Figure S1
Denzler et al.

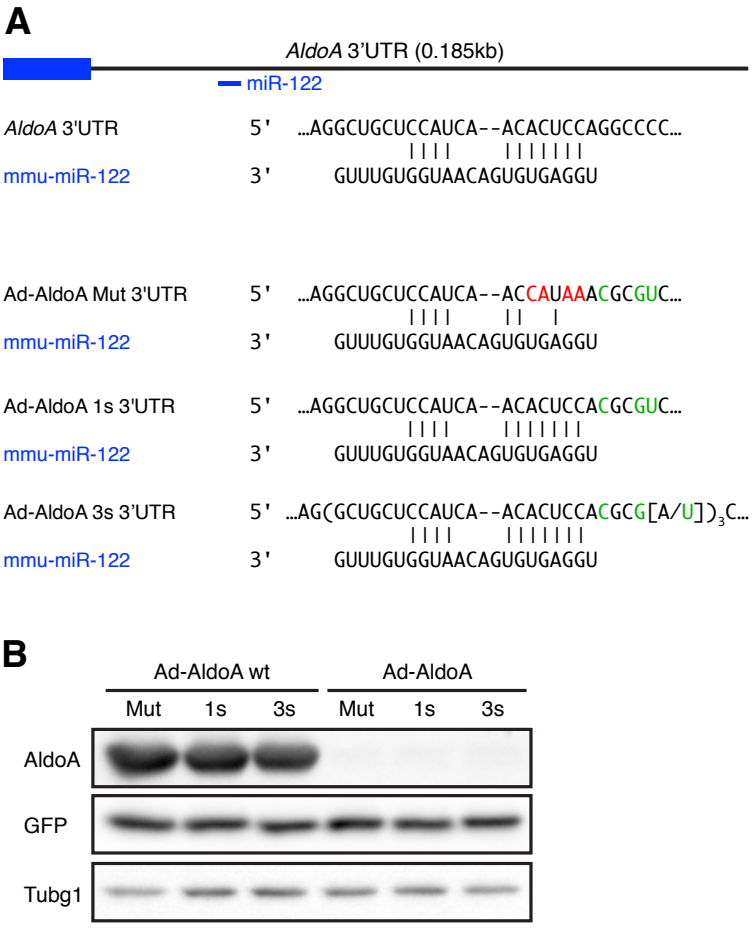
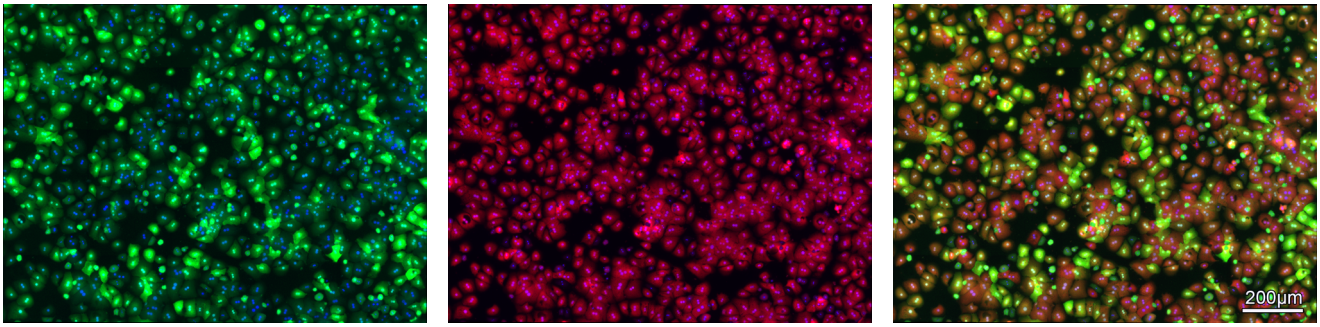


Figure S1: *AldoA* miR-122 sites and adenovirus characterization. Related to Figure 1.

