

Supplementary Information

A hepatocyte–macrophage miR-34a axis governs lipid accumulation and inflammation and reveals IL-6 as a steatosis-selective therapeutic target

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This file includes:

Supplementary methods

Supplementary figures

Supplementary Fig. S1. MiR-34a boosts hepatic and macrophage lipid accumulation via IL-6/AMPK and ZFX3

Supplementary Fig. S2. IL-6 administration does not impact the body and liver weights of allograft MASLD mice

Uncropped Western blot images

Supplementary methods

A hepatocyte–macrophage miR-34a axis governs lipid accumulation and inflammation and reveals IL-6 as a steatosis-selective therapeutic target

Transfection and genetic modifications

For generating lentiviruses carrying miR-34a overexpression, pLemiR-34a or pLemiR-NS (scramble vector) were co-infected to HEK293T host cells with pCMV- Δ R8.91 (RNAi Core Academic Sinica, Taipei, Taiwan) and pMD.G (RNAi Core Academic Sinica) using Lipofectamine 3000 Transfection Reagent (#L3000001, Thermo Fisher Scientific, Waltham, MA). Supernatants containing respective lentiviral particles were harvested between 24 h, 48 h and 72 h post transfection, filtered through 0.2 μ M filter, then either used immediately or stored at -80°C. Lentiviruses-containing supernatant was next used to transduce HepG2, Hep3B, PLC5 and Huh7 liver cancer cells using 8 μ g/ml Polybrene Transfection Reagent (#TR-1003-G, Millipore, Burlington, MA). Stable clones were selected with 0.5 μ g/ml puromycin (Millipore). pLemiR-34a plasmids were constructed by cloning pre-miR-34a from pCDNA3.1-miR-34a (gift from Dr. Heidi Schwarzenbach, #78125, Addgene, Watertown, MA) into the XhoI and NotI cloning site using the primers described in our previous research¹. Stable clones carrying miR-34a overexpression in Hepa1-6 cells were generated using identical aforementioned protocols using retroviruses-containing supernatant derived from co-infection of MSCV-miR-34a (gift from Dr. Lin He, #63932, Addgene) or MSCV (scramble vector) (#24828, Addgene) with pCMV- Δ R8.91 and pMD.G to PT67 host cells. To silence miR-34a, control antagomiR or antagomiR-34a¹ were transfected into HepG2 and Huh7 cells using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific). pLemiR-NS and pCDNA3.1-miR-34a plasmids as well as control

antagomiR and antagomiR-34a were gift from Dr. Chih-Chen Hong (NHRI, Zhunan, Taiwan).

HepG2 and RAW264.7 cells carrying AMPK α and *ZFHX3* silencing were generated using pLKO.1 lentiviral vector cloned with shPRKAA1 (#TRCN0000000859 NM_006251) and sh*Zfhx3* (#TRCN0000075408 NM_007496) oligonucleotides, respectively (RNAi Core Academic Sinica). shRNA oligonucleotides were listed in **Supplementary Table S1**. THP-1 macrophages with ectopic FLAG-tagged *ZFHX3* and Strep II-tagged BACH1 were generated by transient transfection of pcDNA3-FLAG-*ZFHX3* (gift from Dr. Jin-Tang Dong, #40927, Addgene) and pcDNA3.1-2xFLAG-2xSTREP-BACH1 (gift from Dr. Michele Pagano, #159130, Addgene), respectively, using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific). Overexpression and knockdown efficiencies were monitored using semi-quantitative reverse transcription PCR (RT-qPCR) or western blotting.

FFA treatment

Sodium oleate (#O7501, Sigma, Saint Louis, MO) and sodium palmitate (#P9767, Sigma) were dissolved in distilled water at concentration of 30 mM as stock solutions. Cells were treated with FFAs for 24 h at final concentration of 0.30 mM in RPMI-1640 or DMEM complete medium containing 2% fatty acid-free bovine serum albumin (FFA-free BSA) (#A6003, Sigma).

Quantification of lipid accumulation

Cells seeded (2×10^5 /100 μ l medium/well) in 96-well plate were treated with FFA-free BSA coupled 0.15 mM palmitate and 0.15 mM oleate combined with either metformin (2 mM) (Sigma) or recombinant human IL-6 (rhIL-6) (20 ng/ml) (PeproTech, Cranbury, NJ) or or tocilizumab (200 μ g/ml) (Chugai Pharma, Taipei, Taiwan) for 24 h. Treatment with rhIL-6 were performed in serum-free DMEM, while metformin and tocilizumab were in DMEM with 10% FBS. After rinsing with PBS, the cells were incubated in BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Thermo Fisher Scientific) at 0.4 μ M in the dark for 30 min at 37°C. Lipid accumulation was quantified using a GloMax Discover microplate reader (Promega, Madison, WI). For lipid accumulation imaging, cells were fixed with 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) after staining with BODIPY 493/503, then imaged with TCS SP5 confocal microscope (Leica-Microsystems, Wetzlar, Germany).

Measurement of fatty acid oxidation (FAO), triacylglycerol (TAG), and IL-6

FAO activity was measured from cell lysate (0.5 mg/ml) using FAO Assay Kit (#E-141, Biomedical Research Service, Buffalo, NY) as per manufacturer's instruction. Red-colored formazan product proportional to FAO activity was quantified using a Tecan Infinite 200 PRO microplate reader at 492 nm absorbance (Männedorf, Switzerland). Hepatic TAG was extracted from homogenized liver (0.1 g) and tested for concentration using Triglyceride Colorimetric Assay Kit (#orb1173225, Biorbyt, Cambridge, UK) as per the manufacturer's instruction by measuring absorbance at 420 nm, which was read using a Tecan Infinite 200 PRO and normalized by the weight of liver. Plasma IL-6 levels were tested mice using ELISA MAX Deluxe Set Mouse IL-

6 (#431315, Biolegend, San Diego, CA) as per the manufacturer's instruction by subtracting measured absorbance at 570 nm from the absorbance at 450 nm.

Cellular ATP production

ATP production was measured using ATP Determination Kit (#A22066, Molecular Probes, Eugene, OR) as per manufacturer's instruction. Luminescence signals resulted from luciferase reactions with ATP was immediately measured using a GloMax Discover microplate reader (Promega). Background luminescence were subtracted from the result then normalized to the amount of the protein quantified using Bradford reagent (Bio-Rad, Hercules, CA).

Exosome isolation

Liver cancer cells at 90% confluence were incubated with serum-free DMEM for 48 h. Exosome was isolated from cell culture media using Total Exosome Isolation Reagent (from cell culture media) (#4478359, Thermo Fisher Scientific) as per manufacturer's instruction. Isolated exosomes were resuspended in serum-free DMEM for either immediate use for culturing THP-1 or RAW264.7 macrophages or storage at -80°C.

Exosome uptake analysis

A synthetic mature miR-34a mimic (hsa-miR-34a, #AM17100, Ambion, Austin, TX) and negative control (scramble) miRNA mimic (#AM17111, Ambion) were labelled with Label IT siRNA Tracker Cy3 Kit (#M7212, Mirus, Madison, WI) as per the manufacturer's instruction. HepG2 cells were transfected with 30 nM of Cy3-labelled miR-34a and scramble miRNA mimics using Lipofectamine 3000 (Invitrogen, Eugene, OR) and incubated for 24 h. HepG2 cells were washed out to remove free dye and

replenished with serum-free DMEM. The culture media were collected after incubation for 24 h for exosome isolation. THP-1 cells (6×10^4) were plated on μ -slides 4 wells coverslip (#80426, ibidi, Gräfelfing, Germany), differentiated into M0 macrophages, then cultured with HepG2/Cy3-miR-34a and HepG2/Cy3-scramble exosomes for 24 h. THP-1 were fixed in 4% paraformaldehyde (Alfa Aesar) for 15 min, blocked with 1% BSA (Sigma) for 30 min, and stained with anti-CD63 antibody (1:200) (#A5271, ABClonal, Woburn, MA) for overnight at 4°C. Alexa-Fluor 488-conjugated anti-rabbit IgG goat serum (1:500) (#A11034, Invitrogen) were used as a secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) (1:10,000) stained nuclear DNA. Images were captured by Leica Stellaris confocal microscope (Leica Microsystems). Incorporated Cy3-miR-34a into THP-1 was measured as percent area showing all pixels positive for both Cy3-miR-34a and Alexa-Fluor 488-conjugated CD63 using the co-localization algorithm in LAS X software (Leica Microsystems).

Monocyte and macrophage invasion

Monocyte and macrophage invasion were examined using Corning BioCoat Tumor Cell Invasion System (Corning, Bedford, MA) as per the manufacturer's protocol. THP-1 monocytes, M0, M1 and M2 macrophages (1×10^5) were seeded in the serum-free RPMI-1640 on the upper chamber. Cells invading to the lower chamber containing 0.15 mM palmitate (Sigma) and 0.15 mM oleate (Sigma) in RPMI-1640 growth medium were fixed with 4% paraformaldehyde (Alfa Aesar), stained with 0.25% crystal violet and imaged using a Leica CH9435 Flexacam C1 (Leica Microsystems) with a 10x objective. For M1 THP-1 invasion in response to HepG2 cells, the lower chambers were plated with HepG2 cells (5×10^5) pre-treated with either FFA-free BSA (Sigma) or 0.15 mM palmitate (Sigma) and 0.15 mM oleate (Sigma) in DMEM growth medium.

M1 RAW264.7 invasion towards FFA-treated Hepa1-6 cells were conducted using the same protocols.

RT-qPCR

Liver cancer cells and THP-1 cells were washed with ice-cold PBS and subjected to RNA extraction with TRIzol Reagent (Invitrogen, Eugene, OR) as per the manufacturer's protocol. Extracted RNA samples (2 µg) were used for oligo-dT primed reverse-transcription using the FIREScript RT cDNA synthesis kit (Solis BioDyne, Tartu, Estonia). For quantifying miR-34a and miR-26a levels, cDNA synthesis was performed with miR-34a and miR-26a stem-loop RT primers (**Supplementary Table S1**). cDNA samples (100 ng) were then mixed with HOT FIREPol SolisGreen qPCR Mix (Solis BioDyne) and subjected to RT-qPCR using a ViiA 7 Real-time PCR System (Thermo Fisher Scientific) and analyzed using comparative Ct methods ($2^{-\Delta\Delta C_t}$). miR-34a levels were normalized to RNU6B as an internal control, whereas exosomal miR-34a were normalized to miR-26a. Other results were normalized to β -actin. $\Delta\Delta C_t = [(C_t \text{ target gene} - C_t \text{ internal control}) \text{ of treated cells} - (C_t \text{ target gene} - C_t \text{ internal control}) \text{ of untreated cells}]$. NCBI Probe Database were used to design primer sequences (**Supplementary Table S1**), except microRNAs which were designed using sRNAPrimerDB².

