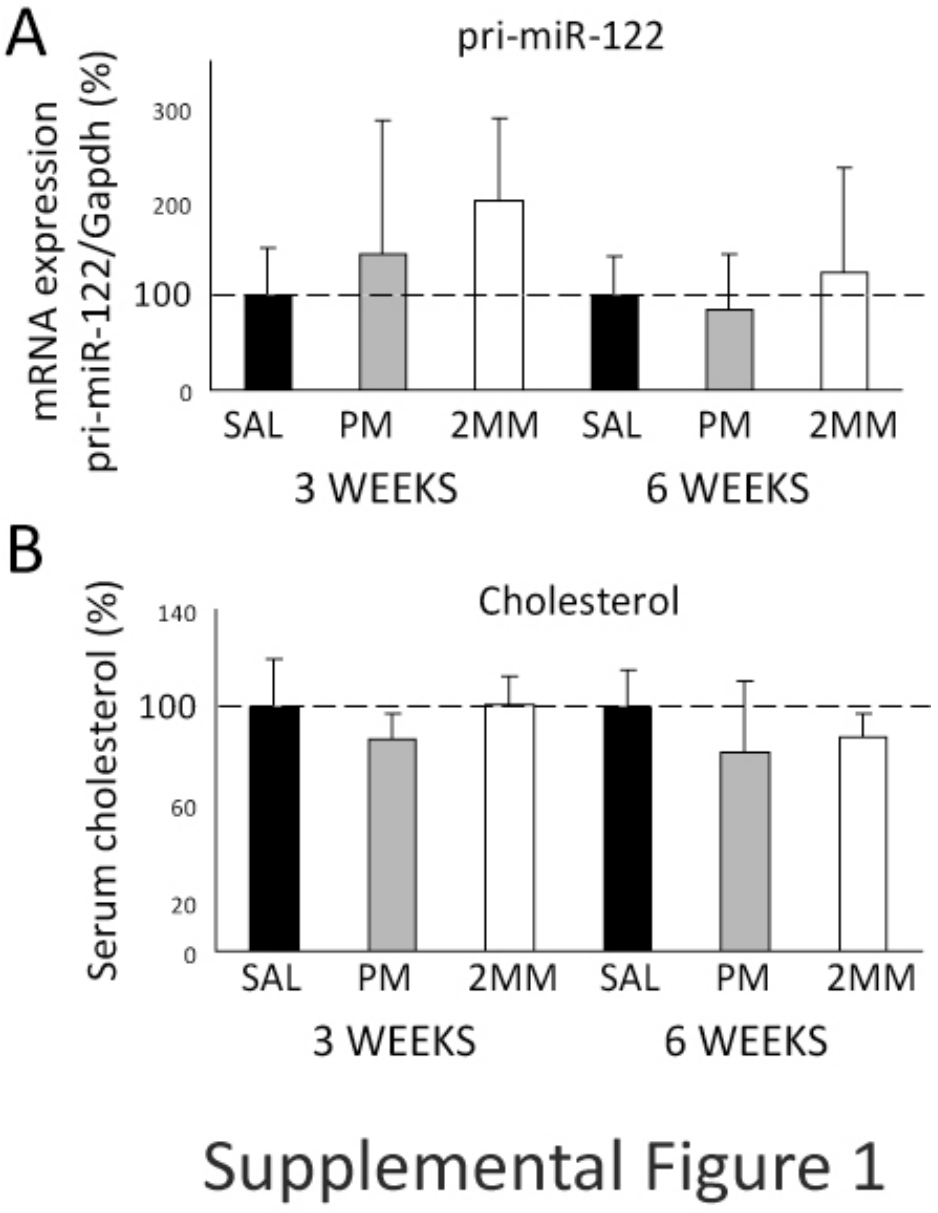
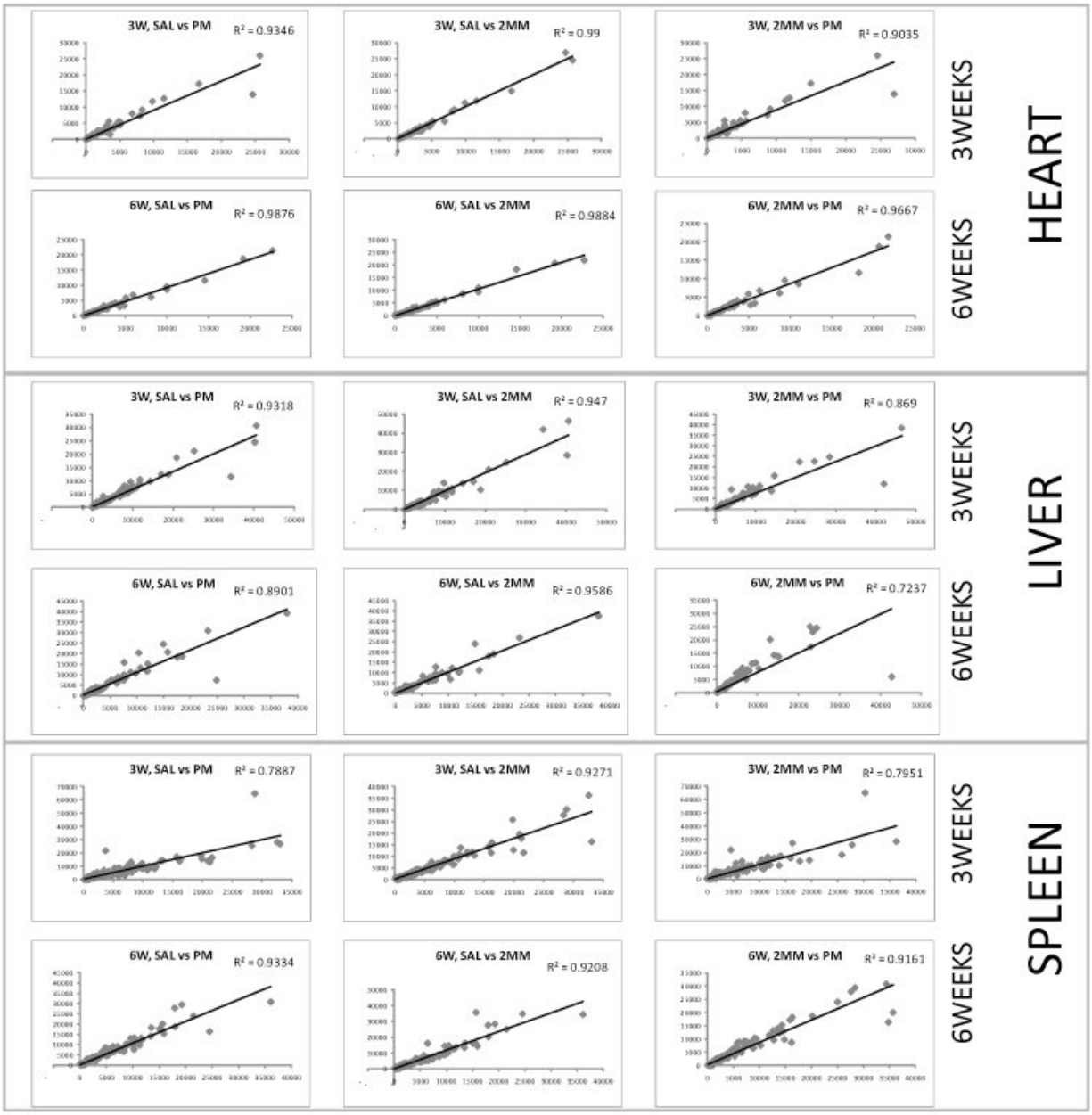