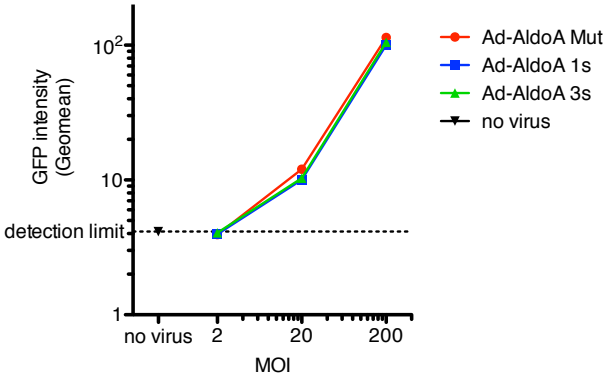
(A) Position and complementarity of the miR-122 binding site within the mouse *AldoA* 3′UTR (top) and the respective site(s) within the Ad-AldoA adenoviruses (bottom). Mutations in the seed match are shown in red, and additional differences compared to the endogenous *AldoA* 3′UTR are in green. **(B)** Western blot of primary hepatocytes infected with Ad-AldoA and Ad-AldoA expressing the full-length protein (Ad-AldoA-wt) with a mutated (Mut), one (1s) or three (3s) miR-122 binding site(s) at MOI 200.

Figure S2
Denzler et al.

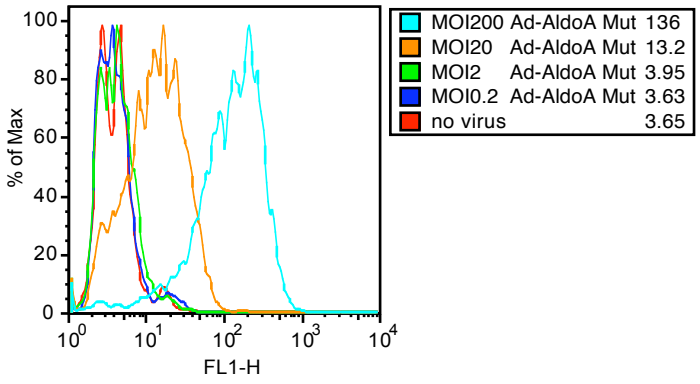
A



B



C



D

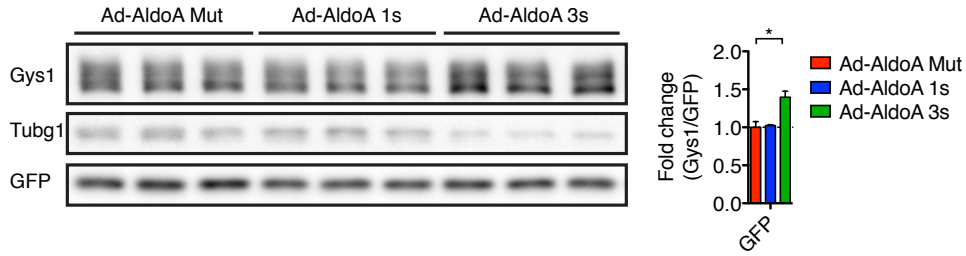


Figure S2: Analysis of adenovirus infection of primary hepatocytes. Related to Figure 1.

(A) Representative light microscopy image of primary hepatocytes infected with Ad-Ctrl at MOI 200 showing nuclear Hoechst 33342 staining (all pictures), viral GFP expression (green, left picture), cytoplasm staining (red, middle picture) and merge (right picture).

(B) Absolute quantification of GFP intensity of primary hepatocytes infected with Ad-AldoA with one (1s), three (3s) or a mutated (Mut) miR-122 binding site at MOI 0.2, 2, 20 or 200 and no virus. Data represent mean ± SEM (n = 3).

(C) Representative histogram showing flow cytometry data for the Ad-AldoA Mut at the indicated MOIs. Numbers at the right of the key indicate geomean intensity values.

(D) Western blot and respective quantification showing levels of proteins detected in primary hepatocytes infected with Ad-AldoA constructs at MOI 200. Data represent mean ± SEM (n = 3). *P < 0.05, unpaired t test.

Figure S3
Denzler et al.

