assessed by commercially available transcription factor assay kit (Abcam). Briefly, a dsDNA sequence containing the concensus HIF-1 alpha binding element (5'-ACGTG-3'), was immobilized to the wells of a 96-well plate. Nuclear protein extract from liver (10 μg) was incubated overnight at 4C. The HIF transcription factor-DNA complex was then detected by addition of a specific primary antibody directed against HIF-1 alpha complex. Calorimetric measurement was obtained using a secondary antibody conjugated to HRP followed by an acid stop. Absorbance was measured at 450 nm. For the cold competition reaction, a double-stranded oligonucleotide was added to the reaction mixture 20 minutes before adding the nuclear extracts.

Histopathological analysis

Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin (H&E), or Sirius Red and assessed for histological features of steatosis, inflammatory cell invasion, and fibrosis. The H&E and Sirius Red stained sections were independently examined by two pathologists, Dr. Garlick and Dr. Jin-Kyu Park in a blinded manner (see acknowledgments). Steatosis, inflammatory cell infiltrate and Sirius Red staining was evaluated on a 0-5 scale. Immunohistochemistry staining for GRHL2 (Atlas antibodies, HPA004820) were performed on formalin-fixed, paraffin-

embedded livers according to the manufacturer's instructions. ImageJ (NIH) threshold measurement was used for image analysis.

Construction of MIR122 antagonist and overexpression plasmids

The scAAV-anti-MIR122 TuD and scAAV-anti-Scr constructs were made as previously described[13]. The BamHI fragment carrying anti-MIR122 TuD was replaced with the pri-MIR122 sequence amplified from C57/BL6 mouse genomic DNA to generate the scAAV-pri-MIR122 construct (pri-MIR122F, 5'-GCGGGATCCGACTGCAGTTTCAGCGTTTGG-3' and pri-MIR122R, 5'-CGCGGATCCAAAAAAGACTCTAGGGCCCGACTTTACA-3')[13].

ChIP

Recombinant human GRHL2 with a GST-tag was expressed in Huh-7 cells. Chomatin immunoprecipitation was performed according to the manufacturer's instructions (Millipore). Briefly, nuclear lysates were sonicated using Biorupter (Diagenode). Sheared chromatin was incubated over night at 4oC with either Rabbit IgG (sc-2027, Santa Cruz Biotechnology) or Anti-GST (ab19256, Abcam) antibody. The following day, pulldown of the antibody-chromatin complex was performed using Bio-Rad Protein A Surebeads. DNA purification was performed using QIAquick PCR Purification Kit (28104, Qiagen). qPCR was performed using primers specific for the putative GRHL2 human MIR122 promoter locus.

MiRNA analysis

Reverse transcription (30 min - 16°C; 30 min - 42°C; 5 min - 85°C) was performed in an Eppendorf Mastercycler (Eppendorf, New York, USA) using 10 ng RNA, TaqMan primers and miRNA Reverse Transcription Kit (Applied Biosystems)

followed by quantitative RT-PCR in CFX96 (Bio-Rad) using TaqMan Universal Probes Master Mix (Bio-Rad). All tissue results were normalized to snoRNA202, or U6 expression based on Normfinder (http://moma.dk/normfinder-software) results. MIR122-FAM, U6-FAM, and sno202-FAM primer sets were purchased from Applied Biosystems.

Construction of MIR122 promoter plasmids

The human MIR122 promoter element was made by insertion of a 1.5 kb amplified sequence from human genomic DNA into a PGL4 plasmid.