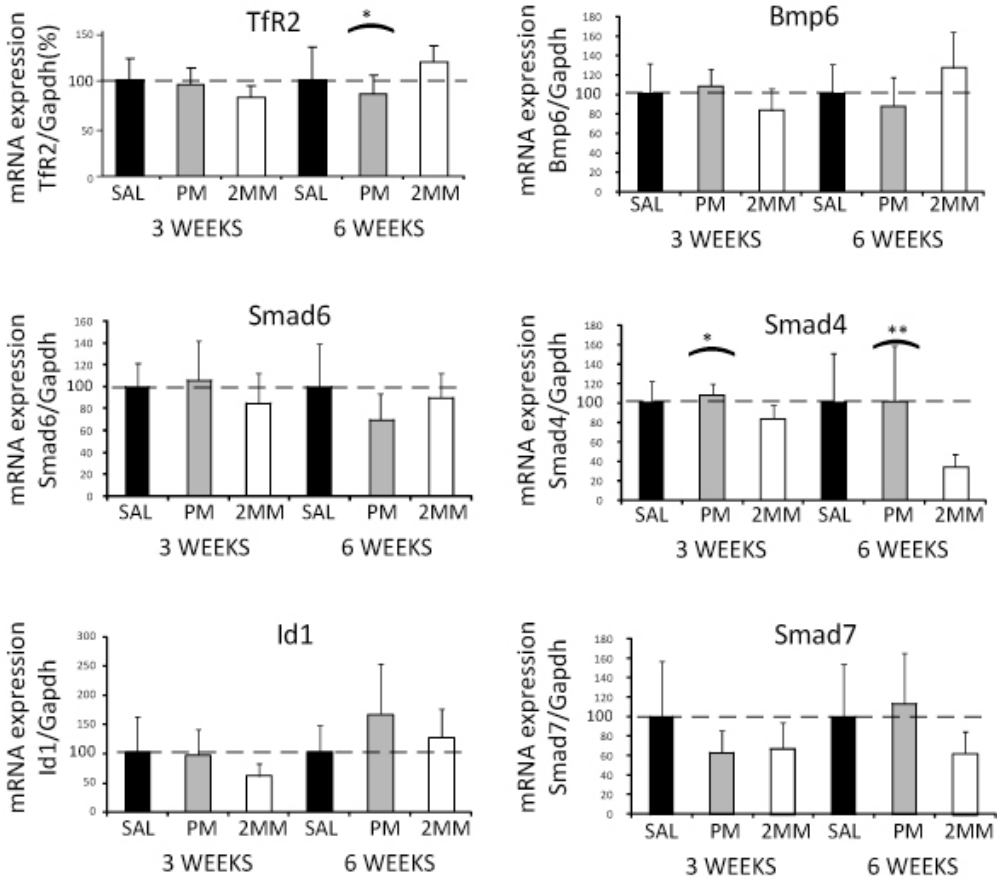
Supplemental Figure 1

AntimiR-122 administration does not alter pri-miR-122 expression levels. (**A**) Analysis of pri-miR-122 expression three (p=0.205) and six (p=0.611) weeks after antimiR administration. Data were normalized to mRNA expression of a reference gene, *Gapdh*. Data are represented as mean ± SD (n=8), whereby data from the SAL-treated group were set to 100%. Total serum cholesterol is reduced in PM_antimiR-122 mice. (**B**) Total serum cholesterol was analyzed as previously described (30) three (p=0.116) and six (p=0.197) weeks after antimiR administration. Data are represented as mean ± SD (n=8), whereby data from the SAL-treated group were set to 100%. Statistical significance was determined by using one-way ANOVA, *p < 0.05. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122.