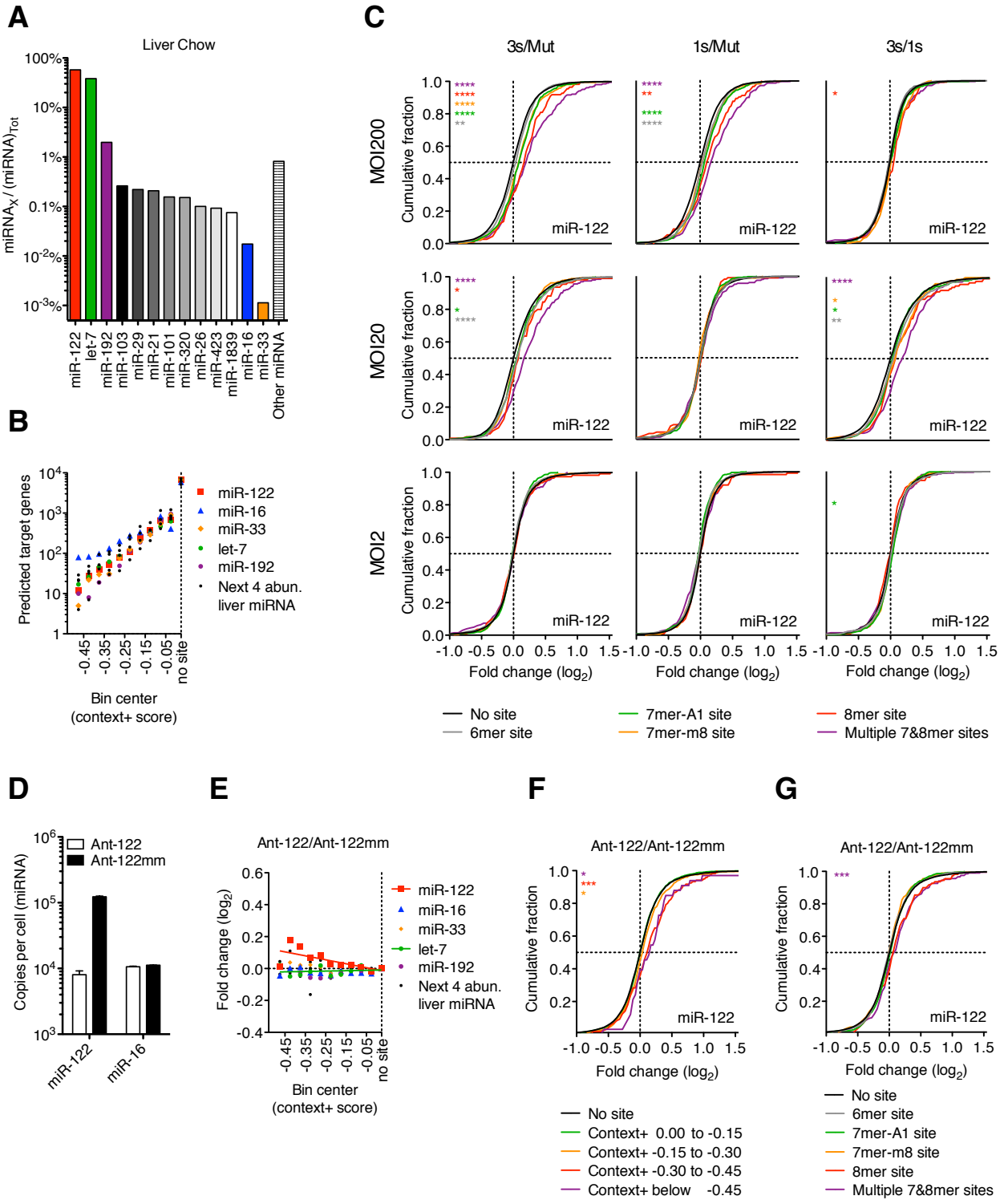


Figure S3: Amount of derepression correlates with predicted site efficacy and number of added *AldoA* MREs. Related to Figure 3.

(A) Relative expression miRNA families in wildtype livers as indicated by small-RNA sequencing. Represented are the 11 miRNA families most abundant in liver, plus miR-16 and miR-33.

(B) Number of predicted targets for the indicated miRNAs in each bin of Figure 4A.

(C) RNA-seq data from primary hepatocytes infected with MOI 2, 20 and 200 of Ad-AldoA Mut, 1s or 3s shown in Figure 1C-H and Figure 4B. Cumulative distributions of mRNA changes for genes with no miR-122 site (black) or predicted target genes with the indicated site type (color). Number of genes per bin: no site, 6444; 6mer, 1001; 7mer-A1, 398; 7mer-m8, 269; 8mer, 110; multiple, 203. *P < 0.05, **P < 0.01, ***P < 0.001, ****, P < 0.0001, one-sided Kolmogorov–Smirnov (K–S) test.

