Dysregulated autophagy and lysosome function are linked to exosome production via miR-155 in alcoholic liver disease

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Supplementary Methods

Animal studies

C57BL/6 wild-type (WT) and breeding pairs of miR-155 knockout (KO) mice were purchased from Jackson Laboratory. Colony of miR-155 KO mice was maintained in the UMMS animal facility. Upon arrival to the UMMS animal facility, wild type mice from Jackson Laboratories were co-housed in cages using a mix of fresh alum and alum of miR-155 KO mice (approx. 1:1 ratio) for 2 weeks. This approach has been used in the literature by another investigator (1, 2). Briefly, C57BL/6 mice were fed a control (PF) or ethanol diet (8-10 mice per group) as described previously (3). After 10 days on the Lieber-DeCarli diet, a group of mice received intraperitoneal injection of chloroquine (65mg/kg) followed by single acute gavage 9h prior to sacrifice. Control mice received

saline injection intraperitoneally and sucrose solution gavage. After experimental treatments, blood and liver samples were collected, processed immediately, and stored at -80˚C for further analysis.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed according to standard protocols (4). Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated. Then tissue sections were heated in a microwave in 0.01mol/L sodium citrate buffer for antigen retrieval. After treating with 3% H_2O_2 for 10 min and washing 3 times with PBS, the slides were blocked using 5% BSA in PBS. Following incubation with LAMP2 antibody, detection was performed using a biotinylated secondary antibody and streptavidin-Horseradish peroxidase, followed by colorimetric detection using DAB. Similarly, for immunofluorescence slides were incubated with primary antibody at 4°C, overnight. Following incubation of slides with corresponding primary antibody, the slides were incubated with fluorescent secondary antibodies (green for LC3 and red for p62 proteins)

Isolation of primary hepatocytes and Kupffer cells from mouse livers

Primary hepatocytes and Kupffer cells (KCs) were harvested from mice following liver perfusions as previously described (4-7). Briefly, mice were anesthetized with ketamine (100 mg/kg) and livers were perfused with saline solution for 5 minutes followed by *in vivo* digestion with liberase enzyme (20 mg/L) for 5 minutes at 37°C. The hepatocytes were separated by centrifugation for 5 minutes at 200*g*, while the non-hepatocyte content was loaded on the top of a 50%-25% Percoll gradient and centrifuged for 30 minutes at 800*g* (5, 8). Primary murine hepatocytes were seeded on collagen-coated plates and

maintained in Dulbecco's Modified Eagle Medium (DMEM) low glucose medium supplemented with 10% fetal bovine serum (FBS) and a 1% insulin, transferrin, selenium solution similar to a previous reports (4, 5). Primary liver mononuclear cells (LMNCs) were seeded in 24-well plates and cultured in DMEM with 10% FBS (4, 5). The non-adherent fraction was washed, and the adherent KC population was used for the experiments.

Cell lines and alcohol treatment

For *in vitro* studies, immortalized mouse Hepa1-6 hepatocytes and RAW264.7 macrophage cell lines were used. Immortalized cell lines and primary cells were maintained in culture medium supplemented with exosome-depleted FBS (10%) (SBI, USA). Exosome-depleted FBS was used to exclude contamination with bovine exosomes. Alcohol treatment of cells involved supplemented cell culture complete medium (DMEM + 10% exosome-depleted FBS +1% penicillin and streptomycin) with 50mM ethanol. Subsequently, culture media was replaced, and stimulation was performed as indicated in the figure legends.

Western blotting analysis

For Western blotting, membranes were probed with specific antibodies for the following proteins: Beta-Actin (cat. # ab49900), LAMP2 (cat # ab13524), Rheb (cat. # ab25873), and TFEB (ab2636) from Abcam. LC3 I/II (cat. # 12741S), p62 (cat. # 39749S), Beclin1 (cat. # 3738), Atg3 (cat. # 3415), Atg7 (cat. # 2631), mTOR (cat. #2983S), and Rab7 (cat. #9367S) were from Cell Signaling. LAMP1 (cat. # AF 4320) was from Millipore.

Antibodies for S6, phospho-S6 (ser235/236), 4EBP1, and phospho-4EBP1 (ser65) were from Cell Signaling.

Real time quantitative PCR (RT-qPCR)

For gene expression analysis, we used RT-qPCR methods as described (5). RNA was extracted using QIAzol Lysis reagent (Qiagen, USA) and total RNA was extracted using the miRNeasy isolation kit (Qiagen, USA). Nanodrop analysis was used to determine the concentration and purity of RNA. TaqMan miRNA Assay (Applied Biosystems) was used to analyze the miRNA from primary hepatocytes and KCs using cellular SnoRNA-202 as an internal control (5). For nucleic acid analysis from exosomes, an exogenous spiked-in cel-miR-39 was used as an internal control.