Chromatin immunoprecipitation (ChIP)

RAW264.7 M1 macrophages (10×10^6) were cross-linked with 1% formaldehyde (Alfa Aesar), quenched with 1.25 M glycine, and lysed with high salt lysis buffer (800 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA pH 8, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 1x protease inhibitor cocktail (Sigma)).

Chromatin was sheared using Q700 sonicator assembled to a cup horn (Qsonica, Newtown, CT) then immunoprecipitated with pre-mixed Dynabeads Protein A/G (Millipore) and ZFH3 antibody (#LS-B12870, LSBio, Shirley, MA) at 4°C overnight on a circular rotator. After thrice washing and elution, RNA was removed using 20 µg/ml RNase A (Thermo Fisher Scientific), protein was digested with 200 µg/ml Proteinase K (Thermo Fisher Scientific) and DNA was purified using spin columns (Qiagen, Germantown, MD). Purified genomic DNA fragments of RAW264.7 cells were amplified using qPCR with primers flanking *Bach1* promoter region (-1 500 to 100 bp relative to transcriptional initiation site) containing potential ZFH3 binding site (**Supplementary Table S1**). Potential ZFH3 binding site was identified by in silico approach as previously described³. Enrichment of *Bach1* promoter region was calculated as relative fold change to IgG (#PP64B, Millipore).

Western blotting and antibodies (Abs)

Cells lysates were extracted using RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% (sodium dodecyl sulphate) (SDS)) mixed with phosphatase and protease inhibitor cocktails (Calbiochem, San Diego, CA). The protein samples (40 µg) was prepared in reducing sample buffer (8% SDS, 10% 2-mercaptoethanol, 30% glycerol, 0.25 M Tris-HCl pH 6.8, 0.02% bromophenol blue) and heated at 95°C for 10 min. For exosomes, protein samples were prepared in non-reducing sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.1% bromophenol blue). Proteins were then resolved in a 4-12% SurePage Bis-Tris polyacrylamide gels (GenScript, Piscataway, NJ), electroblotted using a semi-dry system onto Amersham Protran Supported Nitrocellulose membrane with 0.45 µm pore size (GE Healthcare, Uppsala, Sweden), probed with the indicated Abs. The

immune-reactive proteins were detected by autoradiography upon incubation with Immobilon Western Chemiluminescent HRP substrate (Millipore).

Abs against Arginase-1, IL-10, SIRT1, phospho-AMPK (p-AMPK)-Thr172, AMPK, PGC-1 α , phospho-p53 (p-p53)-Ser15, FASN, phospho-p38 (p-p38)-Thr180/Tyr182 and p38 α were from Cell Signaling Technology (Danvers, MA); Ab against CD163, iNOS and α -tubulin were from Abcam (Cambridge, UK); Ab against β -actin were from GeneTex (Irvine, CA); Ab against p53 was from Santa Cruz Biotechnology (Dallas, TX); Ab against phospho-ACC1 (p-ACC1)-Ser79, CD63, and Strep II-tag were from ABclonal (Woburn, MA); Ab against ACC was from Elabscience (Houston, TX); Ab against FLAG-tag was a gift from Dr. Tsung-Hsien Chuang (NHRI); Abs against Alix, HSP70, and GM130 were constituents of The Exosome Essentials Antibody Kit (#PK30023, Proteintech, Rosemont, IL).

Isolation of Kupffer Cells (KCs)

HFD HFD-fed C57BL/6J allograft mice treated with or without rmIL-6 were anaesthetized using isoflurane anesthetic vaporizer (#VIP3000, Midmark, Versailles, OH). To remove the blood and loosen the cell junctions, mouse liver was subsequently perfused with PBS through the inferior vena cava using a peristaltic pump (#LEAD-2, LongerPump, Hebei, China). KCs were isolated from the liver according to an established protocol with minor modifications⁴. The liver was minced and incubated with digestion buffer (0.2 mg/ml collagenase D (#11088858001, Sigma, Saint Louis, MO), 0.25 mg/ml DNase I (#DN25, Sigma) in DMEM medium supplemented with 10% FBS for 30 min at 37°C under continuous agitation. Digested liver was homogenized by resuspension with a syringe pre-loaded with a 18G needle and filtered using a 100- μ m cell strainer. After centrifugation at 100 x g for 2 min, the pellet containing

hepatocytes was discarded and supernatant was transferred into a new tube. The cell pellet containing the immune cells resulting from another round of centrifugation were then lysed with red blood cell lysis buffer (155 mM NH_4Cl , 12 mM NaHCO_3 and 0.1 mM EDTA) and fixed with 4% (v/v) paraformaldehyde for flow cytometry analysis. To analyze KCs, single cell suspension (1×10^6) was incubated with anti-CD16/CD32 (1:2,000) (#553142) for 15 min at 4°C to block nonspecific binding, stained with BB515-conjugated anti-CD11b antibody (#564454), BB700-conjugated anti-F4/80 (#746070) and PE-conjugated anti-CD80 antibody (#553769) at 1:25 dilution. BB515- (#564421), PE-(#550085) and BB700-conjugated (#566413) rat IgG2a κ isotype controls were used as negative controls. All antibodies and isotype controls were from BD Biosciences (Franklin Lakes, NJ). Flow cytometry experiments were performed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo v10 (BD Biosciences).

Isolation of murine primary hepatocytes

Murine primary hepatocytes were isolated from 8-weeks old C57BL/6J male mice as per previously described with minor modifications⁵. Briefly, mouse was anaesthetized using isoflurane anesthetic vaporizer (Midmark). Mouse liver was perfused through the inferior vena cava with perfusion buffer (0.5 mM EDTA in PBS) to wash out the blood, then further perfused with digestion buffer (25 $\mu\text{g}/\text{ml}$ collagenase D (Sigma) dissolved in DMEM/F-12 medium (#12400024, Life Technologies, Grand Island, NY) using a peristaltic pump (LongerPump). Liver was removed from the mouse and gently ruptured using a cell scraper to release the hepatocytes. Suspension containing the hepatocytes were filtered using a 70- μm cell strainer prior to centrifugation at 100 x g for 1 min. Pelleted hepatocytes (4×10^5) were plated on a 6-well plate pre-coated with

15 µg/ml rat-tail collagen (#C7661, Sigma) and incubated for 6 h in DMEM high glucose medium (Life Technologies) to allow adherence, then treated with FFAs for additional 18 h. For miR-34a overexpression, adherent primary hepatocytes were transduced with lentiviruses from either MSCV-miR-34a (Addgene) or MSCV scramble vector (Addgene) for 24 h.

Immunohistochemistry (IHC) and immunofluorescence staining

Whole slide images from IHC assays were acquired using a digital microscopic slide scanner Panoramic Midi II (3DHistech, Budapest, Hungary). Steatosis and fibrosis were evaluated by microscopic examination of H&E-stained liver sections under a 10x (for human samples) and 20x objective (for mouse samples) magnification. Steatosis segmentation was performed using machine learning toolkit Waikato Environment for Knowledge Analysis (WEKA)⁶ and the percentage of segmented area was quantified using ImageJ/Fiji. Scoring for steatosis (0 to 3) follows a validated system from NASH Clinical Research Network⁷. Liver fibrosis and/or cirrhosis were identified using Masson's Trichrome Stain Kit (#VB-3016, VitroVivo Biotech, Rockville, MD) as per manufacturer's instruction. After deconvolution, the total percentage of liver fibrosis per total area from five randomly selected sections was quantified by ImageJ. Fibrosis stage was determined using the Metavir scoring system⁸. Diameter of microscopic tumor foci was measured using an automatic distance annotation tool in Panoramic Viewer (3DHistech) after a manual identification of the largest linear dimension in focal lesion.

For GPC3 expression as histochemical marker for diagnosing early-stage of HCC^{9,10}, liver sections were stained with anti-GPC3 antibody (#E-AB-53438, Elabscience) (1:60) then immunodetected using EnVision Gl2 Doublestain System

Rabbit/Mouse (DAB+/Permanent Red) Kit (#K5361, Dako, Santa Clara, CA) as per the manufacturer's instruction. The percentage of positively stained cells and the scoring of staining intensity (0 to 3) were used to calculate H-score of GPC3 = (0 x [percentage of negatively stained cells]) + (1 x [percentage of positively stained cells at intensity 1]) + (2 x [percentage of positively stained cells at intensity 2]) + (3 x [percentage of positively stained cells at intensity 3]). The automated scoring of staining intensity and the percentage of cells for each staining intensity were performed via IHC Profiler¹¹.

CD80, ZFHX3, and BACH1 co-expression on liver sections were examined by immunofluorescence staining with anti-CD80 (#66406-1-Ig, Proteintech) (1:100), and anti-ZFHX3 (#LSBIO-2870, LSBio) (1:100) or anti-BACH1 antibodies (#14018-1-AP, Proteintech) (1:100) and followed by incubation with Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (#A-11037, Thermo Fisher Scientific) (1:400) and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (#A-11029, Thermo Fisher Scientific) (1:400), counterstained with DAPI (1:1,000) and mounting with Shandon Immu-Mount (#9990402, Thermo Fisher Scientific) as per previously published protocol¹². Images were acquired by Evident SlideView VS200 (Evident, Tokyo, Japan). Pixel colocalization of CD80 and ZFHX3 or BACH1 was evaluated by calculating Pearson correlation coefficient (PCC) above the threshold set by Costes method¹³ using Coloc 2 plugin of ImageJ/Fiji.