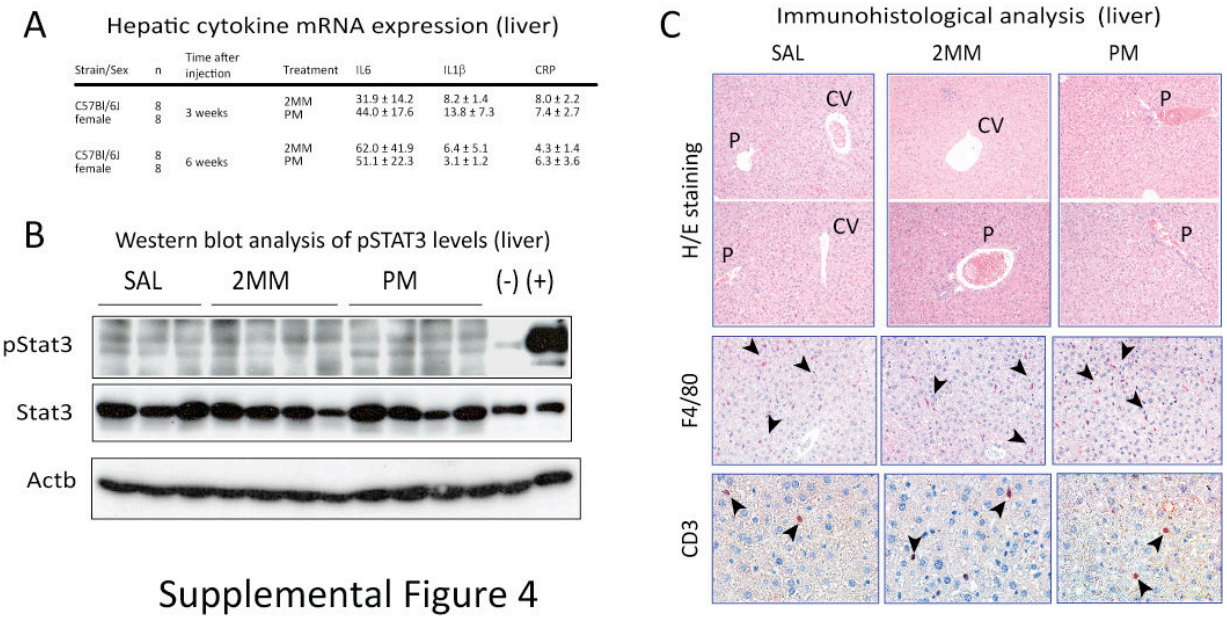
Supplemental Figure 2

AntimiR administration specifically and exclusively affects expression of miR-122 in the liver. Scatter plots indicating miRNA expression in the heart (**top panel**), in the liver (**middle panel**) or in the spleen (**lower panel**) of antimiR treated mice three and six weeks after antimiR administration. Tissue miRNAs were scored as significantly changed when altered more than two-fold in PM_antimiR-122 mice compared to the controls [saline (SAL) and 2MM_antimiR-122 (2MM)]. The linear relationship between the X and Y variables (i.e. samples) was calculated by using the correlation coefficient R^2 (An R^2 of 1.0 indicates that the regression line perfectly fits the data).



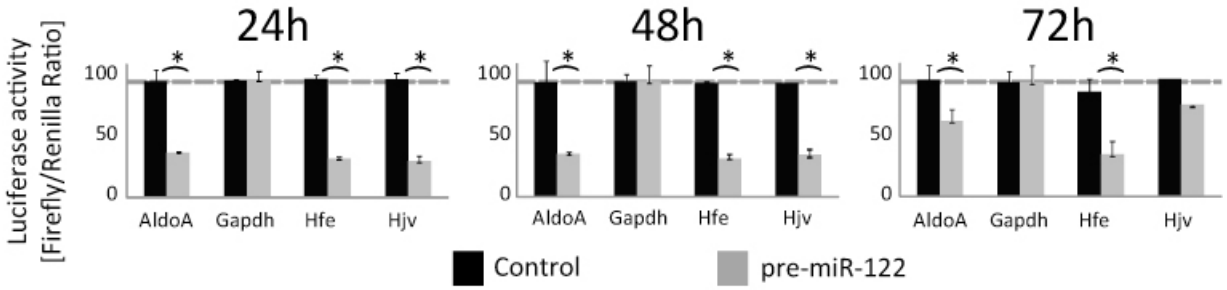
Supplemental Figure 3

mRNA expression of genes involved in hepcidin activation or suppression in PM_antimiR-122 mice. Analysis of hepatic mRNA expression of transferrin receptor 2 (TfR2; p=0.0467 at six weeks time point), Smad family member 6 (Smad6), Bone morphogenetic protein 6 (Bmp6), Smad family member 4 (Smad4; p=0.024 and p=0.0087 at three and six weeks respectively), inhibitor of DNA binding 1 (Id-1) and Smad family member 7 (Smad7) was analyzed by qPCR three and six weeks after antimiR administration. Data were normalized to mRNA expression of a reference gene, Gapdh. Data are represented as mean ± SD (n=8), whereby data from the SAL-treated group were set to 100%. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122. %. Statistical significance was determined by using one-way ANOVA, *p < 0.05, **p < 0.01.