Exosome isolation and quantification

Exosomes from serum and cell culture supernatants were isolated similarly to previous reports (9). Briefly, the serum samples were centrifuged at 1,500*g* for 10 mins to remove cells and 10,000*g* for 20 mins to remove residual cellular debris. Samples were filtered through 0.8 and 0.22μm filters (Millipore, USA). For exosome precipitation, we used ExoQuick-TC™ reagent according to the manufacturer's guidelines (SBI, USA). After exosome precipitation, exosome pellets were resuspended in sterile phosphate buffered saline (PBS) then quantified using NanoSight Tracking Analysis. Before performing the experiments, the instrument was calibrated with 100 nm polystyrene beads (Thermo Scientific, USA). The samples were captured for 30 s at room temperature. We used the NTA software to process the video captures and determine the concentration

(particles/mL) and size distribution (in nanometers) of the measured particles. From every individual sample, three independent captures were taken. Exosomes were characterized and exosome markers were assessed by Western blot analysis as previously described (9).

Transfection

For miR-155 overexpression, macrophages (RAW 264.7) and hepatocytes (Hepa 1-6) were transfected with either negative control mimic or miR-155 mimic (5). Macrophages were transfected with 5 nM and hepatocytes with 1nM (Applied Biosystems) using RNAi MAX transfection reagent (Thermo Fisher Scientific). Cells were harvested 24h after transfection and used for qPCR and Western blot analyses. For LAMP1/LAMP2 knockdown, macrophages (RAW 264.7) and hepatocytes (Hepa 1-6) were plated onto 6-well plates at 200,000 cells/well one day before transfection. Lipofectamine RNAiMAX (Invitrogen, USA) was used to deliver LAMP1/LAMP2 siRNA or control siRNA into macrophages. Cells were harvested 48h after transfection and used for Western blot. LAMP1 and LAMP2 Silencer® Select and the control siRNA were purchased from Ambion Inc (USA).

Results were analyzed from three independent experiments.

Lactate dehydrogenase (LDH) assay

The release of LDH due to cell membrane damage was quantified using the LDH assay kit following the manufacturer's protocol (Abcam, USA). The cells were treated and incubated at 37 °C as indicated in the figure legends. The supernatants were centrifuged

at 10,000*g* for 15 min at 4 °C. Supernatants (50 µL) were transferred to the assay plate and a mixed detection kit reagent (50 µL) was then added to each of the assay wells. NADH served as standard. The absorbance was taken after 30 min at 450 nm using a multiwell assay plate reader.

Supplementary References

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Supplementary Figure Legends

Suppl. Fig 1. Autophagy induction during alcoholic liver disease in mice and humans depends on the mTOR pathway. Total liver proteins were extracted from pair-

fed (PF) or chronic alcohol diet (EtOH) fed mice (n=8-10/group) and analyzed by Western blot for Atg3 (A). Liver protein extracted from pair-fed (PF) or chronic alcohol diet (EtOH) fed mice (n=8-10/group), control subjects, and ALD patients (n=6-8) were analyzed by Western blot for Rheb (B) and total as well as phosphorylated levels of S6 (Ser235/236) (C) and 4EBP1 (D). β-actin was used as a loading control. The densitometry analysis is shown as bar diagrams. * $p<0.05$.

Suppl. Fig 2. Alcohol-induced increase in LC3 and p62 positive autophagosomes in WT mice was attenuated in alcohol-fed miR-155 KO mice

Total liver mRNA was extracted from pair-fed (PF) or chronic alcohol-fed (EtOH) WT and miR-155 KO mice (n=8-10). p62 mRNA levels were quantified by qPCR and normalized to 18s * $p<0.05$. (A). Immunohistochemistry images of LC3 and p62 in liver sections from pair-fed (PF) or chronic alcohol diet-fed (EtOH) WT and miR-155 KO mice. (B). A larger inset of the LC3 and p62 colocalization (C). Composite images of LC3 and p62 colocalization (D). The nuclei were stained using DAPI.

Suppl. Fig 3. miR-155 KO mice are protected from alcohol-induced liver injury and steatohepatitis.

Total liver protein was extracted from pair-fed (PF) or chronic alcohol fed (EtOH) WT and miR-155 KO mice (n=8-10) and an Enzyme-Linked Immunosorbent Assay (ELISA) was performed to determine the protein levels of the main liver pro-inflammatory cytokines p62 (A) TNF-α (B) IL-1β (C) and MCP-1 (D). Measurements were corrected for the protein content of the tissue samples using results from the Bradford protein assay. Serum ALT

levels were used to determine the level of liver damage (E) and H&E liver histology (F) was performed to determine steatosis. * $p<0.05$.

Suppl. Fig 4. Overexpression of miR-155 in macrophages and hepatocytes.

Macrophages (RAW 264.7) (A) or hepatocytes (Hepa 1-6) (B) were untreated or transfected with either a miR-155 mimic or a control mimic and cells were harvested 24 hours after transfection. Total RNA was used to quantify miR-155 expression and SnoRNA-202 was used as internal control. *, # $p<0.05$.

Suppl. Fig 5. miR-155 targets the 3'UTR region of LAMP1 and LAMP2.