Chromogenic in situ hybridization (CISH) for miR-34a was subjected to paraffin tissue slides containing tissue sections from MASLD-HCC patients using double-digoxigenin (DIG)-labeled miRCURY Locked Nucleic Acid (LNA) miR-34a Detection Probes (#339111 YD00618250-BCG, Qiagen, Germantown, MD). Briefly, tissue slides

were deparaffinized, demasked using proteinase K buffer (15 µg/ml proteinase K (Thermo Fisher Scientific), 5mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM NaCl) for 10 min at 37°C, dehydrated, then incubated with either negative control or 80 nM miRCURY LNA miR-34a probes for 1 h at 53°C. After rinsing with pre-heated sodium citrate buffer, the slides were blocked against unspecific binding using 2% sheep serum (#013-000-121, Jackson ImmunoResearch) and 1% BSA (Sigma), labeled with sheep anti-DIG antibody conjugated with alkaline phosphatase (1:800) (#11093274910, Sigma) for 1 h at room temperature, reacted with a mixture of 1-Step NBT/BCIP plus Suppressor Substrate Solution (#34070, ThermoFisher Scientific) for 2 h at 30°C, washed in KTBT buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM KCl), counterstained with FastRed nuclear staining (#H-3403-500, Vector Laboratories), rinsed, dehydrated and mounted under coverslip with Shandon Immu-mount (#9990402, Thermo Fisher Scientific). H-score of miR-34a was calculated based on the fraction (%) of positively stained cells and the scoring of staining intensity (0 to 3), as in GPC3, after color deconvolution.

Statistical analysis and data mining

FIB-4 index was calculated using FIB-4 calculator based on aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, platelet count, and age of patients¹⁴. Correlation of miR-34a H-score with steatosis grade, fibrosis stage, and FIB-4 index was analyzed using Spearman's correlation analysis. Sample size for correlating miR-34a with MASLD histopathological score was estimated using a priori power analysis. Sample size of animal studies was determined based on the previous experiments. The cohorts from GSE193066 were divided into *MIR34A*^{high} (n = 27) and *MIR34A*^{low} (n = 42) groups according to the upper/lower quartile of miR-34a expression levels and the relationship between miR-34a expression and risk for

prognostic liver signature (PLS)-MASLD¹⁵ was analyzed using Chi-square test. MiRWalk¹⁶ was used to search both miR-34a putative and validated targets as well as miR-34a binding site on the *Zfhx3* 3' UTR. GSE166504¹⁷ was used to identify downregulated genes in KCs of MASLD mouse model. ChIP-seq data peaks (GSE231134) was retrieved from the ENCODE Consortium¹⁸, whereas differentially expressed genes (DEGs) from myeloid *Zfhx3*-deficient mice were obtained from GSE183922¹⁹. Statistical tests were selected to be appropriate for the data properties (normality of distribution and homogeneity of variance) as shown in the figure legends and performed with GraphPad Prism 10 (GraphPad Software, San Diego, CA). TIMER2.0 platform was used to visualize the association of tumor-infiltrating immune cells and/or tumor purity with the expression of *ZFH3* (in log2 transcripts per million (TPM)) for TCGA-Liver Hepatocellular Carcinoma (LIHC) cohorts using Estimating the Proportion of Immune and Cancer Cells (EPIC) and xCell algorithms and analyzed using Spearman's correlation analysis²⁰. Because the pooled summary data did not contain a measure of variance/error, no statistical test was performed in GSE99010²¹, which was used to examine *Zfhx3* expression in a diet- and chemical-induced murine MASH model. Results were representative from at least two independent experiments.

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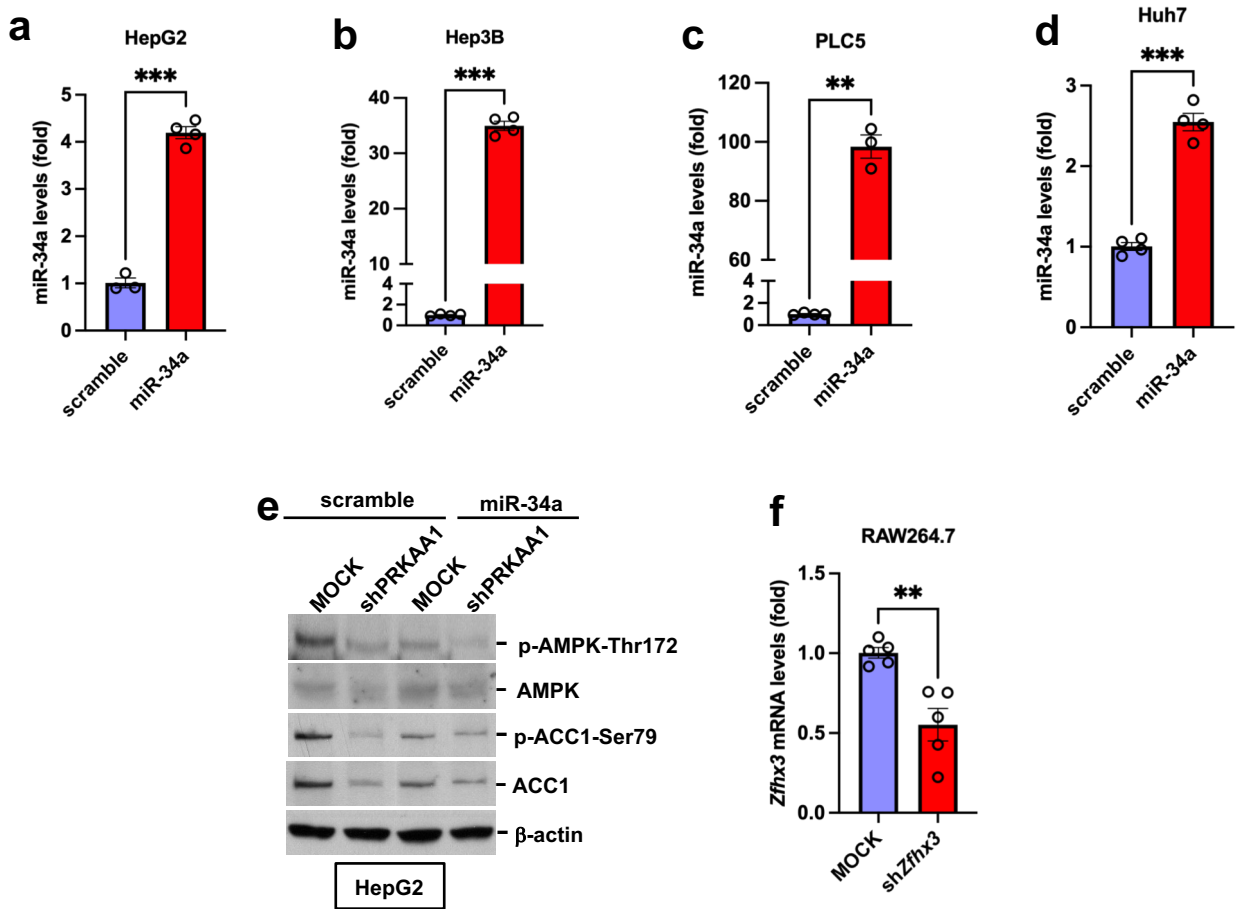
Supplementary figures

A hepatocyte–macrophage miR-34a axis governs lipid accumulation and inflammation and reveals IL-6 as a steatosis-selective therapeutic target

Supplementary Fig. S1. MiR-34a boosts hepatic and macrophage lipid accumulation via IL-6/AMPK and ZFH3

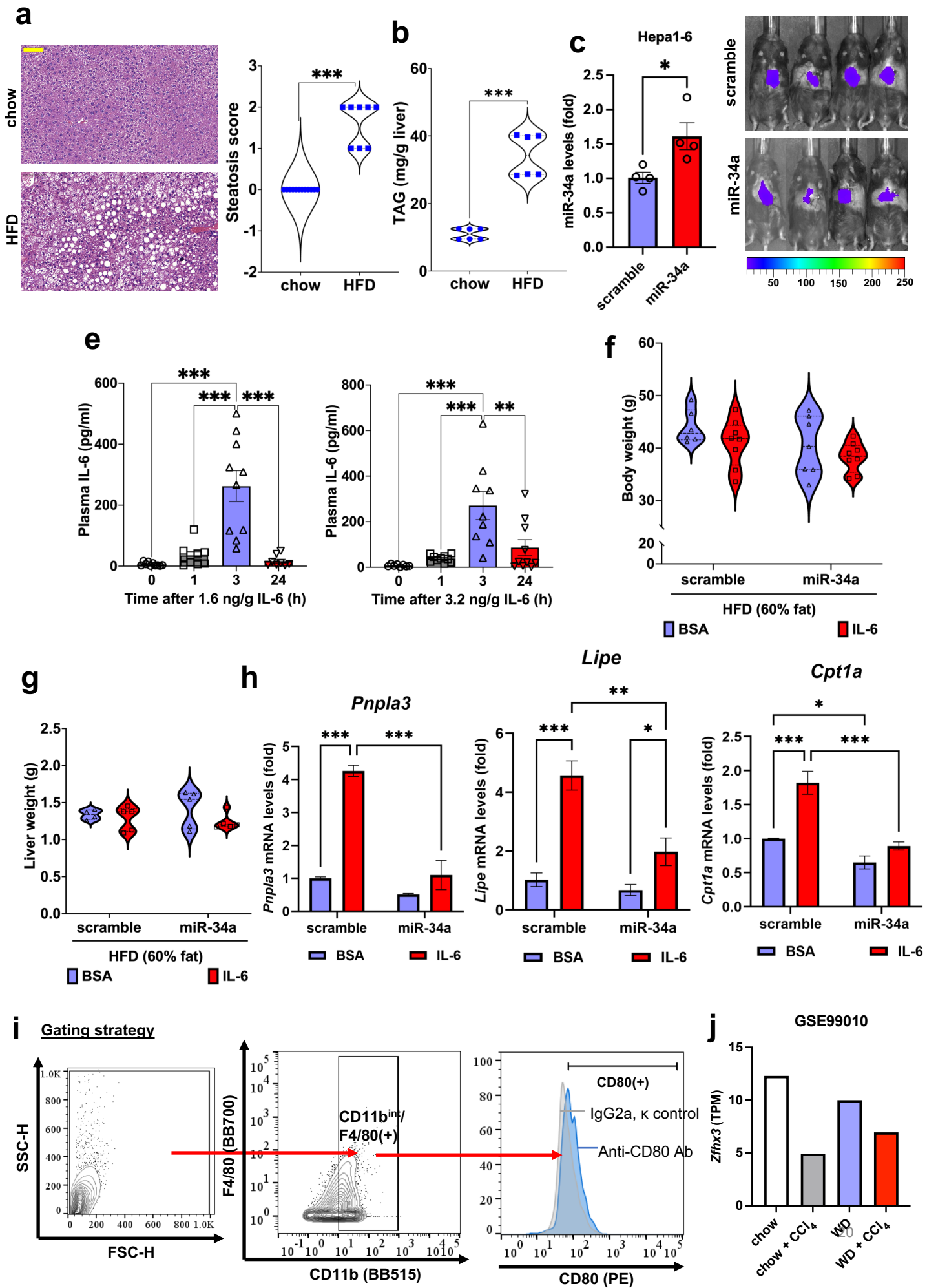
Supplementary Fig. S2. IL-6 administration does not impact the body and liver weights of allograft NAFLD mice

Supplementary Fig. S1



Supplementary Fig. S1. MiR-34a boosts hepatic and macrophage lipid accumulation via IL-6/AMPK and ZFH3. (a-d) miR-34a overexpression in HepG2 (a), Hep3B (b), PLC5 (c), and Huh7 (d) HCC cell lines (n = 3~4) were verified using RT-qPCR. (e) Inhibition of AMPK and its downstream target, ACC, in HepG2 cells (miR-34a vs. scramble) upon transfection with shPRKAA1. (f) *Zfhx3* inhibition in RAW264.7 cells upon transfection with sh*Zfhx3* construct (n = 5). *RNU6B* and β -actin were used as the internal control in (a-e) and (e-f) respectively. Data represent the mean $\pm$ SEM. Analyses were performed using a two-tailed unpaired Student's *t*-test (a-d, f). ***p*<0.01; ****p*<0.001.