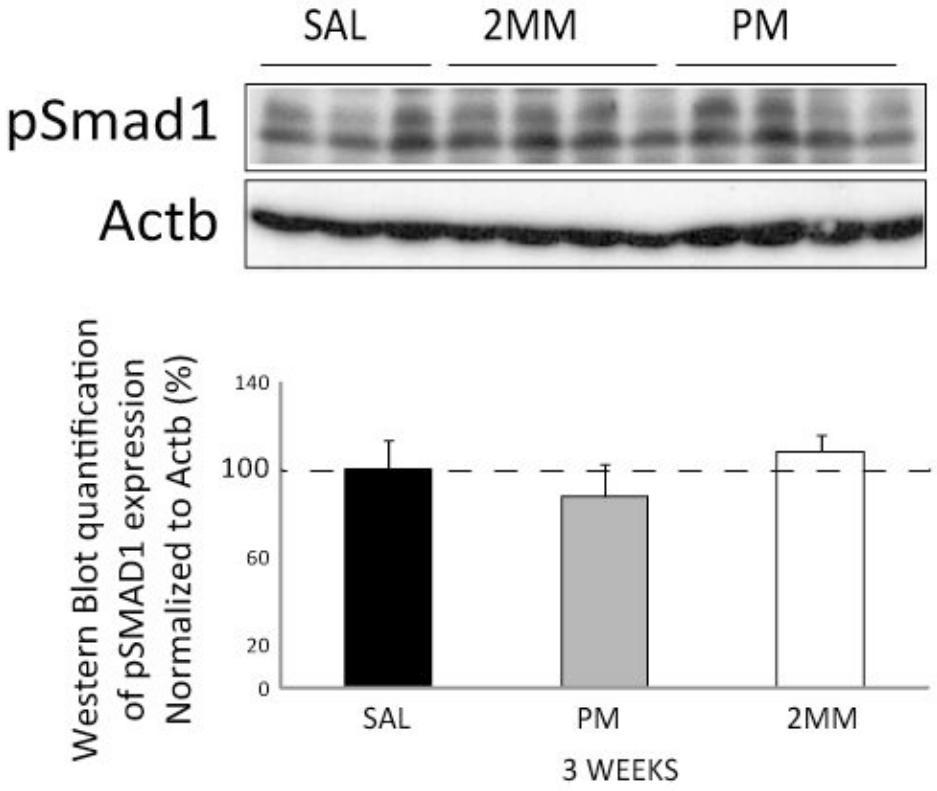
Supplemental Figure 4

(**A**) Cytokine mRNA expression in response to hepatic miR-122 inhibition. mRNA expression of the cytokines IL6 (Interleukin 6) and IL1b (Interleukin 1b) and of the target gene CRP (C-reactive protein), was analyzed by qPCR of liver total RNA from C57BL/6J mice three and six weeks after PM_antimiR-122 or 2MM_antimiR-122 administration. The number of mice analyzed is indicated. For qPCR data normalization Gapdh, Actin B and Tubulin A were used. Data are shown as mean ± SD. PM: PM_antimiR-122; 2MM: 2MM_antimiR-122; (+) positive control; (-) negative control. (**B**) Western Blot analysis of pStat3 and total Stat. Phospho Stat3 (pStat3) and total Stat3 (Stat3) was assessed by western blot analysis in the liver of mice three weeks after PM_antimiR-122 injection. ActinB (Actb) is shown as loading control. As a positive control (+) primary hepatocytes were treated with 5 ng of IL6 for 30 minutes. As a negative control (-) primary hepatocytes were treated with the vehicle solution. Immunohistological analysis of liver morphology. (**C**) To investigate whether antimiR-122 administration induces morphological lesions we performed histochemical stains (hematoxylin-eosin, **E/H**) on liver sections using conventional microscopy (3µm). Hepatic morphology was similar in the two control groups and in the PM_antimR-122-injected mice. **P** indicates, the Portal vein, **CV** the Central vein. To exclude that antimiR-122 administration results in the invasion of immune cells into the liver we performed conventional microscopy analysis of immunohistological stains. We did not identify any significant difference in the number of cells positive for macrophage/Kupfer cell markers (**F4/80, arrowheads**) or in the number of cells positive for T-cell markers (**CD3, arrowheads**). All samples were analyzed three weeks after PM_miR-122 injection. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122. [The immunohistological method is described in detail in Wang S et al. Dis Model Mech. 3(1-2):92-103, 2010.



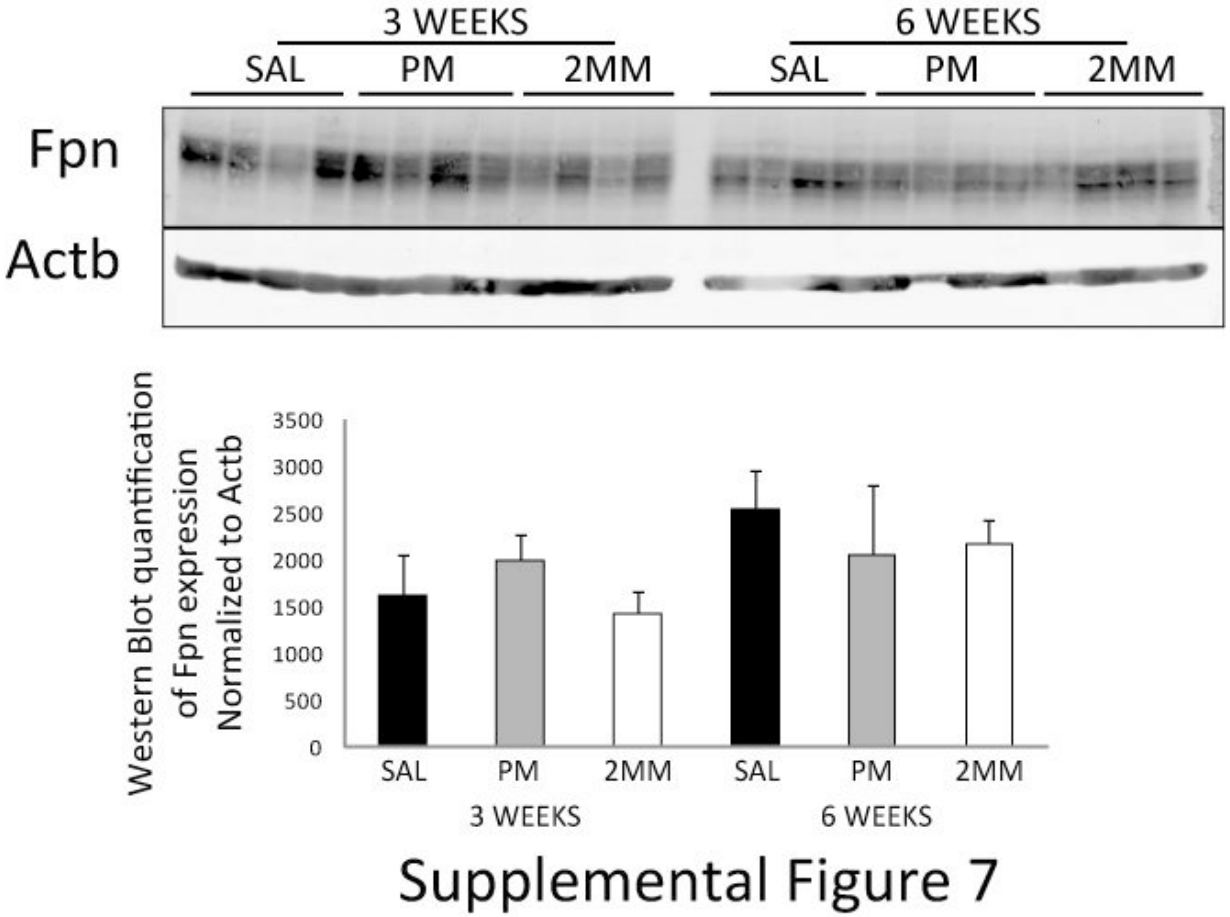
Supplemental Figure 5

Analysis of sequences predicted to bind miR-122 within the Hfe or Hemojuvelin 3'-UTRs. Hepa1-6 cells were cotransfected with luciferase reporter vectors (pMIR) linked to the 3'-UTRs of the murine Aldolase A (pMIR-mAldoA, AldoA), mGapdh (pMIR-mGapdh; Gapdh), Hfe (pMIR-mHfe, Hfe) or Hemojuvelin (pMIR-mHjv, Hjv) genes and a miR-122 mimic. Luciferase activity was measured 24, 48 and 72 hours later. The luciferase reporter assays are presented as normalized firefly luciferase counts ± SD of the pre-miR-122 and control cells. Experiments were performed at least in triplicate and results of the luciferase reporter assay are presented as fold change ± SD of the pre-miR-122 and control transfected cells. Statistical significance was determined using two tailed Student's t-test, *$p < 0.05$



Supplemental Figure 6

Phosphorylation of Smad1 (pSmad1) was assessed by western blot analysis in the liver of mice three weeks after PM_antimiR-122 injection. ActinB (Actb) is shown as loading control. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122.



Supplemental Figure 7

Ferroportin (Fpn) protein expression was assessed by western blot analysis in the spleen of mice three and six weeks after PM_antimiR-122 administration. ActinB (Actb) is shown as loading control. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122.

Supplemental Figure 8

miRNAs with altered expression levels in the spleen of PM_antimiR-122 treated mice. Heat map of differentially regulated miRNAs in spleens of PM_antimiR-122 (PM) and control [saline (SAL) and 2MM_antimiR-122 (2MM)] mice. MiRNA expression profiles were determined three and six weeks after antimiR administration and differentially regulated miRNAs were identified by applying Serial Microarray Analysis (SAM). Expression levels are indicated by colors. miRNAs with decreased expression in PM_antimiR-122 were not detected.