Bioinformatics analysis showing the miR-155 seed binding region at the 3'UTR region of LAMP2 (A) and LAMP1 (B) genes.

Suppl. Fig 6. Alcohol-induced decrease in TFEB expression in wild-type mice was prevented in miR-155 KO mice

Total liver protein was extracted from pair-fed (PF) or chronic alcohol fed (EtOH) WT mice (n=8-10) and analyzed by Western blot using β-actin as a loading control. The immunoblot was probed for Rab7 (A) from WT mice. TFEB protein levels were determined from mouse livers (B) and human livers with ALD (C). Total liver protein (D) and mRNA (E) was extracted from pair-fed (PF) or chronic alcohol-fed (EtOH) WT and miR-155 KO mice and a Western blot was performed for TFEB protein using β-actin as a loading control (D). Total RNA was used to quantify TFEB by qPCR (E) and Ct values were normalized to 18s. * $p<0.05$, **p<0.01, *** $p<0.001$, **** p<0.0001.

Suppl. Fig 7. Characterization of exosomes isolated from hepatocytes and Kupffer cells.

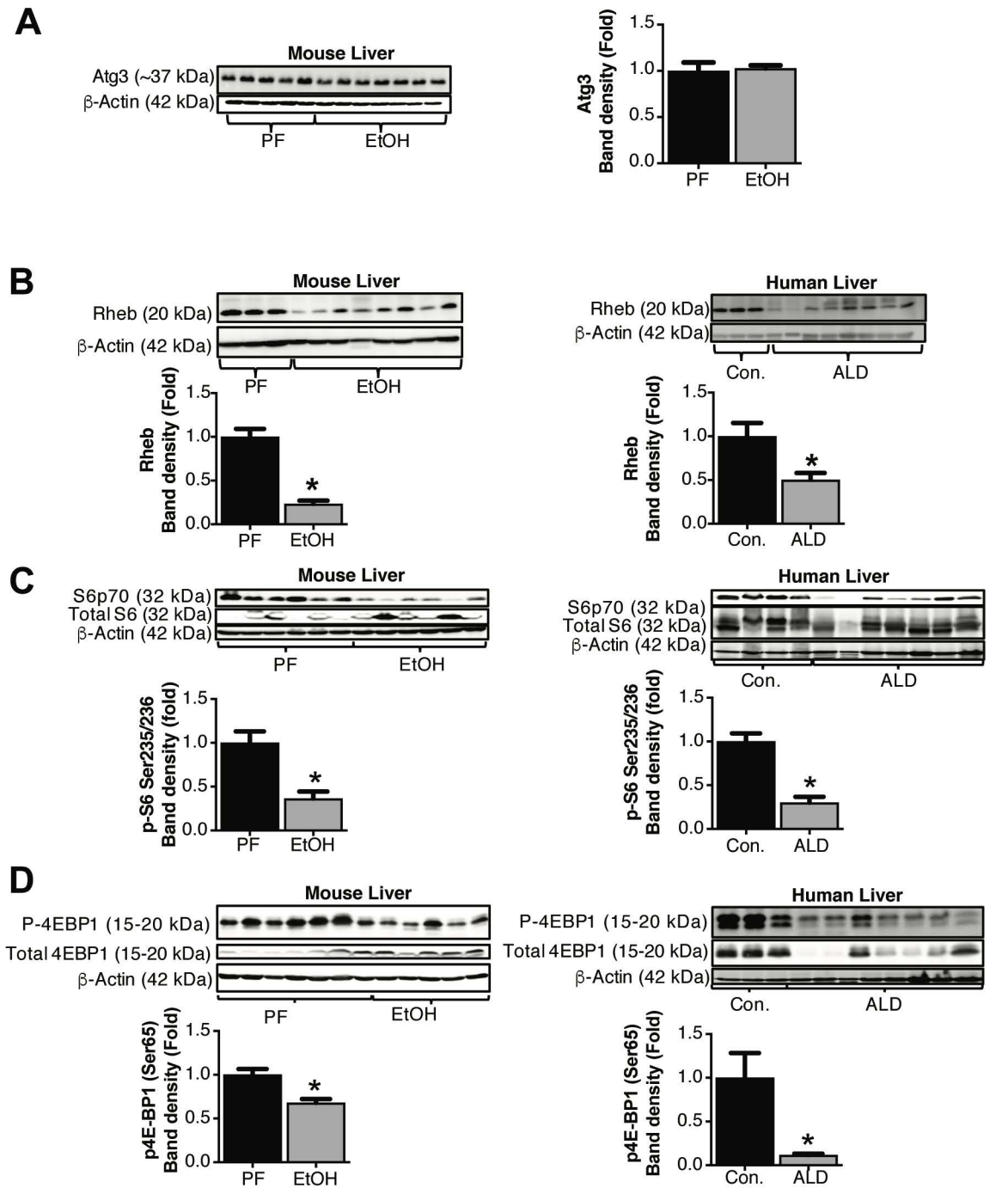
Primary hepatocytes and Kupffer cells from WT pair-fed mice (n=6-8) were isolated by perfusion and cultured. Cell- and debris-free supernatants collected from cultured hepatocytes (A) and Kupffer cells (C) were used to measure the particle size by NanoSight. Exosomes were isolated using ExoQuick TC from the supernatants and lysed with RIPA buffer. Total proteins (~20 µg) from exosomes and cells [hepatocytes (B) and Kupffer cells (D)] were used for Western blot analysis and probed with Alix, Calnexin and CD63 antibodies. Beta actin served as a loading control for cells. * $p<0.05$.