(D) miRNA molecules in primary hepatocytes treated with Ant-122 or Ant-122mm. Mean ± SEM (n = 3).

(E) RNA-seq results from primary hepatocytes of (D) showing mRNA changes of predicted targets after inhibiting miR-122. Predicted targets of miR-122 (red), miR-16 (blue), miR-33 (orange), let-7 (green), miR-192 (purple), or a combination of the next four most abundant liver miRNA families (black) were grouped into ten bins based on their context+ scores. For each miRNA family, the median $\log_2$-fold change is plotted for the predicted targets in each bin. Medians were normalized to that of the bin with genes without sites. Each bin had at least 10 genes; see (B) for group sizes.

(F) Cumulative distributions of mRNA changes in the hepatocytes of (D) for genes with no miR-122 site (black) or predicted target genes with the indicated context+ score bins (color). Number of genes per bin: black, 6629; green, 1693; orange, 434; red, 120; purple, 33. *P < 0.05, **P < 0.01, ***P < 0.001, ****, P <0.0001, one-sided K–S test.

(G) Cumulative distributions of mRNA changes in the hepatocytes of (D) for genes with no miR-122 site (black) or predicted target genes with the indicated sites (color). Otherwise, as in (C).

Figure S4
Denzler et al.

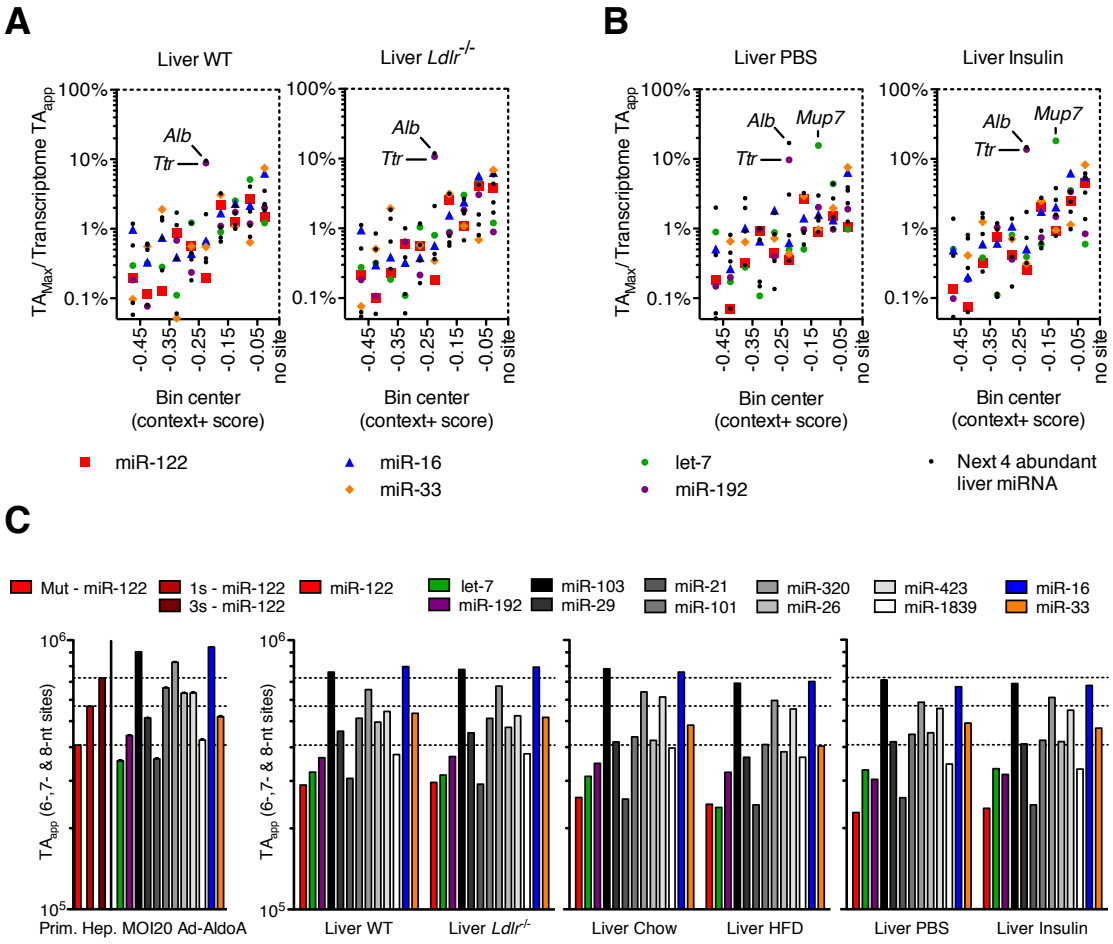


Figure S4: Modest changes in target abundance induced by metabolic stress and disease. Related to Figure 4.