Supplemental Table 1, Haematological parameters

Strain/Sex	n	Time after injections	Treatment	MCV (fl)		MCH (pg)	Reticulocytes (‰)		Rhb (pg)	
C57Bl/6J	8		SAL	46.9 ± 0.8		15.5 ± 0.5	3.9 ± 1.3		14.7 ± 0.4	
female	8	3 weeks	PM	45.5 ± 1.6	*	15.4 ± 0.9	6.8 ± 2.4	*	14.2 ± 0.3	*
	8		2MM	46.7 ± 1.7		16.1 ± 0.3	4.4 ± 1.8		14.4 ± 0.2	
C57Bl/6J	8		SAL	47.9 ± 1.0		15.9 ± 0.6	5.9 ± 3.4		15.4 ± 0.6	
female	8	6 weeks	PM	45.6 ± 1.5	**	15.0 ± 0.9	9.9 ± 6.4		15.0 ± 0.7	
	8		2MM	47.0 ± 2.0		15.6 ± 0.9	5.1 ± 2.4		15.0 ± 0.6	

Strain/Sex	n	Time after injections	Treatment	WBC (/nl)	RBC (/pl)	HGB (g/dl)	HCT (l/l)
C57Bl/6J	8		SAL	278.3 ± 83	6.83 ± 0.5	10.49 ± 0.8	0.32 ± 0.03
female	8	3 weeks	PM	355.5 ± 94.7	6.86 ± 0.4	10.45 ± 0.7	0.31 ± 0.02
	8		2MM	354.2 ± 59.4	6.65 ± 0.8	10.58 ± 1.4	0.31 ± 0.05
C57Bl/6J	8		SAL	295.4 ± 96.2	6.85 ± 0.4	10.84 ± 0.6	0.33 ± 0.02
female	8	6 weeks	PM	252.8 ± 156.8	6.88 ± 0.4	10.36 ± 0.7	0.31 ± 0.02
	8		2MM	353.5 ± 112.6	6.86 ± 0.3	10.75 ± 0.8	0.32 ± 0.02

Strain/Sex	n	Time after injections	Treatment	MCHC (g/dl)	RDW (%)	TRB (/nl)
C57Bl/6J	8		SAL	33.1 ± 0.8	12.4 ± 0.3	601 ± 239
female	8	3 weeks	PM	33.5 ± 2.3	12.6 ± 0.9	434 ± 185
	8		2MM	34.1 ± 2.0	12.6 ± 0.7	486 ± 174
C57Bl/6J	8		SAL	33.0 ± 1.4	12.7 ± 0.3	410 ± 152
female	8	6 weeks	PM	33.5 ± 3.2	13.1 ± 0.7	399 ± 192
	8		2MM	33.4 ± 2.4	12.8 ± 0.6	434 ± 178

Strain/Sex	n	Time after injections	Treatment	UIBC	TIBC		Tf (%)
C57Bl/6J	8		SAL	132.2 ± 27	454.5 ± 84		70.7 ± 6
female	8	3 weeks	PM	139.2 ± 26	416.1 ± 29		66.7 ± 5
	8		2MM	142.0 ± 14	480.7 ± 46		70.3 ± 4
C57Bl/6J	8		SAL	131.0 ± 22	390.7 ± 83		65.8 ± 6
female	8	6 weeks	PM	137.6 ± 27	504.5 ± 58	**	72.6 ± 5
	8		2MM	119.7 ± 20	381.1 ± 56		68.3 ± 5

Complete list of hematological parameters analyzed in antimiR-injected mice. MCV, Mean Corpuscular Volume; MCH, Mean corpuscular hemoglobin; RhB, Hemoglobin content of reticulocytes; WBC, White Blood Cells; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cell Distribution Width; TRB, Thrombocytes; UIBC, unsaturated iron-binding capacity; TIBC, Total iron binding capacity; Tf, Total Iron Transferrin. Number of mice (n) is indicated. Data are shown as mean ± SD. Statistical significance was determined by using one-way ANOVA, *$p < 0.05$; **$p < 0.01$. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122.

Supplemental Table 2A, Haematological parameters & liver iron measurments

Strain/Sex	n	Time after injections	Treatment	MCV (fl)	MCH (pg)	Reticulocytes (‰)	Liver Iron (µg/g)
C57Bl/6J	5		SAL	45.4 ± 0.9	14.9 ± 0.5	4.7 ± 1.2	306.6 ± 103.2
female	5	1 week	PM	45.2 ± 0.4	15.8 ± 0.2	4.3 ± 0.6	291.9 ± 105.5
	5		2MM	45.6 ± 0.6	15.3 ± 0.3	4.8 ± 0.5	366.6 ± 91.7
C57Bl/6J	5		SAL	45.8 ± 1.3	15.8 ± 0.3	4.5 ± 0.3	279.4 ± 21.2
female	5	2 weeks	PM	44.8 ± 0.5	15.3 ± 0.2 **	5.4 ± 0.5 **	330.6 ± 48.3
	5		2MM	45.4 ± 1.2	15.7 ± 0.2	4.6 ± 0.7	343.1 ± 42.4
C57Bl/6J	5		SAL	45.8 ± 0.5	15.5 ± 0.3	4.3 ± 0.4	383.2 ± 91.2
female	5	3 weeks	PM	43.8 ± 0.8 **	14.8 ± 0.4 **	5.0 ± 0.3 **	225.6 ± 57.4
	5		2MM	45.4 ± 0.6	15.5 ± 0.2	4.5 ± 0.4	371.8 ± 93.7

Supplemental Table 2B, tissue iron measurments

Strain/Sex	n	Time after injections	Treatment	Liver Iron (µg/g)	Plasma Iron (µg/dL)
NMRI	7	1 week	SAL	410.2 ± 76.3	457.4 ± 85.3
female	7		PM	367.8 ± 44.5	939.6 ± 58.2
NMRI	7	2 weeks	SAL	405.9 ± 74.5	491.4 ± 174.6 *
female	7		PM	389.4 ± 80.6	345.7 ± 46.2
NMRI	7	3 weeks	SAL	461.1 ± 117.3 *	426.0 ± 93.9 **
female	7		PM	327.0 ± 91.3	306.6 ± 50.1

Data from two additional, independent miR-122 in vivo inhibition experiments applying either altered experimental protocols and/or mice with different genetic background. (**A**) Hematological parameters (MCV, Mean Corpuscolar Volume; MCH, Mean corpuscular hemoglobin) and hepatic iron levels of antimiR injected mice. C57/BL6 WT-mice were injected into the tail-vein with antimiR (25 µg/g/day) for three consecutive days and sacrificed one, two and three weeks after the final injection. (**B**) Liver (µg iron/g dry tissue) and plasma iron content (µg iron/dl) were determined in NMRI wild-type mice one, two and three weeks after injection of antimiR (25 µg/g/day) into the tail-vein for three consecutive days. Number of mice (n) is indicated. Data are shown as mean ± SD. Statistical significance was determined by using one-way ANOVA (Table 2A) or two tailed Student's t-test (Table 2B), *$p < 0.05$; **$p < 0.01$. SAL: Saline; PM: PM_antimiR-122; 2MM: 2MM_antimiR-122.