Suppl. Fig 8. Bafilomycin A treatments did not cause any cellular toxicity. LDH assay was performed from macrophages (A) and hepatocytes (B) supernatants (n=12).

Suppl. Fig 9. LAMP1 and LAMP2 knock down in macrophages and hepatocytes.

Macrophages (RAW 264.7) were untreated or transfected with control or LAMP1 and LAMP2 siRNA and the protein levels analyzed by Western blot for LAMP1 and LAMP2 (A), using β-actin as a loading control (n=6). The densitometry analysis is shown as bar diagrams. Hepatocytes (Hepa 1-6) were untreated or transfected with control or LAMP1 and LAMP2 siRNA and the protein levels analyzed by Western blot for LAMP1 and LAMP2 (B) using β-actin as a loading control (n=6). Macrophages (RAW 264.7) were untreated or transfected with control or LAMP2 siRNA and TFEB (C) levels were analyzed by Western blot using β-actin as a loading control (n=3). * $p<0.05$, **p<0.01, *** $p<0.001$.

Supplementary Figure 1



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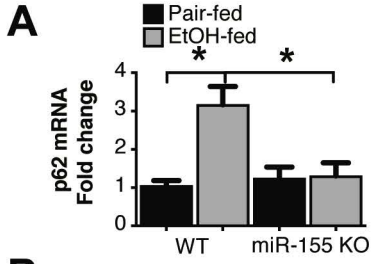
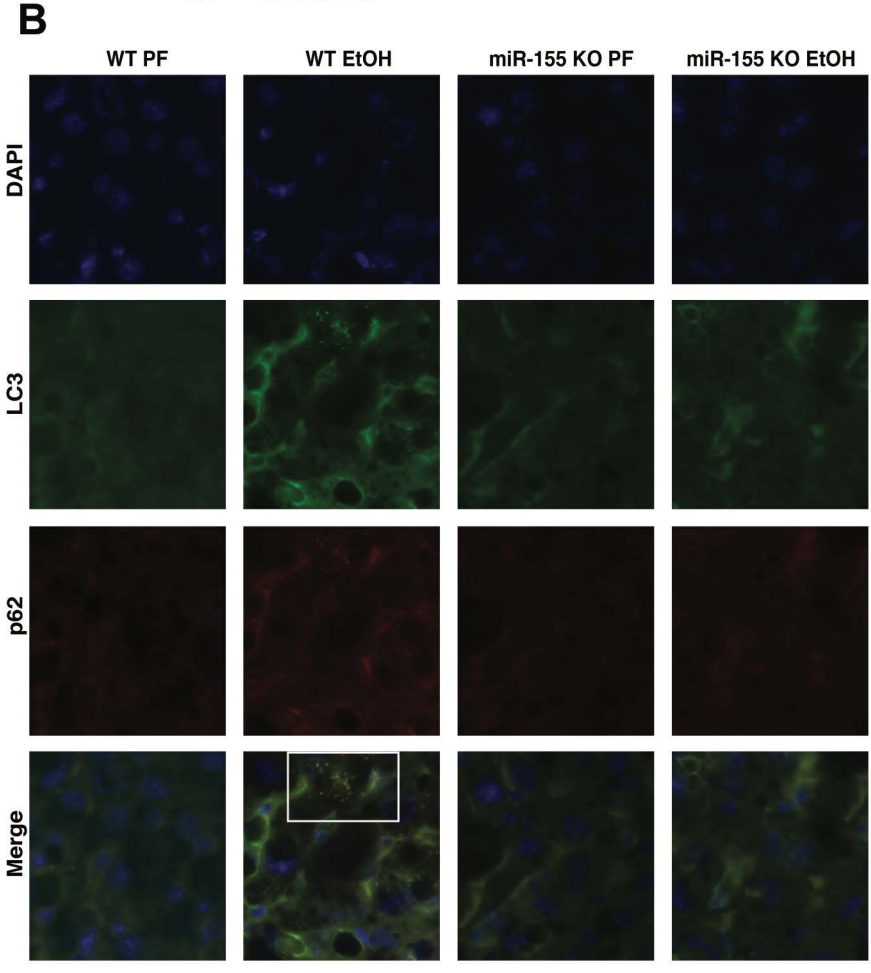
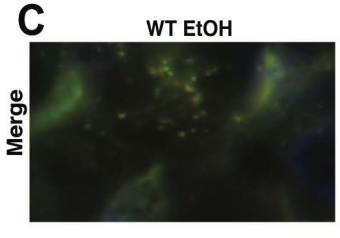
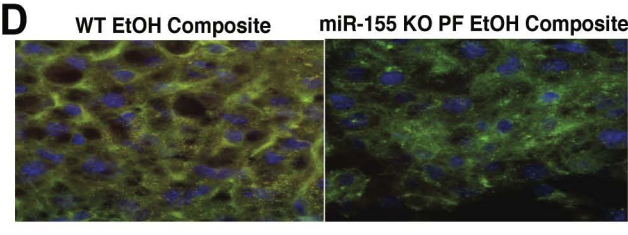
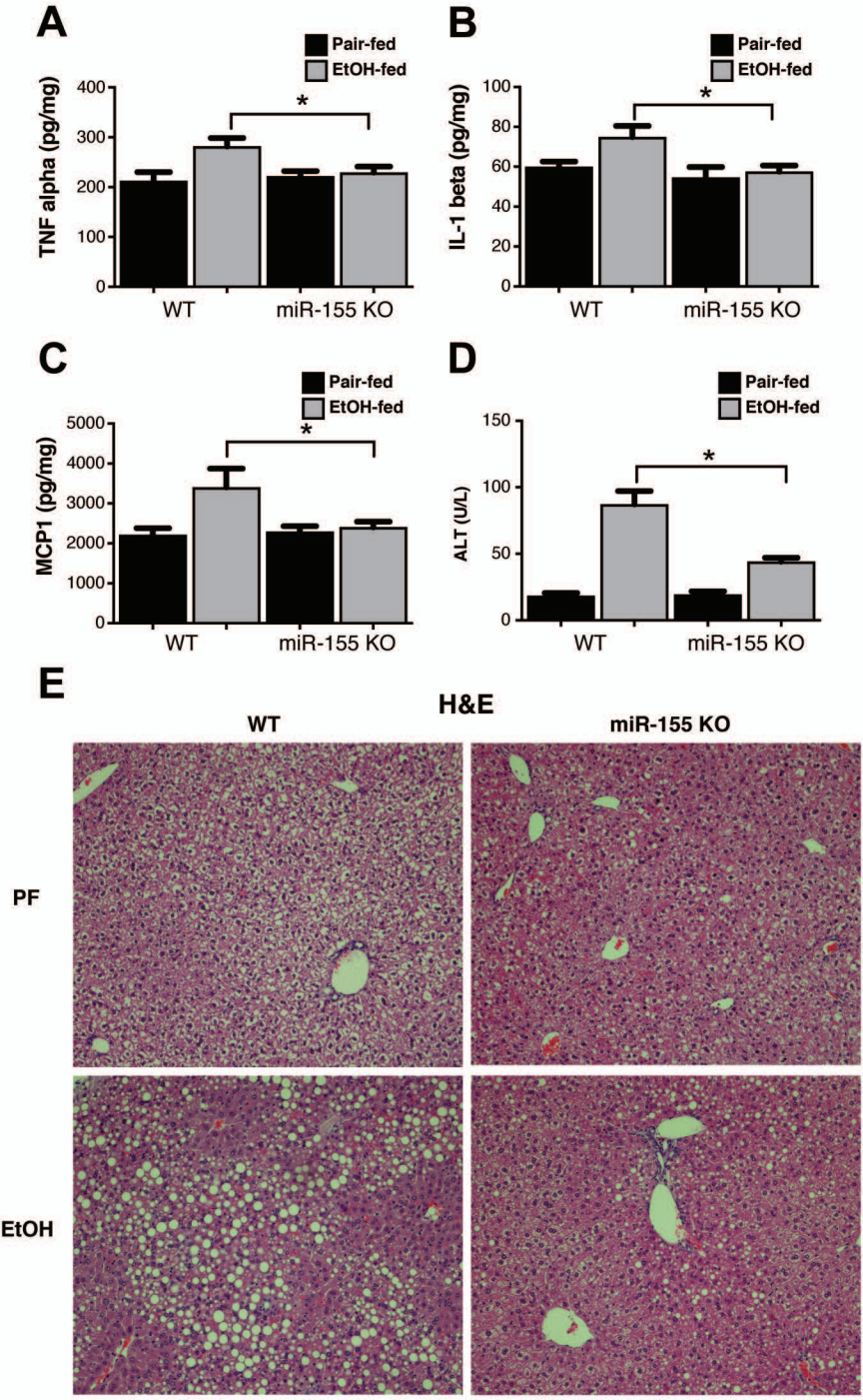