(A–B) Fractional contribution of the largest potential contributors to transcriptome TA_{app} in livers originated from *Ldlr*$^{-/-}$ or wildtype (WT) mice **(A)** or in livers that originated from wildtype mice perfused either with Insulin or PBS **(B)**. Potential contributors were binned by their context+ score, and the top potential contributors are plotted within each bin.

(C) Transcriptome TA_{app} in primary hepatocytes (n = 3) infected with MOI 20 Ad-AldoA or in livers (n = 1) from models of physiological (Insulin) or disease/stress states (*Ldlr*$^{-/-}$ and HFD). Dashed lines indicate miR-122 TA_{app} at MOI 20 of Ad-AldoA Mut, 1s, and 3s.

Table S1: Gene expression levels, fold-changes, and predicted target site efficacy scores (context+ scores) across all hepatocyte RNA-seq samples. Related to Figure 3 and S3).

Table S2: Gene expression levels, fold-changes, and predicted target site efficacy scores (context+ scores) across all liver RNA-seq samples. Related to Figure 4 and S4).

Table S3: Primer sets used in this study

Name	Sequence (5' to 3')
P1 (AldoA 5'UTR)	CAGCTGAATAGGCTGCGTTC
P2 (AldoA 3'UTR)	TTTTTCCCCCTTAAATAGTTGTT
P3 (AldoA MluI site f)	CTCCATCAACACTCCACGCGTCTGCCTACCCACTTGC
P4 (AldoA MluI site r)	GCAAGTGGGTAGGCAGACGCGTGGAGTGTTGATGGAG
P5 (AldoA Mut MluI site f)	AGCTGAACTAAGGCTGCTCCATCAACCATAAACGCGTCTGCCTACCCACT TGCTATTGAAGA
P6 (AldoA Mut MluI site r)	CTCTTCAATAGCAAGTGGGTAGGCAGACGCGTTTATGGTTGATGGAGCAG CCTTAGTTCAGCT
P7 (AldoA miR-122 sites f)	CGCGAGCTGCTCCATCAACACTCCACGCGAGCTGCTCCATCAACACTCCA
P8 (AldoA miR-122 sites r)	CGCGTGGAGTGTTGATGGAGCAGCTCGCGTGGAGTGTTGATGGAGCAGCT
P9 (AldoA Stop f)	CAGCTCCTTCTTCTGCTCTCAGGTCAGTGCTGGGTATGG
P10 (AldoA Stop r)	CCATACCCAGCACTGACCTGAGAGCAGAAGAAGGAGCTG
36B4 f	GCCGTGATGCCCAGGGAAGACA
36B4 r	CATCTGCTTGGAGCCCACGTTG
AldoA f	GCGCTGTGTGCTAAAGATTG
AldoA r	AGGCTCCACAATGGGTACAA
GFP f	GAAGCGCGATCACATGGT
GFP r	CCATGCCGAGAGTGATCC
Gys1 f	GGTGTGAGGACGCAGGTAG
Gys1 r	GCCAACGCCCAAAATACA
Slc7a1 f	ATTTCAGCCGGCCTCCTA
Slc7a1 r	TGCCCACAGTGTCCCTTC
P4ha1 f	CGTGGGGAGGGTATCAAAAT
P4ha1 r	ATGGTAGCGGCAGAACAGTC
Ndrg3 f	TCCTGGCCAACAAGAAGC
Ndrg3 r	CTCATCCATGGTGGGGTACT
Tmed3 f	GGTCACGGCTCTCACTCAG
Tmed3 r	TCACAGTCTTCAGAGCCTCGT
Snrk f	TGCGGGTCTCTTGCATACT
Snrk r	GCCCAGGCTCCATATGTCTA
Dyrk2 f	CTACCACTACAGCCCACACG
Dyrk2 r	TCTGTCCGTGGCTGTTGA
ApoM f	CCCAGACATGAAAACAGACCT
ApoM r	GGGTGTGGTGACCGATTG
Crot f	AGTGAAGGGCATTGTCCAAC
Crot r	TCTTGTGGATATATGTCAATTGTCTG
Chka f	AACAGATTTGCTCTTGCCTCTC
Chka r	TCAAAGTAGGCCTCGAATCTG