Gene Name	Predicted Target Site 1	Predicted Target Site 2	Predicted Target Site 3
Hjv	position 104-125bp 5′...CGGGGACAGGAGACA-ACACTT-A... 3′ G--TTTGTGGTAACAGTGTGAGGT	position 274-295bp 5′...CATACACCTGATAC-CACTCCTA... 3′ GTTTGTGGTAACAGTGTGAGG-T	position 492-513bp 5′...CGGGCATTTCCATC-CCTTCCTA... 3′ GTTTGTGGTAACAGTGTGAGG-T
Hfe	position 53-75bp 5′...GGGGCACACTTGCTC-CACTGTA... 3′ GTTTGTGGTAAC-AGTGTGAGGT	position 249-270bp 5′...GAAGCATCCTATGTTTC-CTTCA... 3′ GTTTGTGGTA-ACAGTGTGAGGT	
Hamp	position 83-104bp 5′...TCAACCCCATTATT-TATTCCTG... 3′ GTTTGTGGTAACAGTGTGAGG-T		
AldoA	position 29-35bp 5′...AAGGCTGCTCCATCA--ACACTCCA... 3′ GTTTGTGGTAACAGTGTGAGGT		

Supplemental Table 3

Putative miR-122 binding sites in the murine Hfe, hemojuvelin and hepcidin -3'-UTRs were identified by computational analysis applying MirTar, (47) or by a manual approach [RNA22; (48)]. The homology to the 3'-UTR of the target gene is indicated. Additionally, the previously validated miR-122 binding site (29, 30) in the AldoA 3'-UTR (TarBase) is shown. Numbering refers to the position in the 3'-UTR whereby the first base following the stop codon is counted as position 1. The nucleotides that have been mutated in the wild type 3'-UTRs are indicated in bold. The newly introduced nucleotides contained in the mutated 3'-UTR sequences are indicated in bold and *italics*.

Table 4A

Primers used for qPCR analysis of mRNA transcripts

Name	Sequence
mmu-Actb F	GCTTCTTTGCAGCTCCTTCGT
mmu-Actb R	ACCAGCGCAGCGATATCG
mmu-Alas2 F	CAGGGGCTTTCCTGTTATCC
mmu-Alas2 R	TGCTGTTGAGTGCTGCATTA
mmu-AldoA F	TGCCAGTATGTTACTGAGAAGGTC
mmu-AldoA R	CATGTTGGGCTTCAGCAA
mmu-BMP2k F	CCGTCCCTTTCATTTCTCAC
mmu-BMP2k R	TTGGAGAATGTTCCGTCGTT
mmu-Bmpr1a F	CAGTTTTATCTAGCCACATCTCTGA
mmu-Bmpr1a R	GGGAGGCTTCCTTACAGAACA
mmu-CRP F	TCAGCTTCTCTCGGACTTTTG
mmu-CRP R	AGGTGTTCAGTGGCTTCTTTG
mmu-Gapdh F	CCCATTCTCGGCCTTGACTGT
mmu-Gapdh R	GTGGAGATTGTTGCCATCAACGA
mmu-Hamp F	CCTATCTCCAGCAACAGATG
mmu-Hamp R	AACAGATACCACAGGAGGGT
mmu-Hba-a1 F	CCTGGAAAGGATGTTTGCTAGCT
mmu-Hba-a1 R	GCCGTGGCTTACATCAAAGTG
mmu-Hfe F	CACCGTCTGTGCCATCTTCTT
mmu-Hfe R	ACATAGCCACCCATGGTTCCT
mmu-Hmbs F	CATACTACCTCCTGGCTTTACTATTGG
mmu-Hmbs R	TTTGGGTGAAAGACAACAGCAT
mmu-Hjv F	CAGCTCCCCGGTTTCGT
mmu-Hjv R	TGGTAGACTTTCTGGTCAATGCA
mmu-Gata-1 F	CCCTGAACTCGTCATACCACT
mmu-Gata-1 R	GGGAACACTGGGGTTGAA
mmu-Gata-2 F	GCTTCACCCCTAAGCAGAGA
mmu-Gata-2 R	TGGCACCACAGTTGACACA
mmu-Smad4 F	ACACCAACAAGTAACGATGCC
mmu-Smad4 R	GCAAAGGTTTCACTTTCCCCA
mmu-Smad6 F	GTTGCAACCCCACTTC
mmu-Smad6 R	GGAGGAGACAAGAATA
mmu-Smad7 F	GCAGGCTGTCCAGATGCTGT
mmu-Smad7 R	GATCCCCAGGCTCCAGAAGA
mmu-Smurf2 F	TTACATGAGCAGGACACACTTACA
mmu-Smurf2 R	GCTGCGTTGTCCTTTGTTC

Table 4B

Primers used for qPCR analysis of miRNAs

Name	Sequence
mmu-miR-122	TGGAGTGTGACAATGGTGTTTG
mmu-miR-17	AAAGTGCTTACAGTGCAGGTAGG
mmu-miR-29b	GCACCATTTGAAATCAGTGTTG
mmu-miR-451	AAACCGTTACCATTACTGAGTTG
pri-mmu-miR-122 F	CCAAACACCATTGTCACACTCCAG
pri-mmu-miR-122 R	CTAGCTGGAGTGTGACAATGGTGTTTGGAGCT