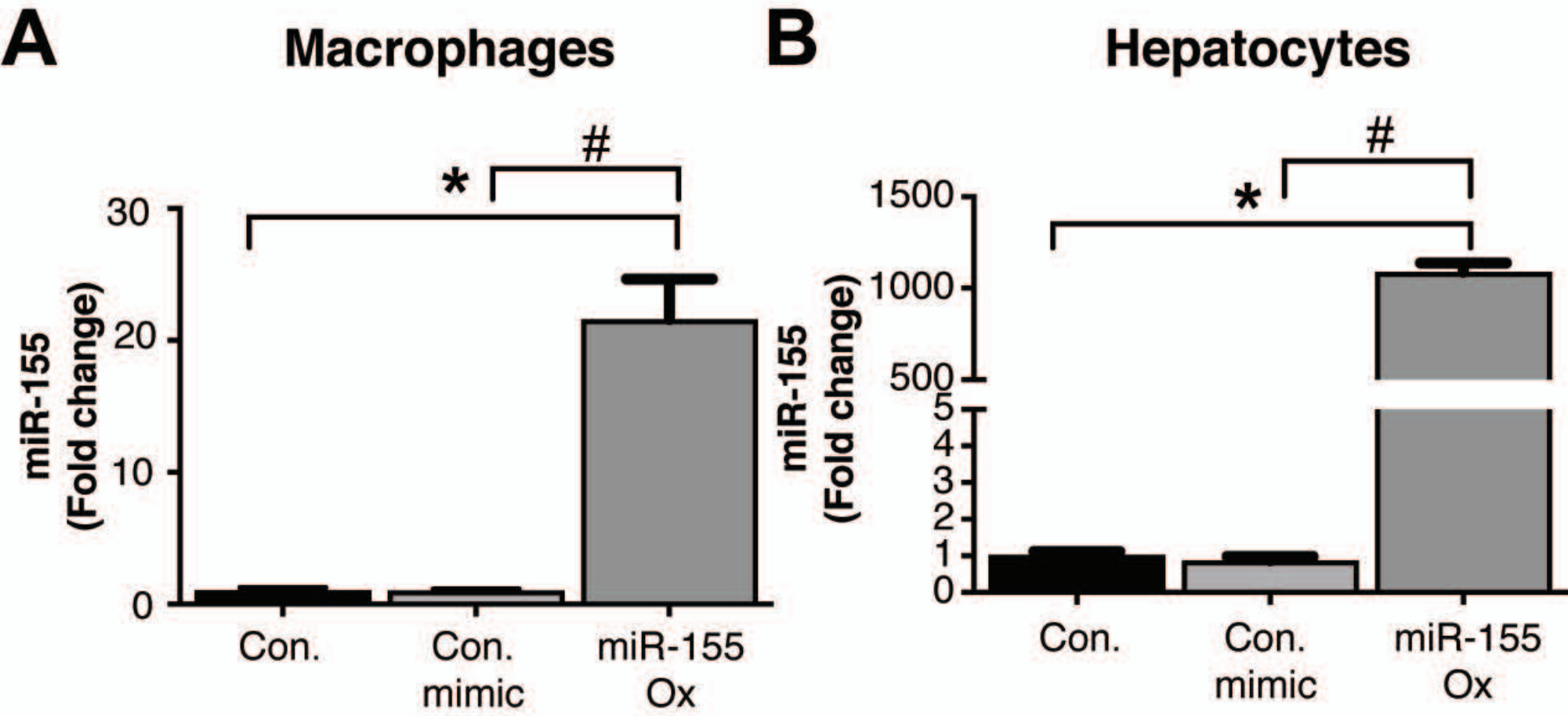




Supplementary Figure 3

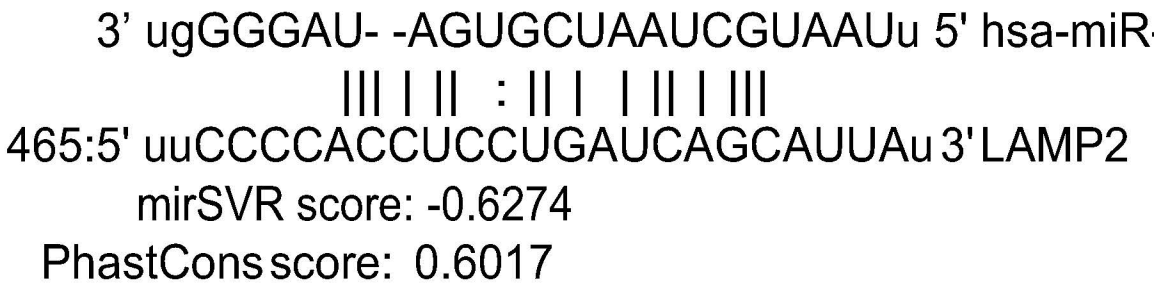


Supplementary Figure 4



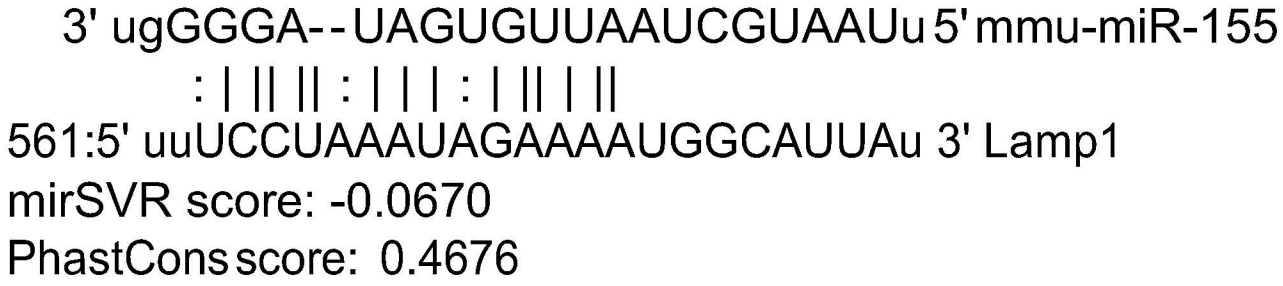
Supplementary Figure 5