Supplementary Fig. S2



Supplementary Fig. S2. IL-6 administration does not impact the body and liver weights of allograft NAFLD mice. (a) Hepatic steatosis was confirmed in C57BL/6J male mice (HFD vs. chow) after 8 weeks of feeding, through steatosis scoring (right) on HE-stained liver sections (left), with a scale bar of 100 μ m. (b) Liver TAG contents were determined by a colorimetric assay. (c) miR-34a overexpression in Hepa1-6 murine HCC cell lines (n = 4) was validated using RT-qPCR. *RNU6B* was used as the internal control. (d) Hepa1-6 cells (miR-34a vs. scramble) were orthotopically injected into subcapsular region of the liver of HFD-fed C57BL/6J mice and verified by *in vivo* bioluminescence imaging immediately after implantation. Representative images were shown (left). Color bar (right) indicates luminescent signal intensity. (e) Plasma IL-6 levels were measured in circulating blood by ELISA before the initial dose (0 h) and 1, 3, and 24 h after the mice were i.p. administered with rmIL-6 at 1.6 ng/g (left) and 3.2 ng/g (right) (n = 9~10). (f-g) Body and liver weights were recorded post-engraftment with Hepa1-6 cells (miR-34a vs. scramble) following 14 weeks of HFD and administration with or without IL-6 (IL-6 vs. BSA) during the final two weeks. (h) RT-qPCR analysis was conducted for hepatic lipolysis genes (*Pnpla3*, *Lipe*, *Cpt1a*) (n = 3), using *Ppia* as the internal control. (i) Flow cytometry analyzed Kupffer cells (KCs) isolated from the liver, assessing cell population integrity via side scatter (SSC) versus forward scatter (FSC) dot plot (left panel). KCs were gated for CD11b intermediate fractions (CD11b^{int}/F4/80(+)) cells (middle panel) using rectangular gating. Fluorophore-matched isotype control antibodies of CD80 (right panel) served as a negative control for background staining to identify CD80(+) subsets. (j) Hepatic *Zfhx3* expression (in normalized Transcript Per Million [TPM] in MASLD-HCC mouse model (GSE99010) (pool of n = 4). Mice were fed with western diet (WD) and/or injected with carbon tetrachloride (CCl₄) for 12 weeks to rapidly mimic MASLD progression to liver fibrosis and HCC. Violin plots (a-b, f-g) illustrate the median (center, dashed line) and the upper/lower quartiles (dotted lines). Data in (g) represent the mean $\pm$ SEM. Analyses were performed using a two-tailed unpaired Student's *t*-test (a-c), one-way ANOVA with Tukey-Kramer test (e), and two-way ANOVA with Tukey-Kramer test (f-h). *p<0.05; **p<0.01; ***p<0.001.

Uncropped Western blot images

A hepatocyte–macrophage miR-34a axis governs lipid accumulation and inflammation and reveals IL-6 as a steatosis-selective therapeutic target

Figure Source Data Fig. 2f (inset)

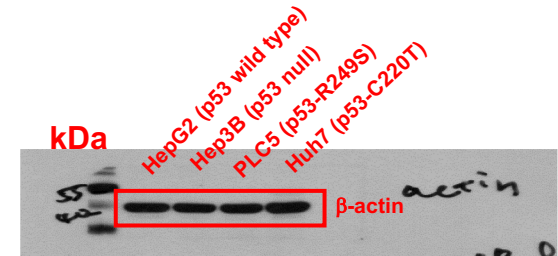
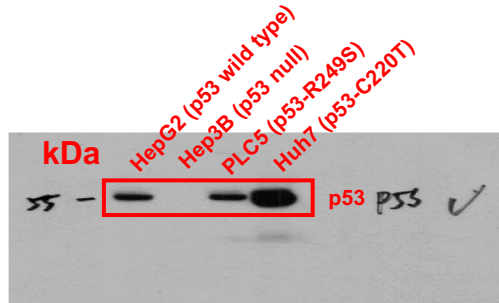
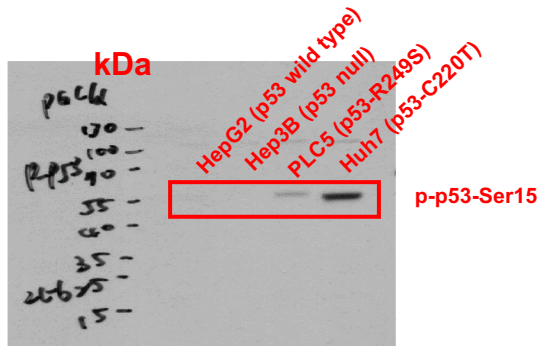


Figure Source Data

Fig. 2i

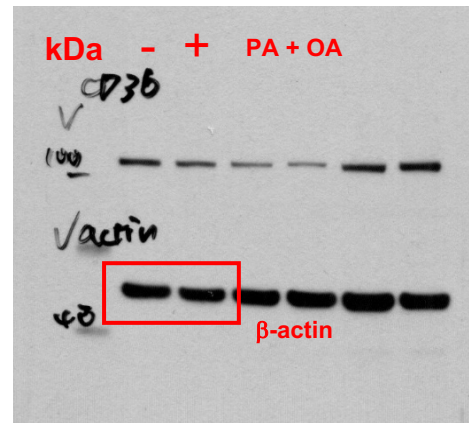
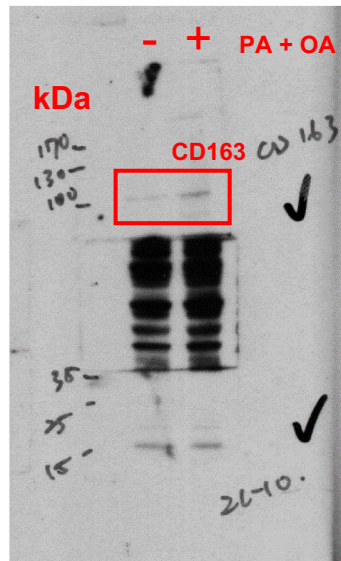
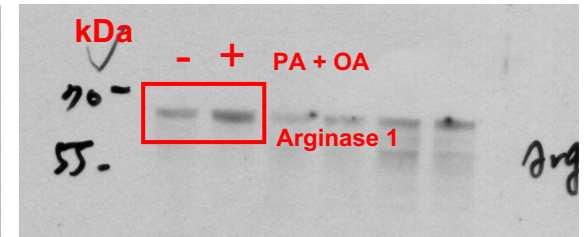
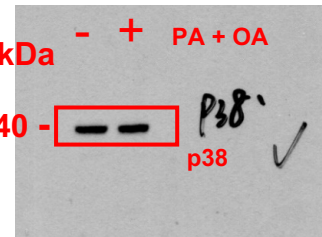
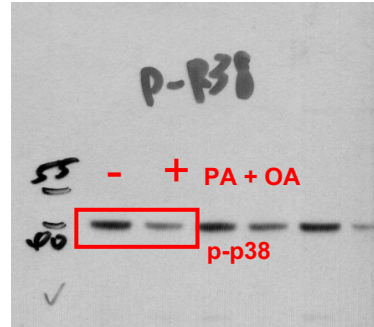
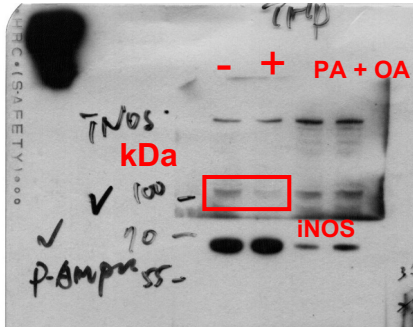