Table 4C

Primers used for the amplification of 3'-UTR sequences

Name	Sequence
mmu-AldoA F	GAGAGCTCCGCAGCCAGTGAATCTCTC
mmu-AldoA R	GAGCTAGCAAATAGTTGTTTATTGGCAGTGG
mmu-Gapdh F	GAGAGCTCACATGGCCTCCAAGGAGTAA
mmu-Gapdh-A	GAGCTAGCGGGTGCAGCGAACTTTATTG
mmu-Hamp F	GAGAGCTCCAACTTCCCCATCTGCATCT
mmu-Hamp R	GAGCTAGCTAAAATCGTCTTTATTTCAAGGTCA
mmu-Hfe F	GAGAGCTCGGAACCATGGGTGGCTATGT
mmu-Hfe R	GAGCTAGCCACAGTTGTGTAGCCTTCATGG
mmu-Hjv F	GAGAGCTCTGTTCTGTGGCTTTGCTTCA
mmu-Hjv R	GAGCTAGCGGAGAACTAATCGCTTTAATAACAGA
pMIR F	CCAAGAAGGGCGGAAAGA
pMIR R	TCATCAATGTATCTTATCATGTCTGC

Table 4D

Primers used to mutagenize the miR-122 seed sequences

Name	Sequence
mHjv_m3-F	CACCAACTCAGAAACGGGGATATCCATCCCTTGGTAATCTGTTATT
mHjv_m3-R	CCCGTTTCTGAGTTGGTGCCAGTCTCC
mHjv_m2-F	CACTCTCCATACACCTGATATCACTGGTACAAGCCTA
mHjv_m2-R	AGGTGTATGGAGAGTGGAATTTCATT
mHjv_m1-F	CCAAAGGAAACAGCGGGTACCGGAGACAACAGTTTCTCAATCAGA
mHjv_m1-R	CGCTGTTTCCTTTGGCACATCTTTG
mHfe_m2-F	AAGACTTGGAGGGGGTACCCTTGCTCCAGAGTAGGACACA
mHfe_m2-R	CCCCCTCCAAGTCTTTGGCTGAGTT
mHfe_m1-F	CTGCTTCTTGGAAGCTTCCTATGTTTCCTTGTTGCACCTAGA
mHfe_m1-R	GCTTCCAAGAAGCAGCCTAAGAATC
mHamp_m1-F	TCAACCCCATTATTTATTGGTGCCCTCCCC
mHamp_m1-R	AATAAATAATGGGGTTGAGGGGCTGCAG
mAldoA_m1-F	TGAACTAAGGCTGCAGCATCAACACTGGAGGCCCCTGC
mAldoA_m1-R	CCTTAGTTCAGCTCTGG

Table 4E

LNA oligos used in this study

Name	Sequence
PM_antimiR-122	CcAttGTcaCaCtCC
2MM_antimiR-122	CcAttCTcaCaCtGC

Supplemental Table 4

List of primers for (**A**) qPCR analysis of mRNA transcripts, (**B**) miQPCR analysis to monitor miRNA expression, (**C**) PCR amplification of selected 3'-UTRs for cloning into the pMIR vector and (**D**) mutagenesis of the predicted miR-122 binding sites within the 3'-UTR of the genes of interest. (**E**) Sequences of the PM_antimiR-122 and 2MM_antimiR-122 LNA oligonucleotides used in this study. Uppercase: LNA; lowercase: DNA.

Supplemental Table 5A: Laboratory findings in patients with C282Y mutations and controls .

No.	Age	HFE	Transferrin (2.0-3.6 g/l)	Transf. Sat. (16-45%)	Ferritin (30-300 µg/l)	AST (<35 U/l)	ALT (<35 U/l)	GGT (<40 U/l)	AP (55-105 U/l)	CHE (4.26-11.25 kU/l)	BIL (<1.0 mg/dl)
1	70	C282Y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	58	C282Y	n.d.	n.d.	2000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	34	C282Y	1.4	100%	2932	46	94	64	49	7.2	2.8
4	48	C282Y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	54	C282Y	n.d.	n.d.	1500	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	48	C282Y	n.d.	84%	1200	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	64	Contr.	n.d.	n.d.	n.d.	27	19	90	29	n.d.	0,5
8	22	Contr.	1.7	n.d.	63	19	15	43	9	8.4	0.9
9	52	Contr.	2.4	48%	100	74	58	139	100	9.4	0.8
10	36	Contr.	2.6	23%	128	32	67	89	177	11.6	0.4

Supplemental Table 5B: Histological evaluation of liver biopsies.

No.1	Siderosis grade	Inflammation	Fatty change	HFE stage	Fibrosis (SSS)	Fibrotic area (%)
1	2	1	2	2	8	5
2	4	1	1	2	4	5
3	4	0	0	2	6	5
4	4	0	0	2	4	<5
5	2	1	3	2	7	5
6	3	1	2	2	2	5
7	0	1	1	n.a.	1	5
8	0	0	0	n.a.	2	<5
9	0	0	0	n.a.	1	<5
10	0	0	0	n.a.	2	<5

(5A): Abbreviations: n.d. = no data available, AST: aspartate aminotransferase; ALT: alanin aminotransferase; GGT: γ-glutamyl transpeptidase; AP: alkaline phosphatase; CHE: choline esterase; BIL: total bilirubin. Contr.: Control patients without a C282Y mutation in the Hfe gene. Normal limits are indicated in brackets. C282Y homozygosity was established by sequencing (see supplemental material and methods)

(5B): Abbreviations n.a. = not applicable; SSS = semiquantitative severity score according Chevallier et al.

Supplemental material and methods

Materials and methods

Detailed protocol for the preparation of murine primary hepatocytes

Primary mouse hepatocytes have been isolated according to a modified standard procedure published by Klingmüller *et al* (2006). If not stated otherwise chemicals were purchased from Sigma Aldrich. Primary mouse hepatocytes were isolated from eight to twelve weeks old male C57BL/6N mice (Charles River). The mice have been anesthetized by intra peritoneal injection of 5 mg per 100 mg body weight ketamine hydrochloride 10 % (w/v) (Bayer) and 1 mg per 100 mg body weight of xylazine hydrochloride 2 % (w/v) (Pfizer). The liver perfusion was performed by an anterograde fashion via the portal vein with EGTA-containing buffer (0.6 % (w/v) glucose, 105 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 26 mM Hepes, 490 µM L-glutamine, 512 µM EGTA, 15 % (v/v) amino acid solution, pH 8.3) and collagenase-containing buffer (0.6 % (w/v) glucose, 105 mM NaCl, 2.3 mM KCl, 1.2 mM KH_2PO_4, 25 mM Hepes, 490 µM L-glutamine, 5.3 mM $CaCl_2$, 12 % (v/v) amino acid solution, 365 µg/ml collagenase type 1-A, pH 8.3). The amino acid solution constituted of 270 mg/l L-alanine, 140 mg/l L-aspartic acid, 400 mg/l L-asparagine, 270 mg/l L-citrulline, 140 mg/l L-cysteine hydrochloride monohydrate, 1 g/l L-histidine monohydrochloride monohydrate, 1 g/l L-glutamic acid, 1 g/l L-glycine, 400 mg/l L-isoleucine, 800 mg/l L-leucine, 1.3 g/l L-lysine monohydrochloride, 550 mg/l L-methionine, 650 mg/l L-ornithine monohydrochloride, 550 mg/l L-phenylalanine, 550 mg/l L-proline, 650 mg/l L-serine, 1.35 g/l L-threonine, 650 mg/l L-tryptophane, 550 mg/l L-tyrosine, and 800 mg/l L-valine, pH 7.6. The perfused liver was subsequently withdrawn and transferred into washing buffer (0.6 % (w/v) glucose, 105 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 26 mM Hepes, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.2 % (w/v) BSA, 15 % (v/v) amino acid solution, pH 7.6). Hepatocytes were collected by disrupting the liver capsule and filtering the suspension using a 100 µm cell strainer (BD biosciences). Cells were then washed two times