A **HSA-miR-155/LAMP2 Alignment**

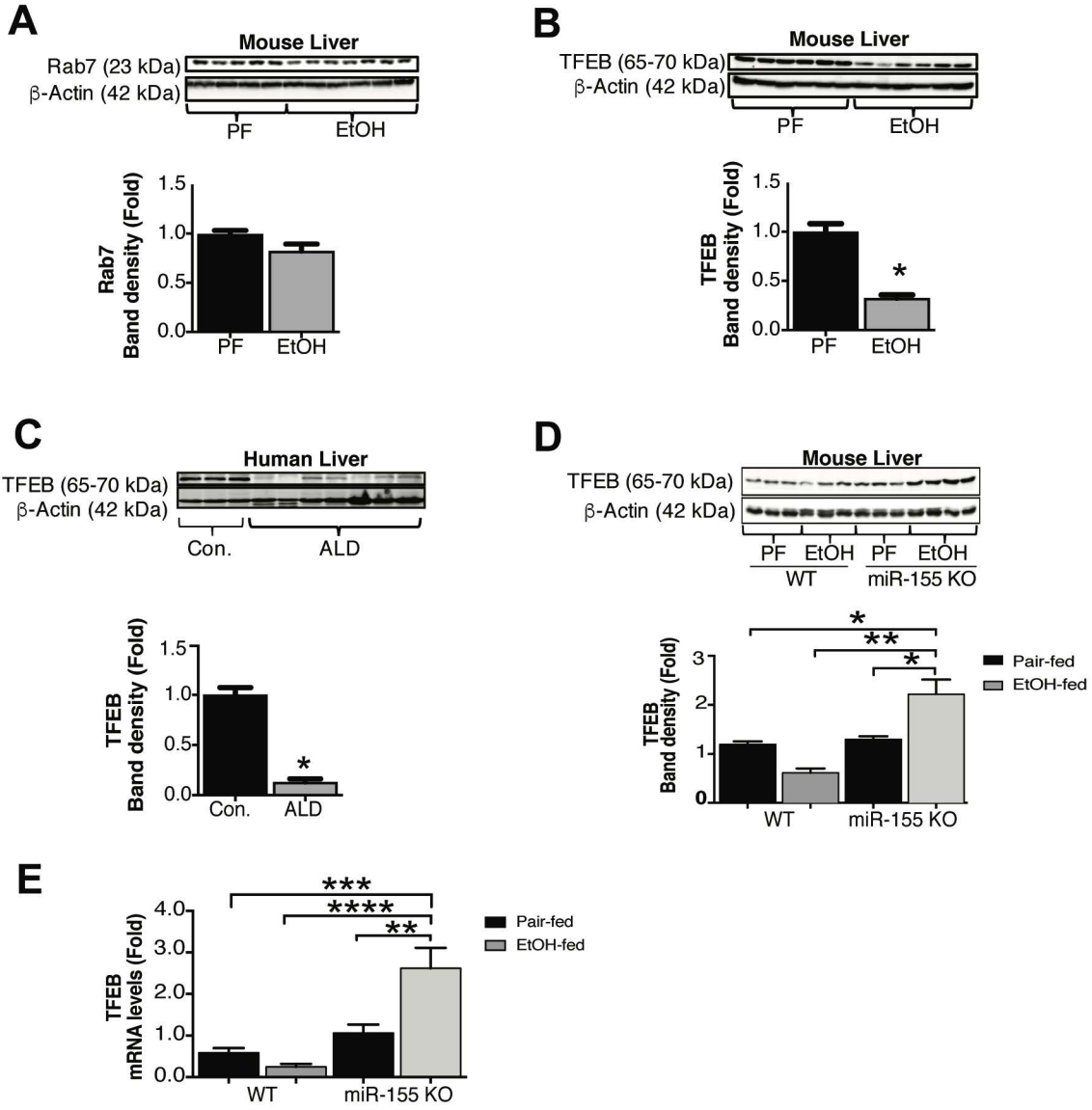


B

mmu-miR-155/LAMP1 Alignment



Supplemental Figure 6



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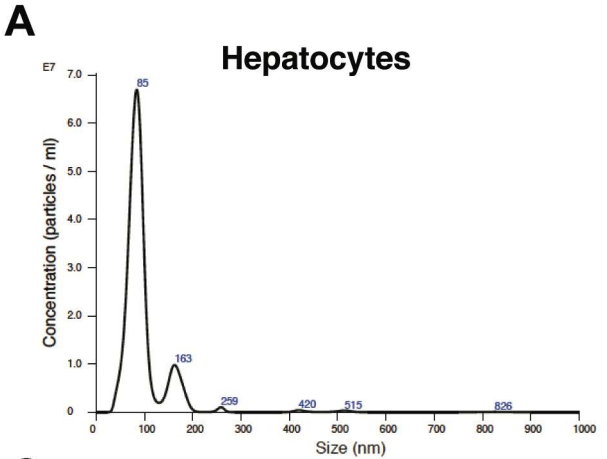