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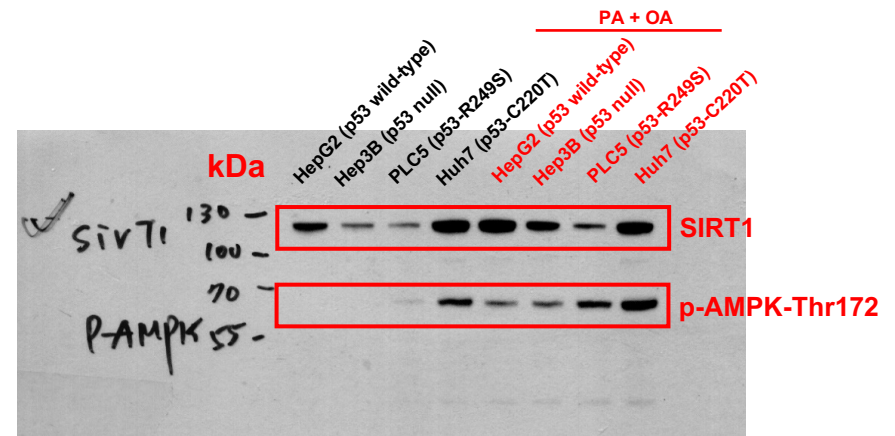
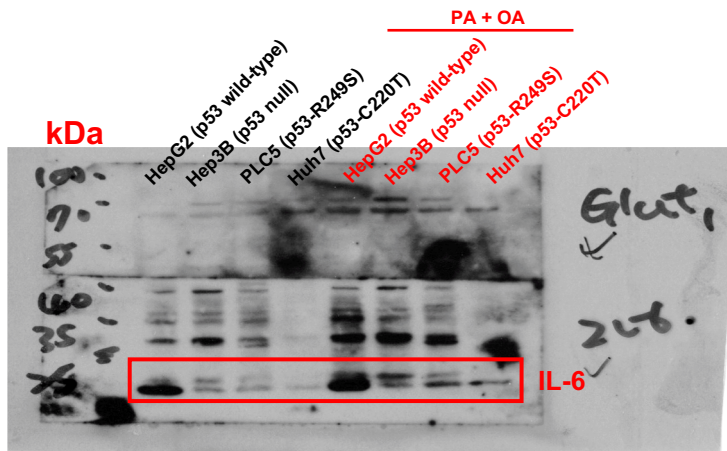
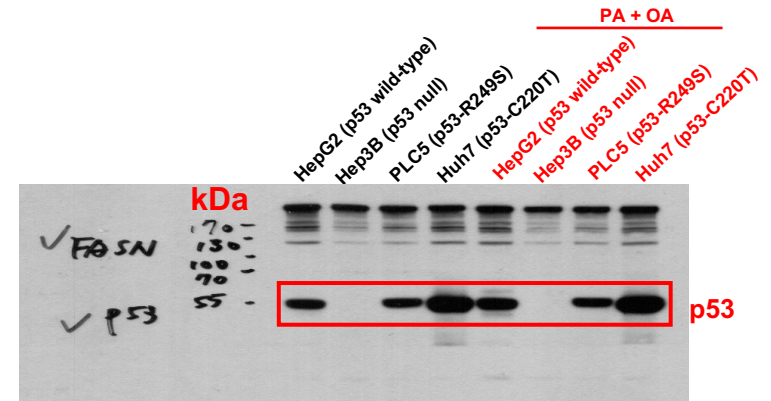
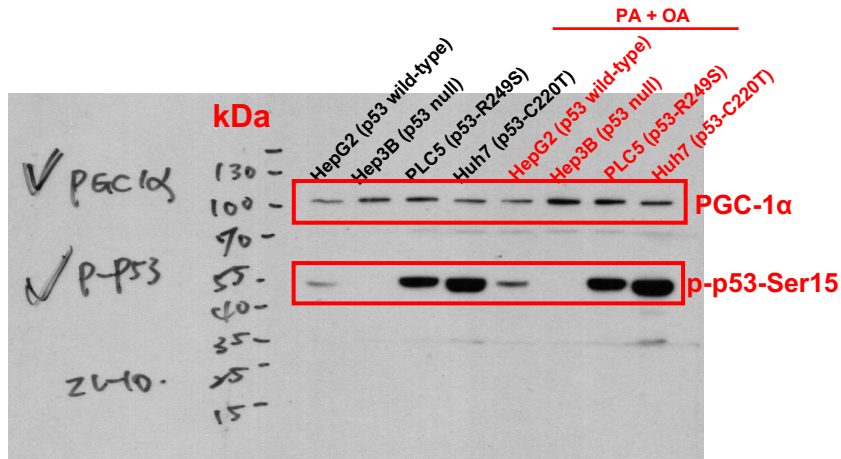


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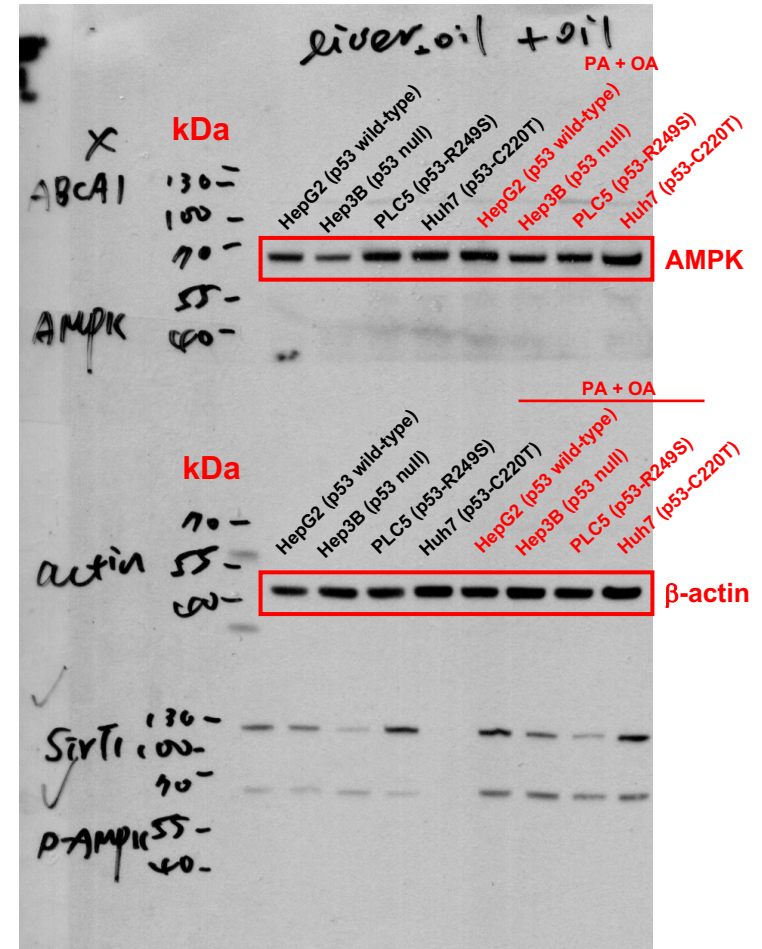
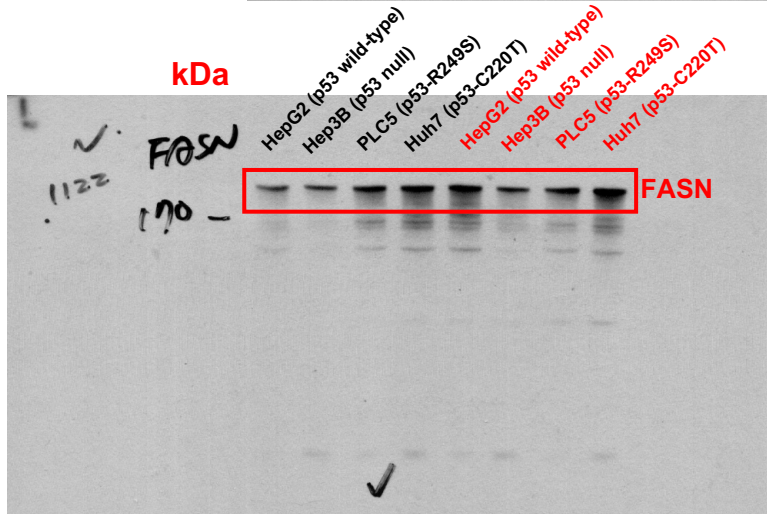
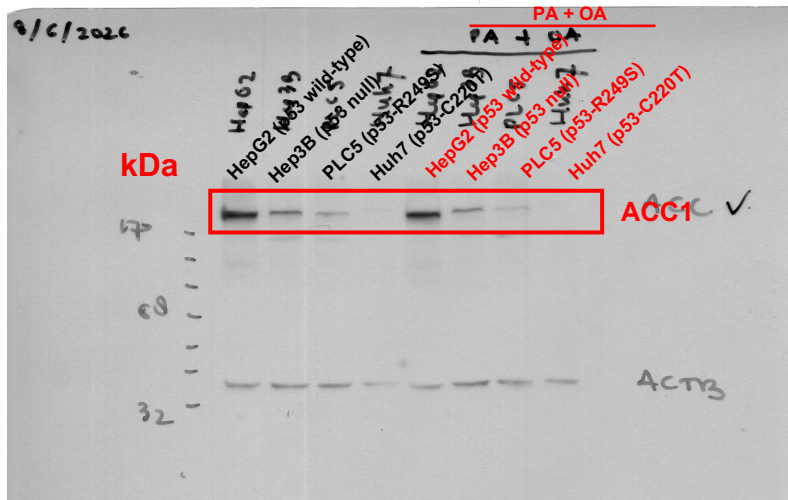
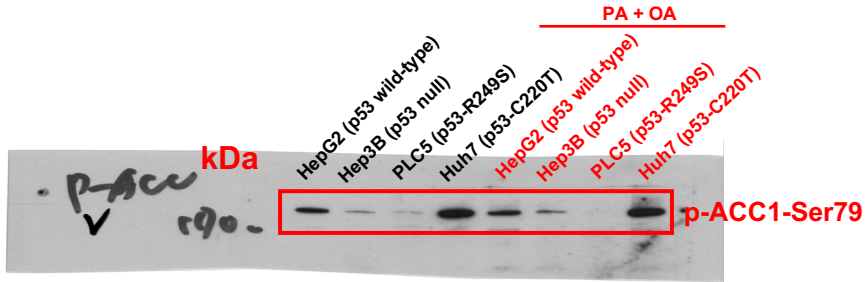