Supp Fig 1

Supplementary Figure 1

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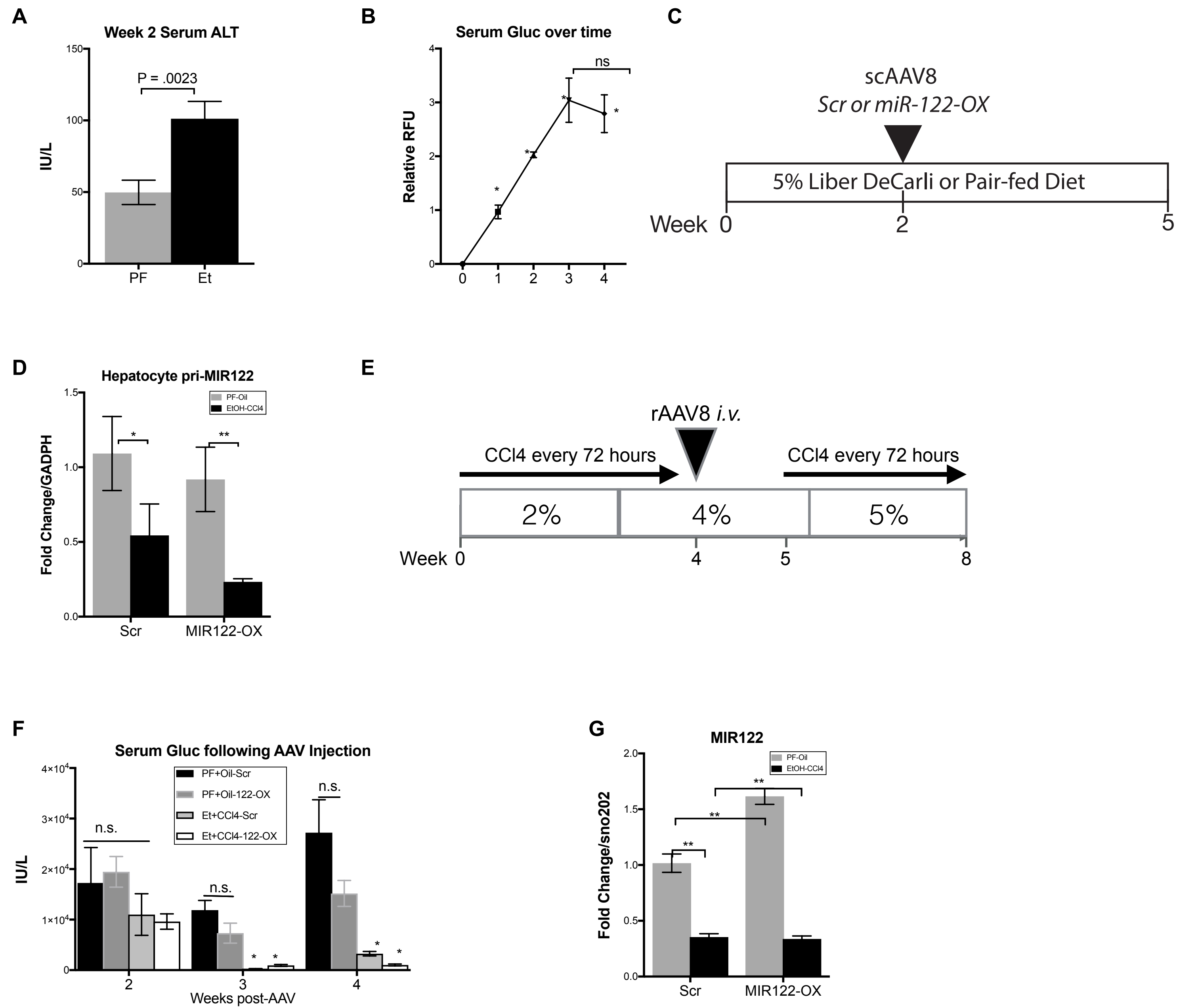

Supp Fig 2

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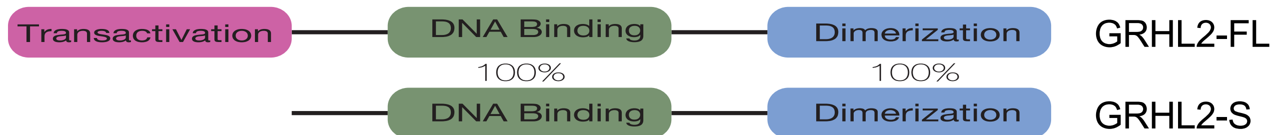
Supplementary Figure 2