EXTENDED EXPERIMENTAL PROCEDURES

Animal Experiments

Animals were maintained on a 12-hour light/dark cycle under a controlled environment in a pathogen-free facility at the Institute for Molecular Systems Biology, ETH Zürich (Switzerland). The *Ldlr* KO mice were originally obtained from the Jackson Laboratory (B6.129S7-*Ldlrtm1Her*/J) and backcrossed for >10 generations into a *C57Bl/6J* background. For the liver insulin perfusion experiments, starved *C57Bl/6J* mice were euthanized and the portal vein was canulated. The liver was perfused with oxygenated Krebs–Henseleit buffer with 0 or 20 ng/ml insulin at 37 °C in a single-pass mode with a total flow rate of 1.5 to 2 ml min^{-1} for 4 hours (Wolfrum et al., 2004). All animal experiments were approved by the ethics committee of the Kantonale Veterinäramt Zürich.

Primary Hepatocytes Isolation and Viral Infections

Primary hepatocytes were isolated based on the method described by Zhang et al. (Zhang et al., 2012) with the following modifications and conditions (additional explanations, images, and videos to primary hepatocyte isolation can be found on http://www.mouselivercells.com). Male 8- to 12-week-old C57BL/6N mice (Charles River) were anesthetized by intraperitoneal injection of 150 ul pentobarbital (Esconarkon US vet) pre-diluted 1:5 in PBS. The liver was perfused by cannulation of the caudal vena cava with the portal vein as a drain. The liver was perfused with pre-warmed Hank's Balanced Salt Solution (Life Technologies) containing 0.5 mM EGTA followed by pre-warmed digestion medium [DMEM 1 g/l glucose (Life Technologies) supplemented with 1% Penicillin-Streptomycin (Life Technologies), 15mM HEPES (Life Technologies) and 30 ug/ml Liberase TM Research Grade medium Thermolysin concentration (Roche)] each for four minutes with a flow rate of 3 ml min^{-1}. The liver was surgically removed, hepatocytes released into 10 ml digestion media by shaking and supplemented with 15 ml ice cold low glucose media [DMEM 1 g/l glucose (Life Technologies) supplemented with 1% Penicillin-Streptomycin (Life Technologies), 10% heat-inactivated fetal bovine serum (Sigma) and 1% Glutamax (Life Technologies)] and filtered through a 100 um Cell Strainer (BD). The suspension was then washed three times with 25 ml of ice-cold low glucose media (50g at 4°C for 2 min). Hepatocytes were counted and plated at 300,000 cells/well in surface-treated 6-well plates (BD Primaria) in low glucose media. 4–6 hours after plating, cells were infected with adenovirus constructs in Hepatozyme media [HepatoZYME-SFM (Life Technologies) supplemented with 1% Penicillin-Streptomycin (Life Technologies),

1% Glutamax (Life Technologies)] and harvested 24 hours post infection. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

RNA Isolation

RNA was extracted using Trizol (Life Technologies) according to the manufacturer's instructions, except for a 30 min isopropanol precipitation at –20°C. RNA integrity was analyzed on an Agilent 2100 Bioanalyzer for all samples that were sequenced.

Cell Number Calculation

By dividing total RNA yield from one 6-well plate well (22 ug RNA) by the cell number obtained from microscopy (300,000 cells per 6-well plate well), one hepatocyte was calculated to yield 73.5 pg RNA.