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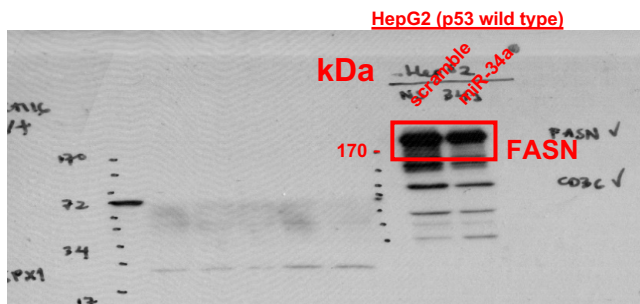
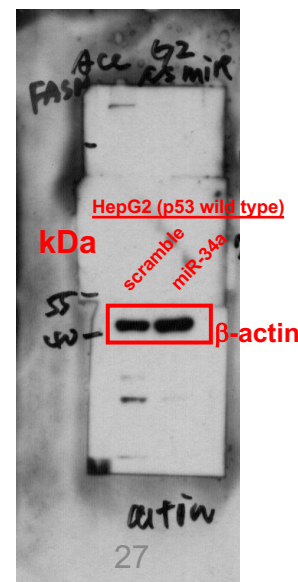
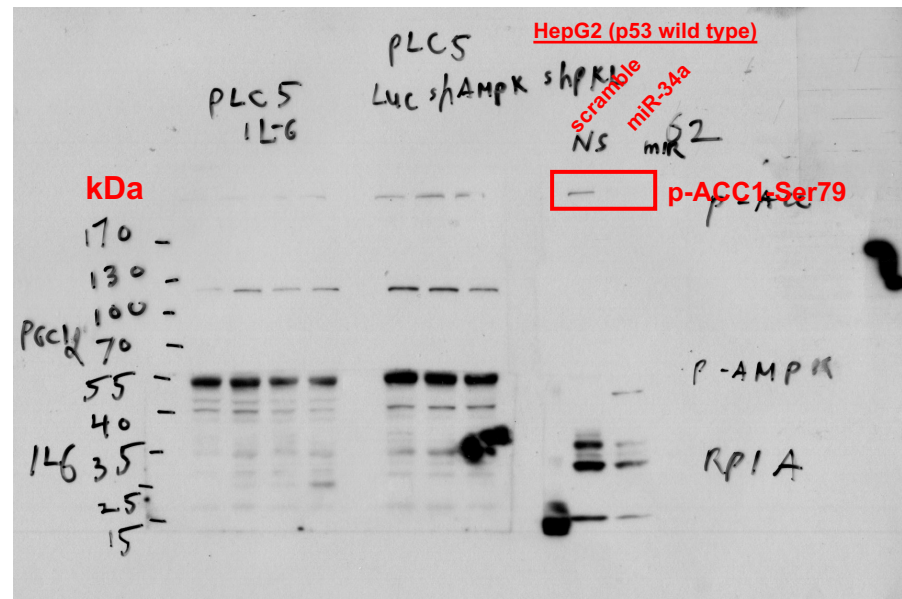
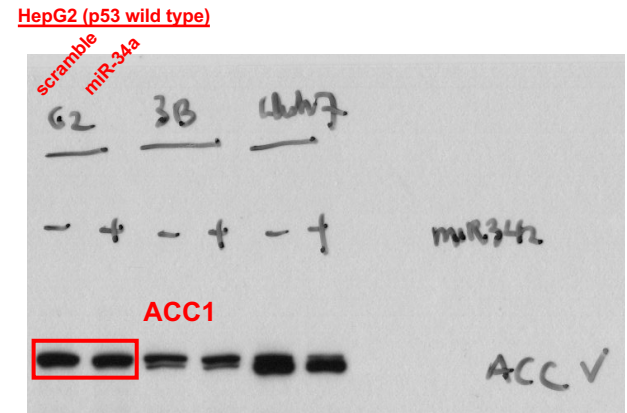
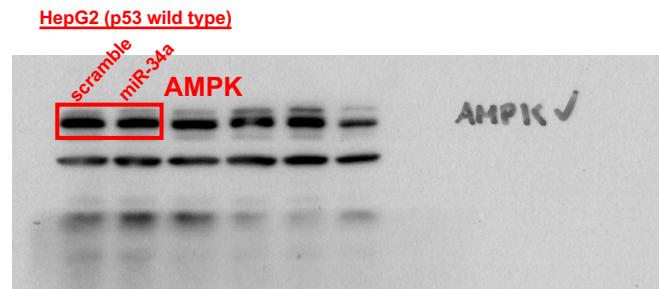
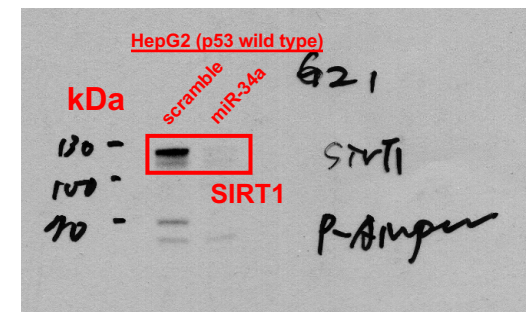
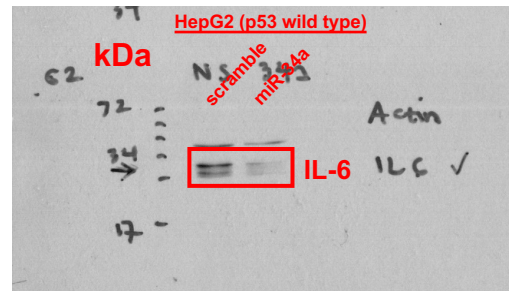
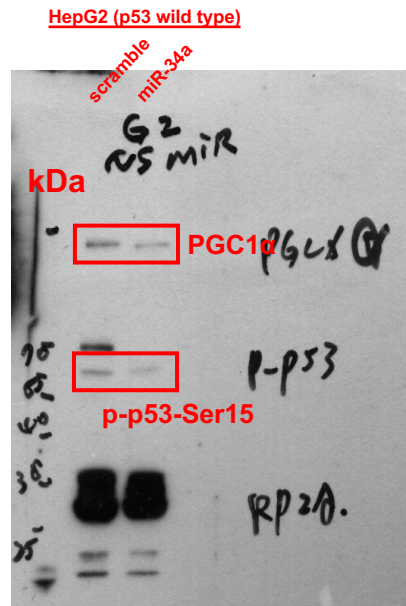