by centrifugation at 50 x g for 2 min. Cell yield and vitality were determined by trypan blue staining, preparations with a vitality > 70 % were used for our studies. For experiments 900000 cells were seeded in 1 ml full medium (phenol red-free Williams E medium (Biochrom) supplemented with 10 % (v/v) FBS (Invitrogen), 1 µM dexamethasone, 10 µg/ml insulin, 2 mM L-glutamine, and 1 % (v/v) penicillin/streptomycin 100x (both Invitrogen)) using collagen I-coated 6well/dishes (BD Biosciences). Primary hepatocytes were cultured at 37 °C, 5 % CO_2 and 95 % rh. Unattached hepatocytes were removed after 4 h of adhesion by washing with PBS (PAN Biotech), the attached hepatocytes were subsequently cultured in cultivation medium (phenol red-free Williams E medium supplemented with 1 µM dexamethasone, 2 mM L-glutamine).

Western blot analysis

Protein isolation and western blotting were performed according to standard protocols. In brief, flash-frozen tissues were homogenized in RIPA buffer complemented with 4 mg/mL PefablocSC, 0.1 mg/mL aprotinin, 20 µg/mL leupeptin, 40 µg/mL E64, 4 µg/ml pepstatin (all from Roche Diagnostic), and soluble proteins were recovered by centrifugation. For immunoblotting, equivalent amounts of protein extract (40 µg) were resolved on a 10% SDS-gel and transferred onto a PVDF membrane. Membranes were incubated with primary antibodies [pStat3, (Cat 9138, diluted 1:500, cell signaling); Stat3(Cat 9132, diluted 1:500, Cell signaling); pSmad1/5/8, (Cat 9511, diluted 1:500, Cell signaling); βActin, (Cat A5316, diluted 1:10000, Sigma] and with a secondary IgG-HRP-conjugate (diluted 1:10000; Sigma). Blots were developed using an ECL (Amersham) chemiluminescence detection system.

Patient characteristics and liver biopsy evaluation:

Pseudonymized male human tissue samples were provided by the Tissue bank of the National Center for Tumor Diseases Heidelberg after approval by the ethics committee. Patient characteristics are depicted in

(Supplemental. Table 5A and 5B) For the evaluation of liver biopsies, serial 3 μm sections were stained with hematoxylin-eosin (H&E), periodic acid Schiff after diastase digestion, Perl´s Prussian blue, and modified Gomori´s stain. Inflammatory changes were recorded semiquantitatively: 0 = none; 1 = mild: few single cell necrosis, mild portal infiltrate, no interface hepatitis; 2 = moderate: some single cell necrosis, moderate portal infiltrate, or interface hepatitis; 3 = group necrosis. Fatty change was graded using a 4-tiered system: 0 = no fat; 1 = <25% fatty change (mild); 2 = 25-50% fatty change (moderate); 3 = >50% (fatty liver). Grading of hepatocellular siderosis and staging of genetic hemochromatosis was performed as reported previously (Scheuer PJ, Williams R, Muir AR (1962) Hepatic pathology in relatives of patients with haemochromatosis. The Journal Pathol Bacteriol 84: 53–64). In brief, hepatocellular siderosis was evaluated as follows: 0 = none; 1 = mild siderosis, hardly visible at magnification 250-fold; 2 = moderate siderosis, visible at magnification 100-fold; 3 = severe siderosis, visible at magnification 25-fold with porto-central gradient; 4 = iron excess. Staging of genetic hemochromatosis: 1 = no fibrosis; 2 = fibrosis; 3 = cirrhosis. For semiquantitative assessment of liver fibrosis the semiquantitative severity score (SSS) according to Chevalier et al. was used (Chevallier M, Guerret S, Chossegros P et al. (1994) A histological semiquantitative scoring system for evaluation of hepatic fibrosis in needle liver biopsy specimens: comparison with morphometric studies. Hepatology 20: 349–355).

cDNA synthesis by miPCR

miQPCR is a novel approach for expression profiling of microRNA by qPCR. In principle, the 3'-ends of single-stranded RNAs are extended uniformly with a specific RNA/DNA linker (named miLINKER). This adapter is used as an anchor to prime cDNA synthesis during reverse transcription and for detecting the selected amplicon during the qPCR. For each sample 50 ng of total RNA were ligated to 5 μM of miLINKER using the T4 RNA ligase (NEB). Ligated samples were reverse transcribed using Superscript II (Invitrogen) following the supplier's instructions. miRNA-specific qPCR requires 100 pg of cDNA, 2.5 μM of universal primer complementary to the linker and 2.5 μM of miRNA specific primer. qPCR reactions are carried out on an AB7500 machine, whereas amplicons are detected using SIBR green I (AB).

PCR design and sequencing

For PCR-amplification of the human Hfe gene, the following primers were used (Hfe-Ex2-F: 5´-GTT TGA AGC TTT GGG CTA CG-3, and Hfe-Ex2-R:´5´- ATG TGA TCC CAC CCT TTC AG-3´ for exon 2

and Hfe-Ex4-F: 5´- TCG AAC CTA AAG ACG TAT TGC CC-3´, and Hfe-Ex4-R: 5´- TTC TCA GCT CCT GGC TCT CAT C-3´). The temperature profile was 94°C for 5 min followed by 32 cycles of 94°C for 30 s. 60°C for 30 s and 72°C for 30 s. A final extension step of 72°C for 5 min was added. PCR-amplification was carried out using *Pfu* DNA Polymerase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer´s instructions. PCR fragments were directly sequenced bidirectionally on an ABI Prism® 377-18 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye® Termination v 1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer´s instructions.