Gene Expression Analysis

2 ug of total RNA was treated with the DNA-free Kit (Life Technologies) and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR reactions were performed with the Light Cycler 480 (Roche) employing a 384-well format, gene-specific primer pairs (see Table S3, designed by http://qpcr.probefinder.com/organism.jsp) and KAPA SYBR Fast qPCR Master Mix (2x) for LightCycler 480 (Kapa Biosystems). Cycles were quantified employing Light Cycler 480 Analysis Software (Abs quantification/ 2^{nd} derivate max). Relative gene expression was calculated using the ddCT method and mouse *36b4* (*Rplp0*) for normalization. For absolute mRNA quantification the pCR2.1 plasmids of AldoA and the coding regions of Carnitine O-octanoyltransferase (*Crot*, NM_023733.3), Choline Kinase alpha (*Chka*, NM_013490) and Apolipoprotein M (*ApoM*, NM_018816) were cloned into plasmids with a T7 promoter. All plasmids were linearized after the poly-A region, transcribed using the T7 Quick High Yield RNA Synthesis Kit (NEB), cleaned up using the RNAeasy MinElute Cleanup Kit (Qiagen), and visualized on a gel to confirm the presence and correct size of the RNA. Molar concentrations of mRNA transcripts were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and the respective molecular weight. To create a standard curve, the mRNA transcripts were serial diluted, spiked into 2 ug of yeast RNA, reverse transcribed and quantified by PCR performed as described above.

miRNA Expression Analysis

150 ng of total RNA was reverse-transcribed using TaqMan MicroRNA Assays (Life Technologies) and TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The RT primers were multiplexed in a dilution of 1:20 as described by the manufacturer. Quantitative PCR reactions were performed with the Light Cycler 480 (Roche) employing a 384-well format, TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies) and TaqMan MicroRNA Assays (Life Technologies). Cycles were quantified employing Light Cycler 480 analysis software (Abs quantification/ 2^{nd} derivate max). Relative miRNA expression was calculated using the ddCT method and mouse snoRNA202 for normalization. For absolute quantification synthetic miRNAs (Sigma-Aldrich) were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and the respective molecular weight. miRNAs were spiked into primary hepatocyte cell lysates and absolutely quantified employing a synthetic miRNA standard curve.

Small RNA sequencing and data analysis

For small-RNA libraries (performed by BGI): Total RNA was size fractionated (18–30 nt), followed by 5′ and 3′ adaptor ligations, RT-PCR, and Solexa sequencing. To quantify miRNA levels, we counted the number of occurrences in which the first 20 nt of the raw sequence matched a known mature *Mus musculus* miRNA sequence deposited in miRBase version 20.

RNA-seq data analysis

To process RNA-seq data, raw reads were aligned to the latest build of the mouse genome (mm10) using STAR v. 2.3.1n (options --outFilterType BySJout --outFilterMultimapScoreRange 0 --readMatesLengthsIn Equal --outFilterIntronMotifs RemoveNoncanonicalUnannotated --clip3pAdapterSeq TCGTATGCCGTCTTCTGCTTG --outStd SAM) (Dobin et al., 2013). The option "--clip3pNbases 2" was additionally used for the libraries with the following codes: ACAGTG-s_6, CAGATC-s_6, CGATGT-s_6, CTTGTA-s_6, GCCAAT-s_6, TGACCA-s_6 due to sequencing errors in the last 2 nucleotides. Pooling all biological replicates of a particular sample, differential expression analysis was performed between two samples of interest using cuffdiff v. 2.1.1 (options --library-type fr-firststrand -b mm10.fa -u --max-bundle-frags 100000000) (Trapnell et al., 2013), using mouse transcript models of protein-coding and long noncoding RNA genes annotated in Ensembl release 72. For data analysis, only genes with FPKM

above 1.0 were considered. Raw files as well as processed files for gene expression measurements and differential expression analysis are deposited in the GEO (accession ID GSE52801).

Target Abundance Calculation

We fit a linear regression function to transform gene expression measurements (measured in FPKM) from RNA-seq data into absolute copy numbers (as determined by quantitative PCR) (Figure 4A). To account for experimental noise in qPCR and RNA-seq measurements, we used the constraints that there be no x or y weighting and that the y intercept equal 0. Copy numbers per cell were determined by multiplying the FPKM with the resulting slope of 3.83. For each miRNA, the copy number of each predicted target gene was weighted by the number of 6-, 7-, and 8-nt 3′UTR binding sites, and these values were summed to yield TA_{app}.