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Fig. 3k

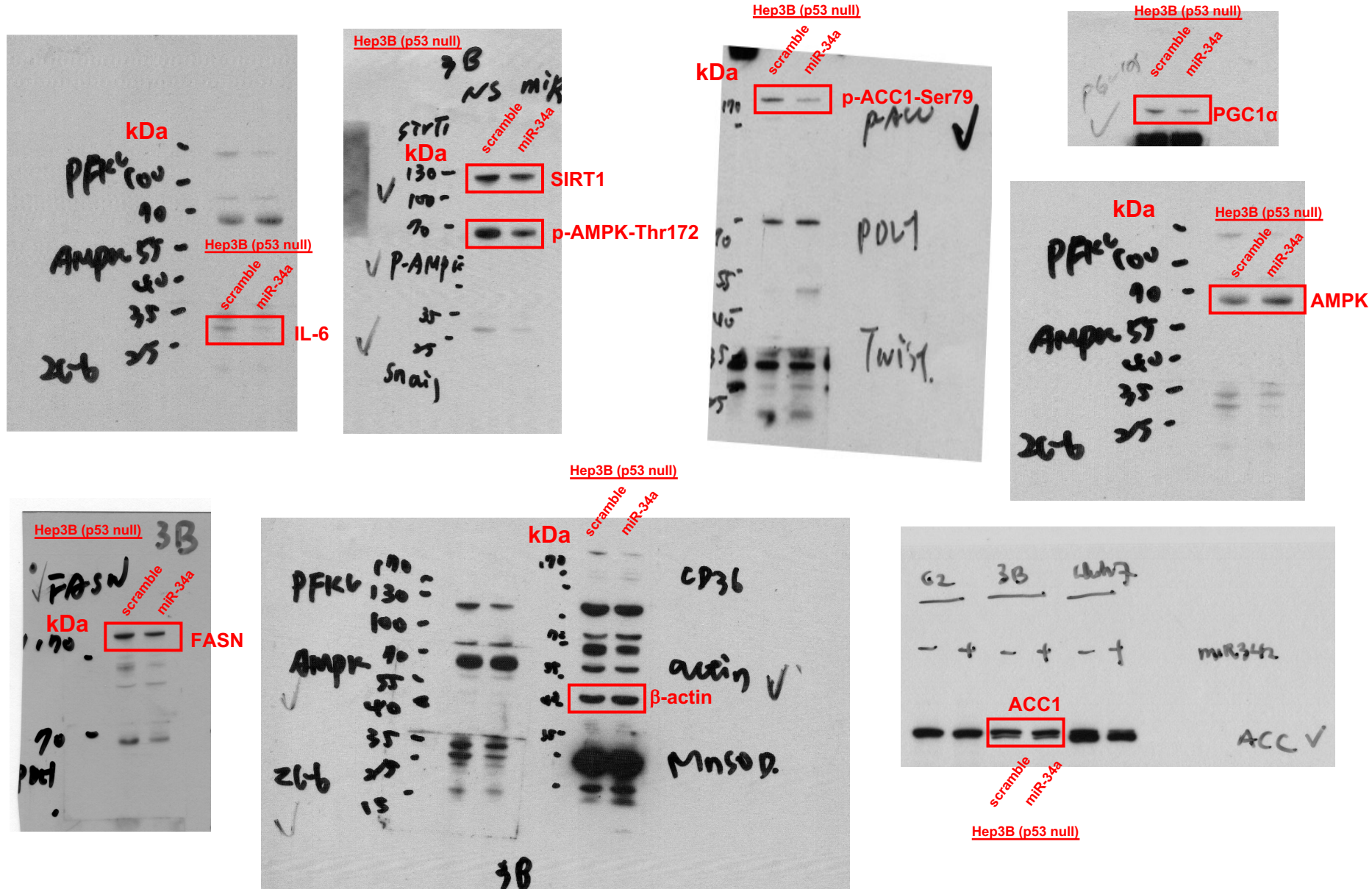


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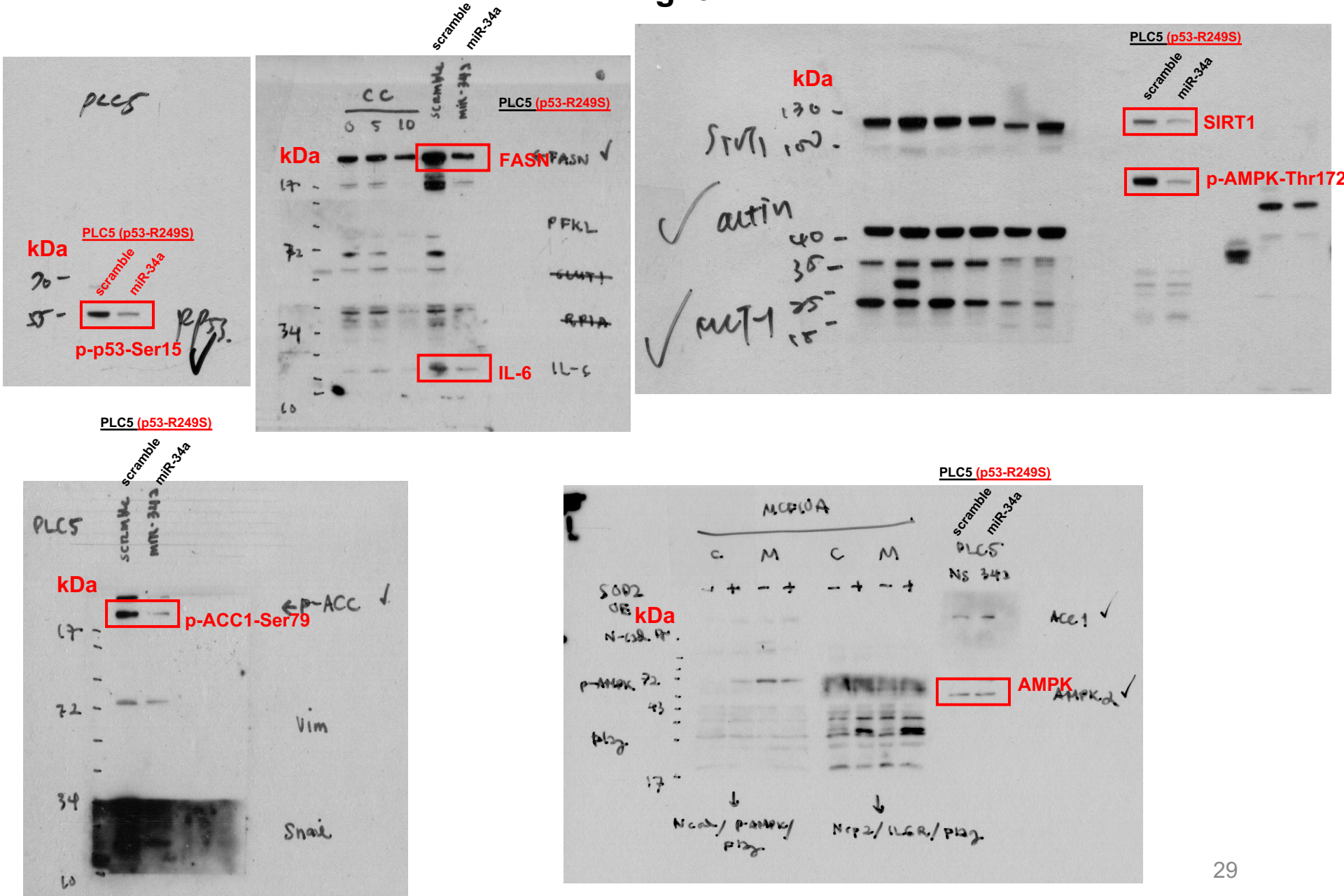


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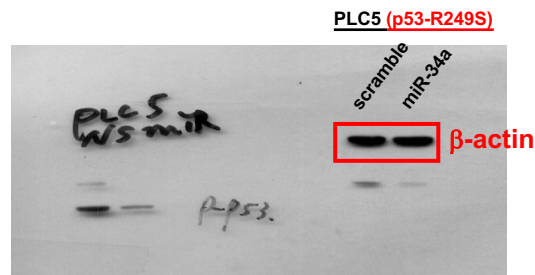
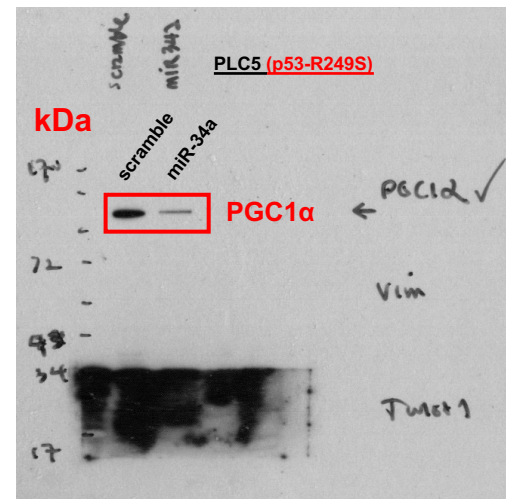
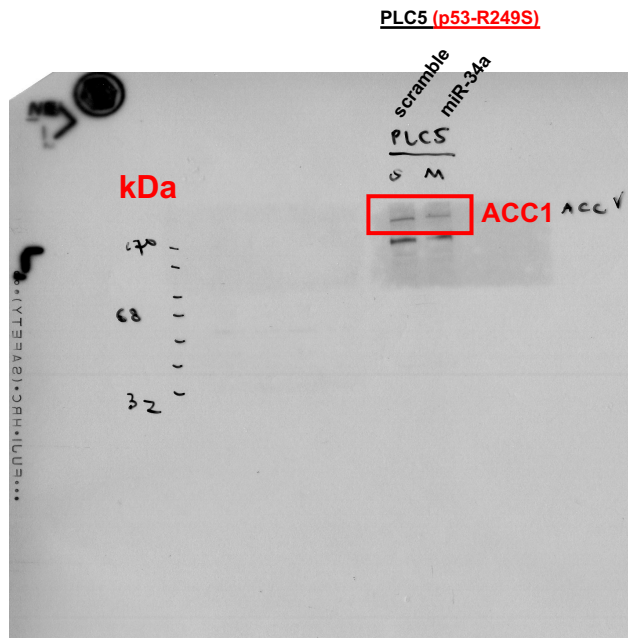


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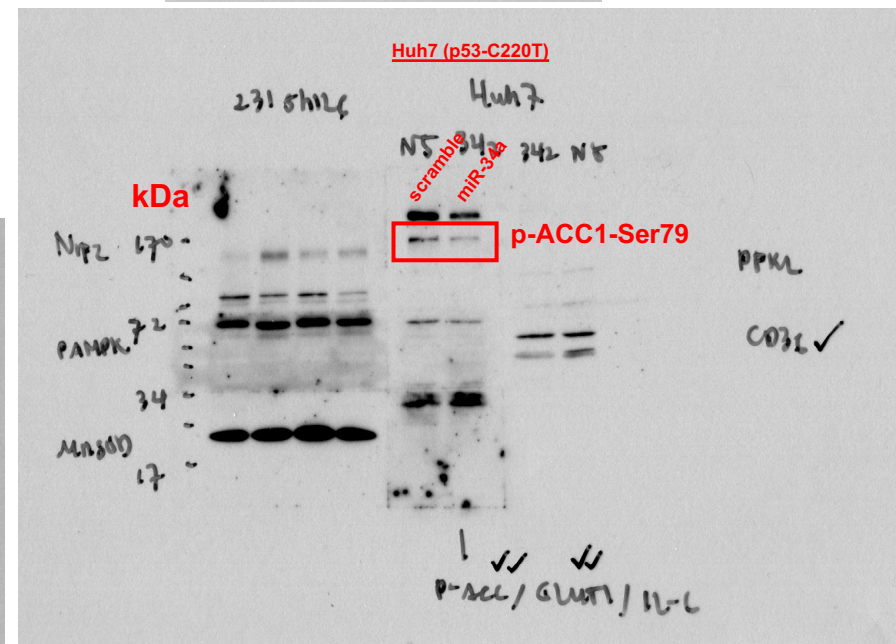
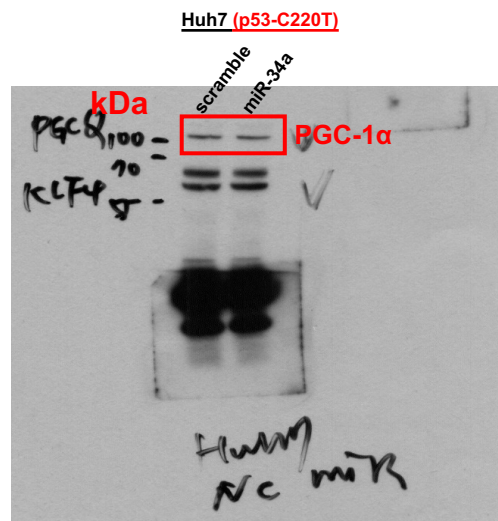
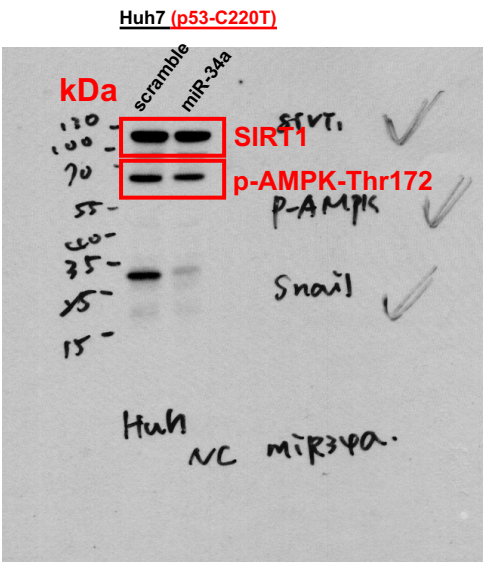
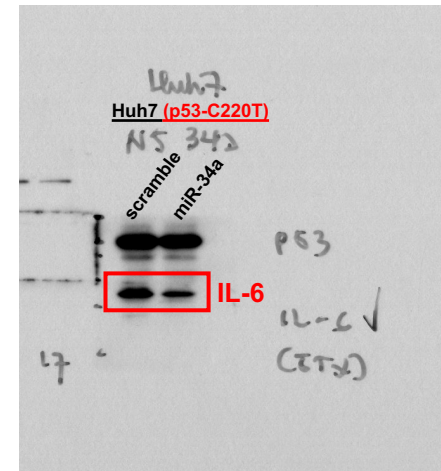
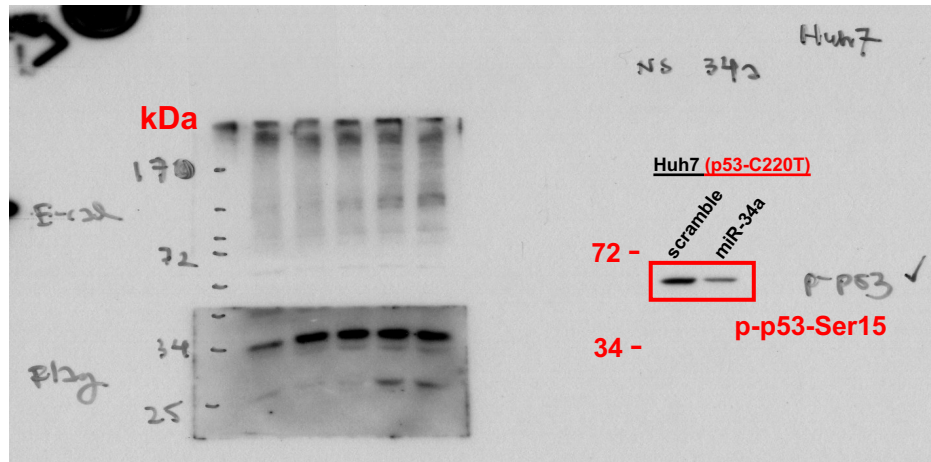