A

B



Supp Fig 3

Supplementary Figure 3

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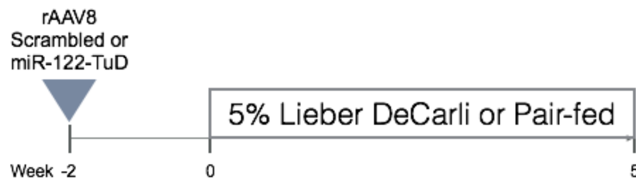
A



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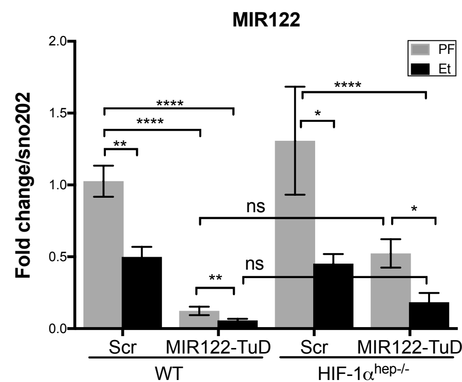
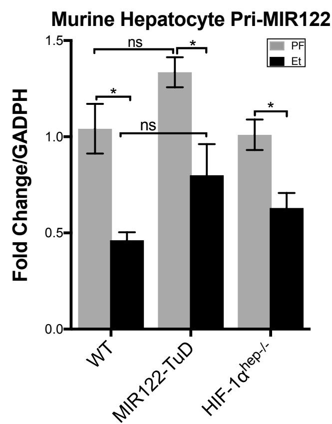


C



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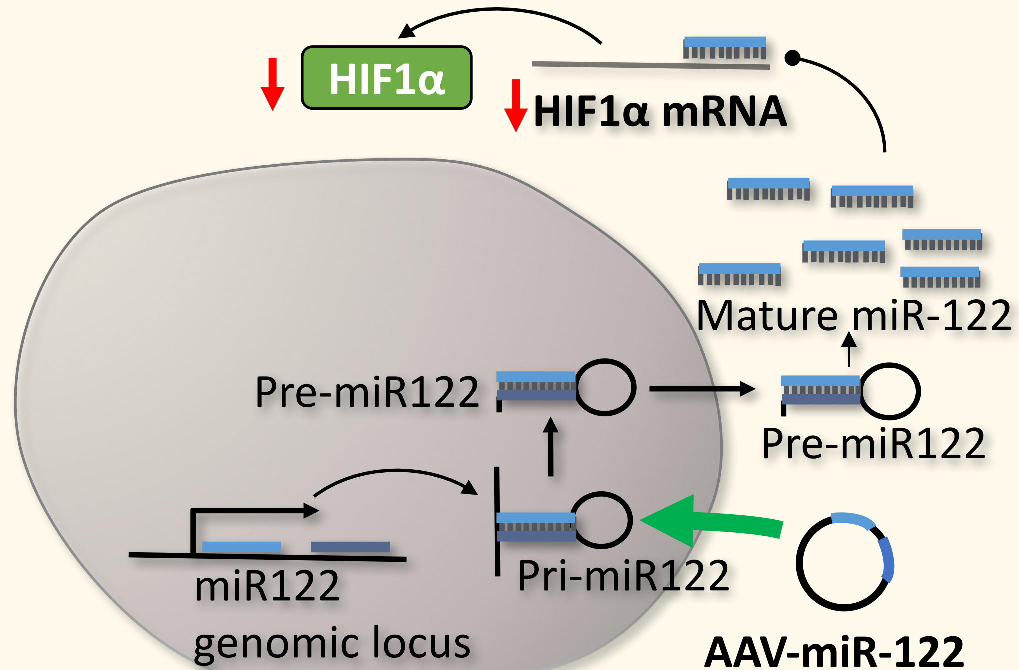