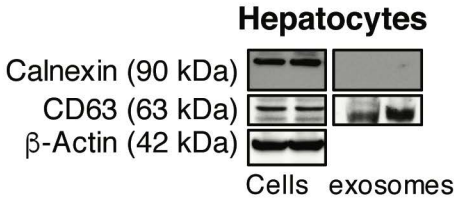

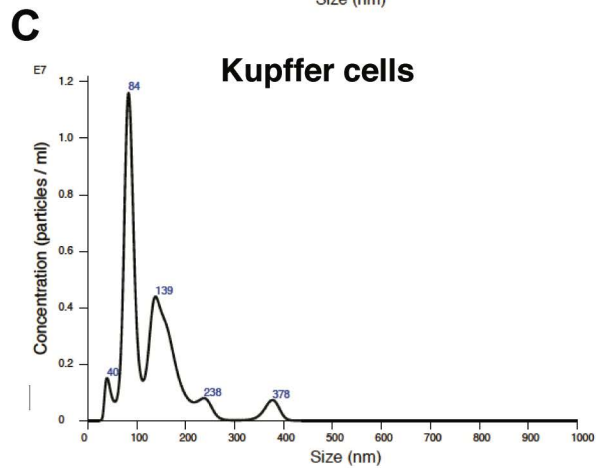

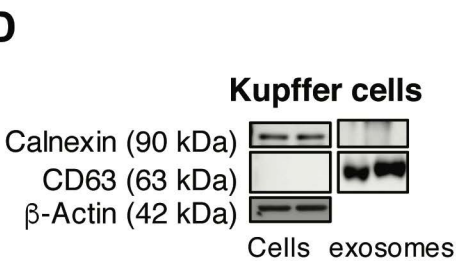

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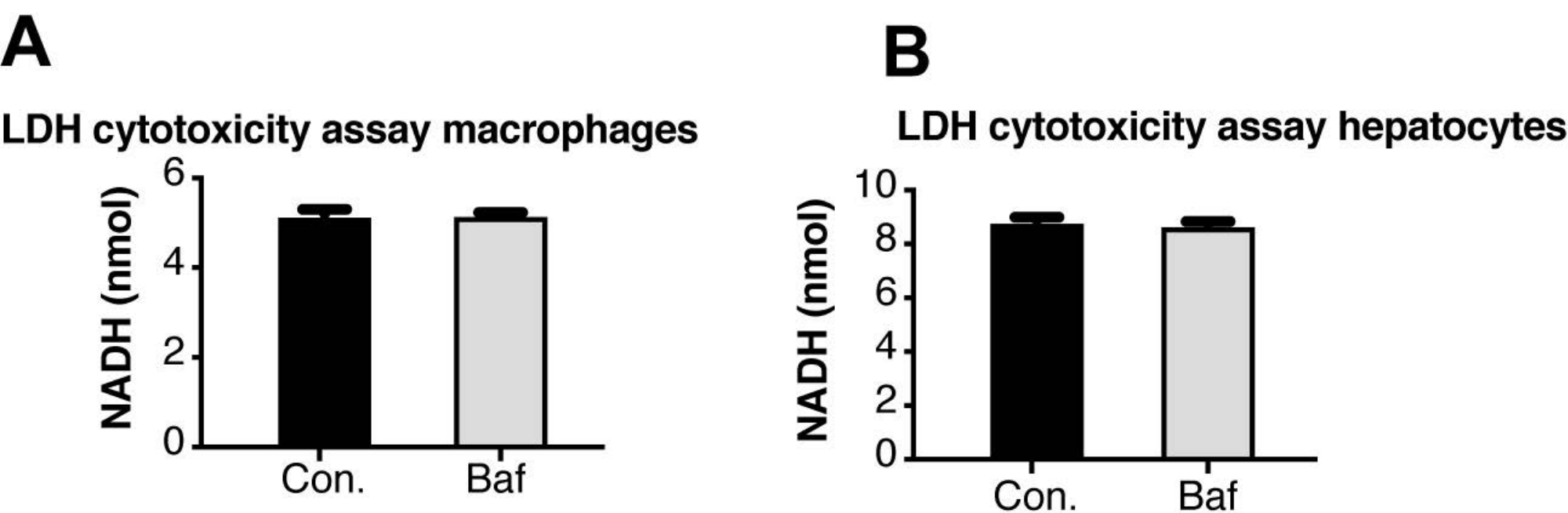

Supplementary Figure 9