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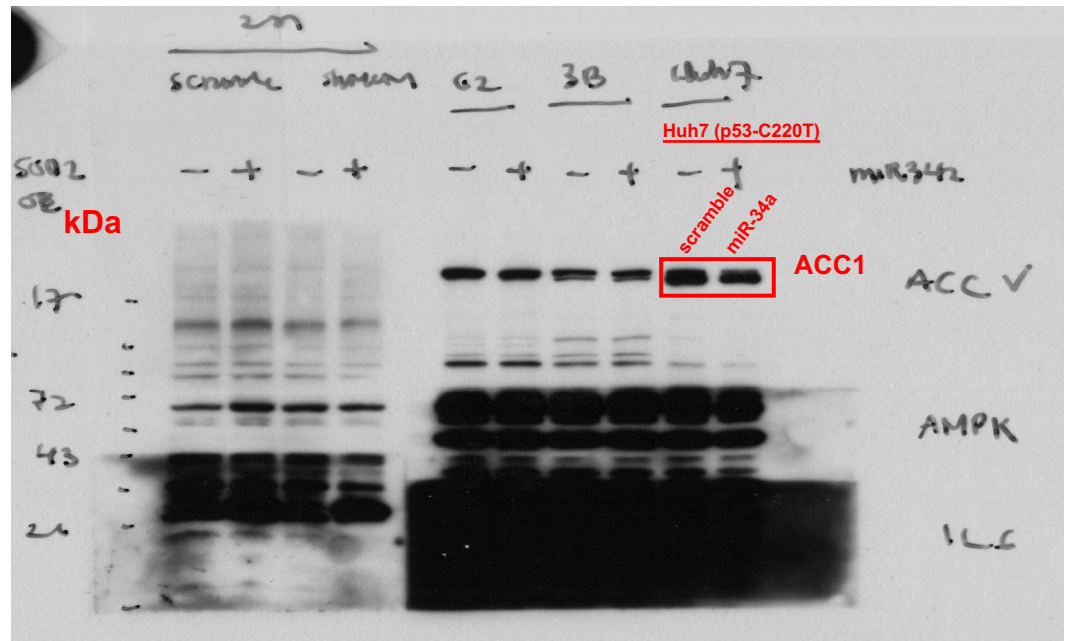
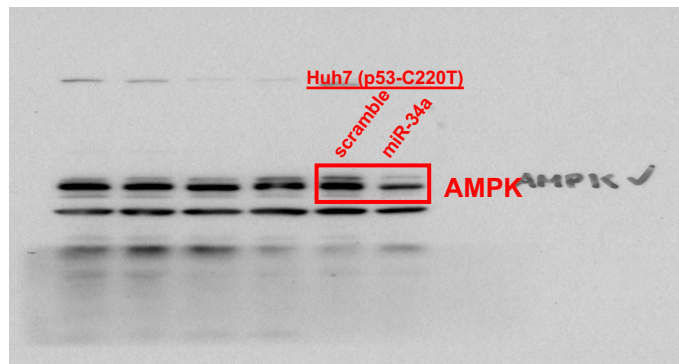


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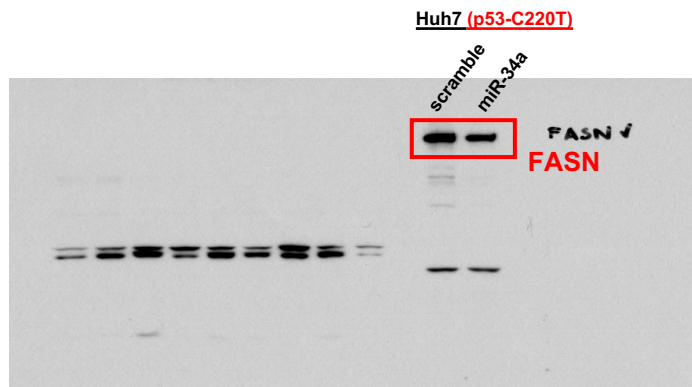


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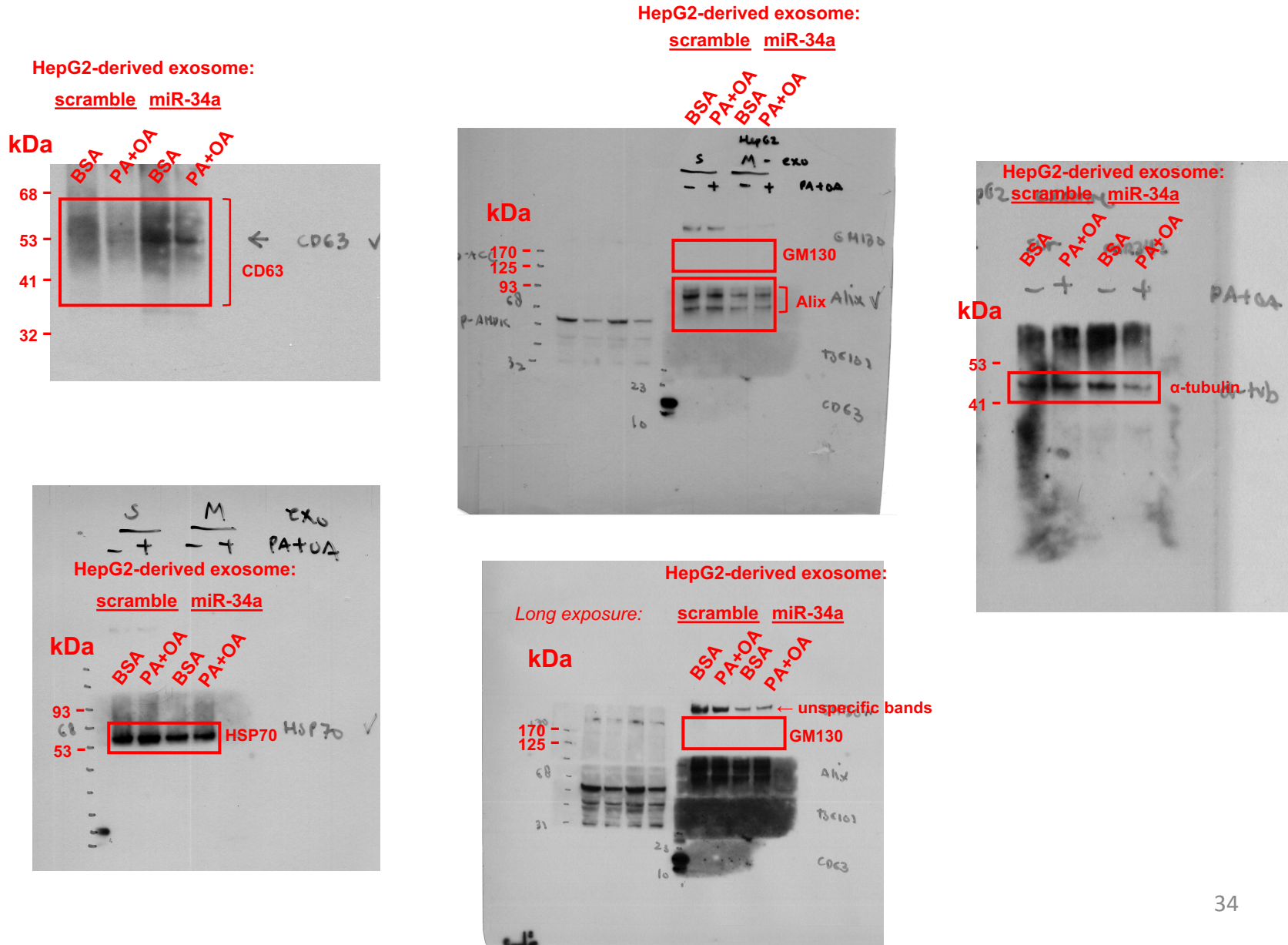


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Fig. 5k

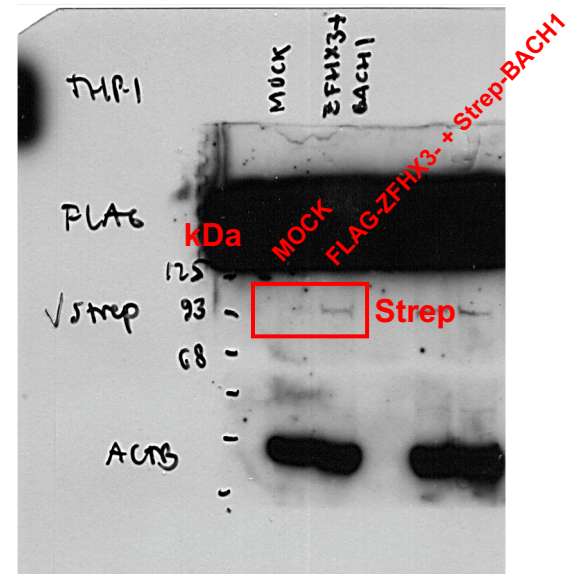
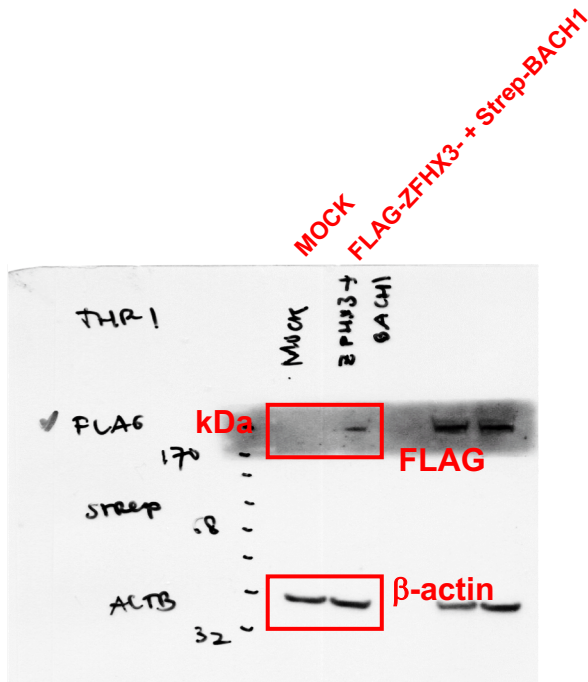


Figure Source Data Supplementary Fig. S1f