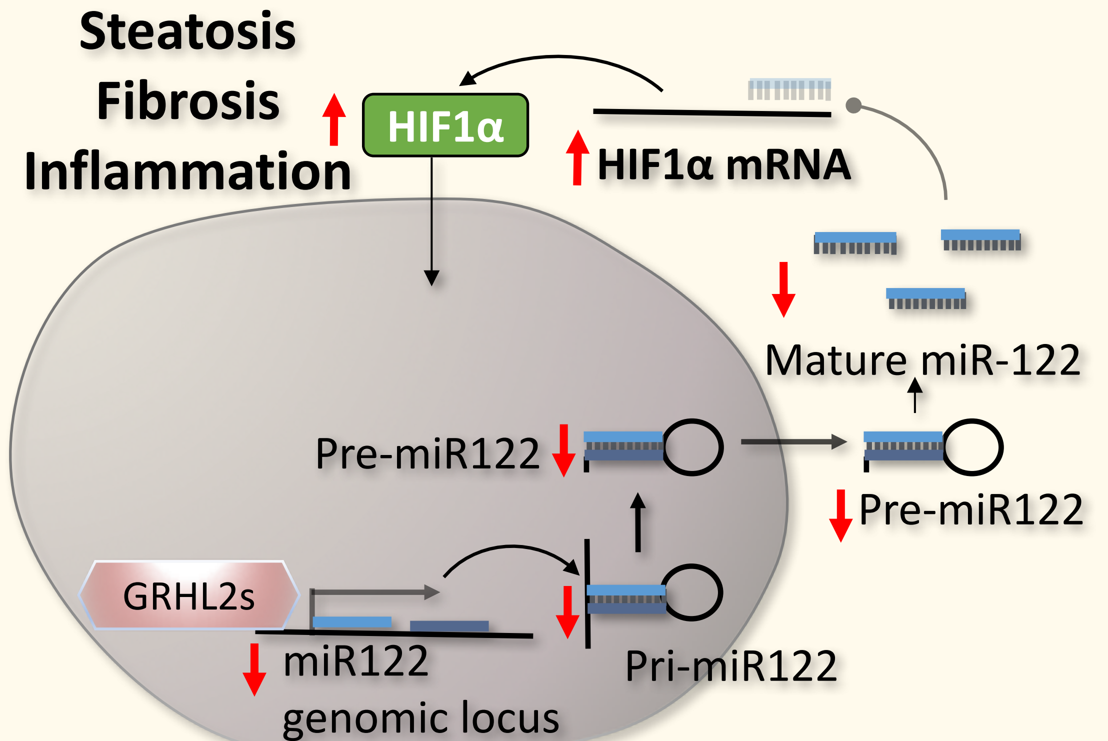

Supplementary Figure 4

Supplementary Figure 5:

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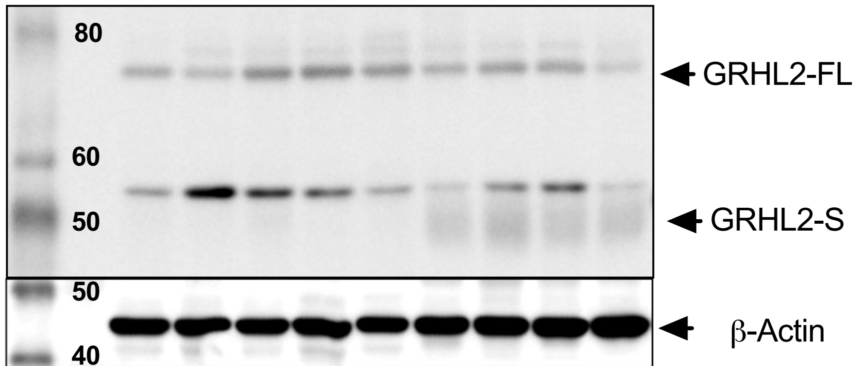
A) **Human Liver**



B) **Murine Liver**

80
60
50
40
GRHL2-FL
GRHL2-S

60
50
GRHL2-FL
GRHL2-S
β-Actin