Generation of adenovirus construct

AldolaseA variant 2 (*AldoA*, NM_007438) was amplified from cDNA with primers P1 and P2 (Table S3) using platinum PCR SuperMix High Fidelity (Life Technologies) and brought into the plasmid PCR 2.1-TOPO (pCR2.1) vector using TOPO TA Cloning Kit (Life Technologies). An MluI restriction site was introduced after the miR-122 binding site (Aldo 1s wt) using QuickChange II Site-Directed Mutagenesis Kit (Agilent) and primers P3 and P4. Analogously an MluI restriction site along with mutations in the seed target region of miR-122 at positions 2, 3, 5 and 6 (Aldo Mut wt) using the primers P5 and P6 were inserted. Two additional miR-122 binding sites were introduced into Aldo 1s wt by inserting a synthetic linker resulting in Aldo 3s wt. The plasmid pCR2.1 Aldo 1s wt was digested with MluI (NEB), dephosphorylated using Antarctic Phosphatase (NEB) and cleaned up with QIAaquick Gel Extraction Kit (Qiagen). For the synthetic linkers an unimolar mixture of linker oligonucleotite P7 and P8 with two miR-122 binding sites were first denatured at 95°C for 5 minutes, annealed by lowering the temperature from 70°C for 10 minutes followed by 60°C, 50°C, 40°C, and 20°C for 3 minutes each (using a ramping temperature of 0.07°C/s) and phosphorylated using T4 Polynucleotide Kinase (NEB). The linker and pCR2.1 Aldo 1s wt backbone were then ligated at room temperature for 1 hour using T4 DNA Ligase (NEB), transformed in Mach1 cells (Life Technologies), screened for insertion length and then sequenced. The three plasmids (pCR2.1 Aldo Mut wt, 1s wt, and 3s wt) were then re-cloned into pVQAd CMV K-NpA (pVQAd, Viraquest) using the restriction sites BamHI and XhoI (NEB). Finally, a stop codon was introduced at amino acid position ten using QuickChange II Site-Directed Mutagenesis Kit

(Agilent), primers P9 and P10 and plasmids pVQAd Aldo Mut wt, 1s wt, and 3s wt resulting in plasmids pVQAd Aldo Mut, 1s, and 3s. All pVQAd plasmids constructs were sent for adenovirus production to Viraquest Inc., USA.

Antibodies

Antibodies used for Immunoblotting: AldoA (rabbit, 1:1000, Cell Signaling, #3188), GFP (rabbit, 1:1000; Life Technologies, A11122), Tubg1 (mouse, 1:5000, Sigma-Aldrich, T6557), Gys1 (rabbit, 1:300, Cell Signaling, #3893).

Immunoblotting

Cells were lysed with 250 ul RIPA lysis buffer (50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 1% NP-40, 1% sodium deoxycholate, 1% triton-X 100 and protease inhibitor cocktail) for 5 min on ice. Protein concentration was determined using the Bicinchoninic Acid Kit (Sigma-Aldrich). Equal protein amounts were boiled in Laemmli buffer (1.7% SDS, 5% glycerol, 0.002% bromophenol blue, 60 mM Tris-HCl pH 6.8, 100 mM DTT) for 5 min at 98°C, separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting in a wet chamber (Bio-Rad). The membranes were blocked for one hour with 5% non-fat dry milk TBS-0.1% Tween (Sigma-Aldrich), incubated with the primary antibodies overnight at 4°C, followed by a one hour incubation with a horseradish peroxidase-conjugated secondary antibody (Calbiochem). Blots were then developed by chemiluminescent detection with a Fujifilm analyzer (LAS-4000) and quantified using ImageJ (Schneider et al., 2012).

Immunohistochemistry

Cells were fixed on ice for 45 min with 4% paraformaldehyde, permeabilized for 15 min at room temperature with PBS containing 0.1% Triton-X and stained with PBS containing 1:10’000 HCS CellMask Red stain (Life Technologies) and 1:2000 Hoechst 33342 (Life Technologies) for 30 min at room temperature. Plates were imaged with Zeiss Axio Observer Z1 at 20x magnification and cell numbers assessed using cell profiler software (Kamentsky et al., 2011).

Flow cytometry

Primary hepatocytes were trypsinized, fixed at room temperature for 15 min with 4% paraformaldehyde and resuspended in FACS buffer (2% FBS, 5 mM EDTA, and 0.02% NaN3 in PBS). Samples were analyzed counting 10,000 events per sample using a BD FACSCalibur flow cytometer and the FlowJo software package.

Plasma cholesterol levels

Cholesterol was measured from mouse serum using a commercial kit (Roche Diagnostics).

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