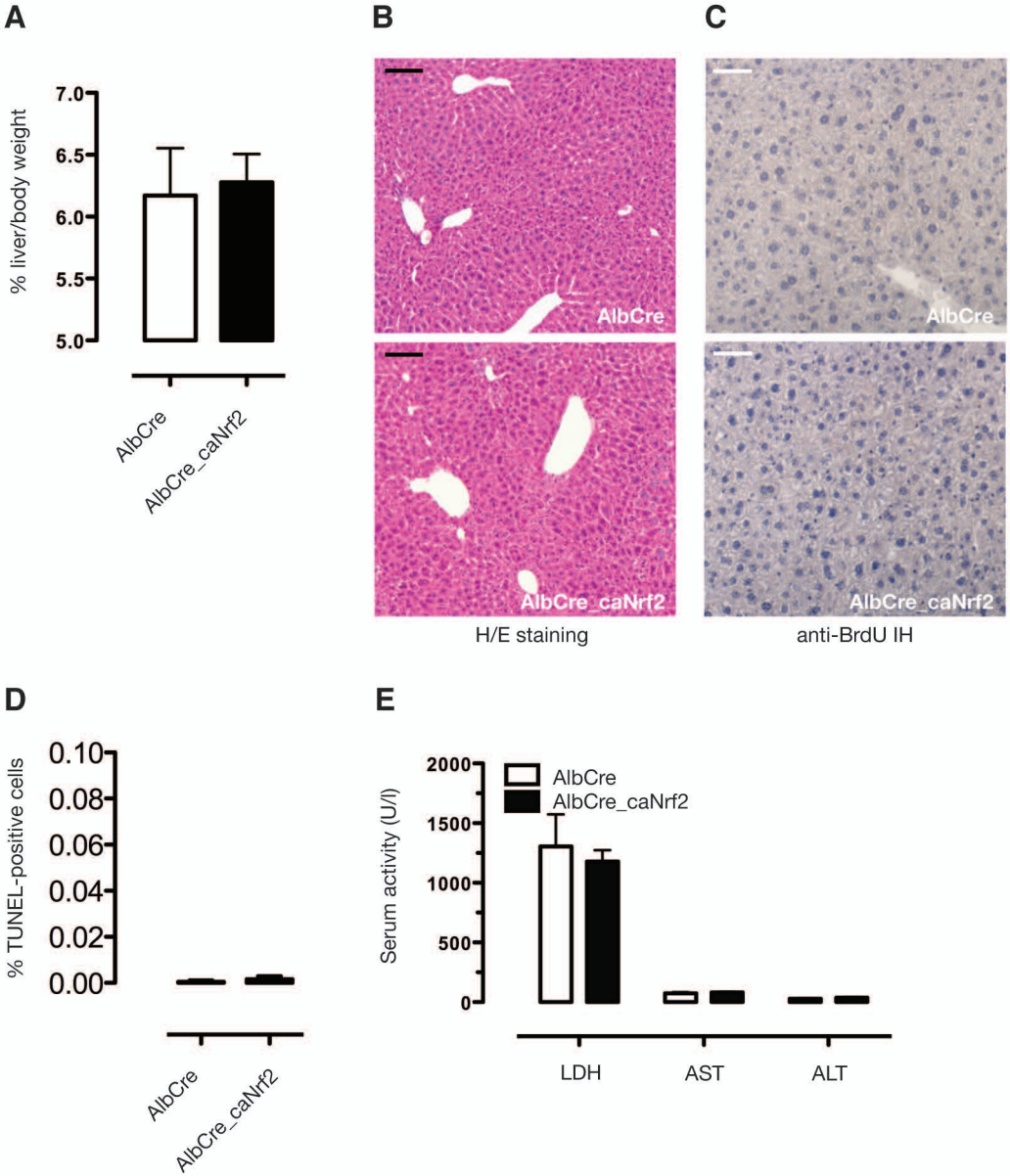
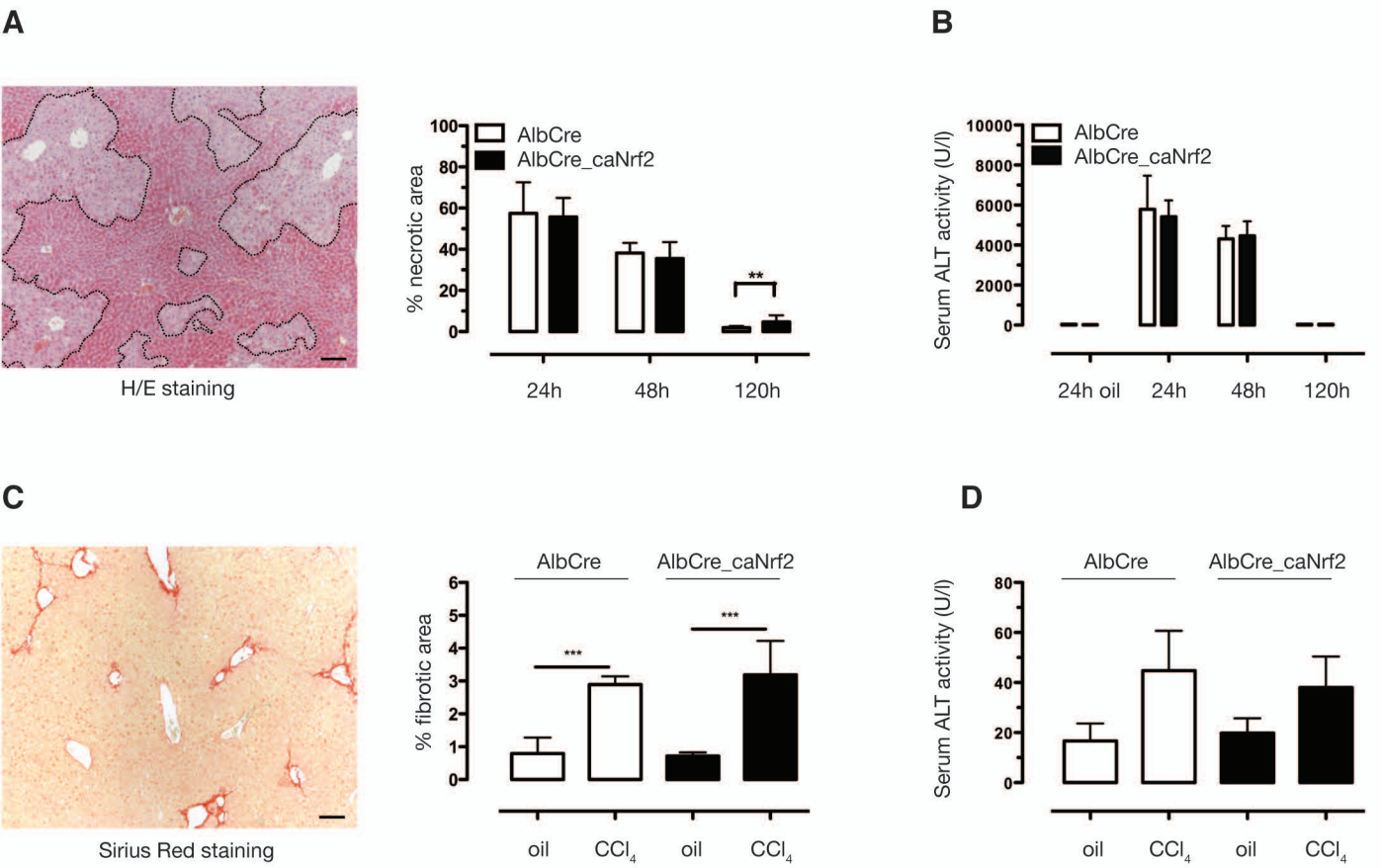
Köhler et al, Suppl. Fig. S1



Köhler et al, Suppl. Fig. S2



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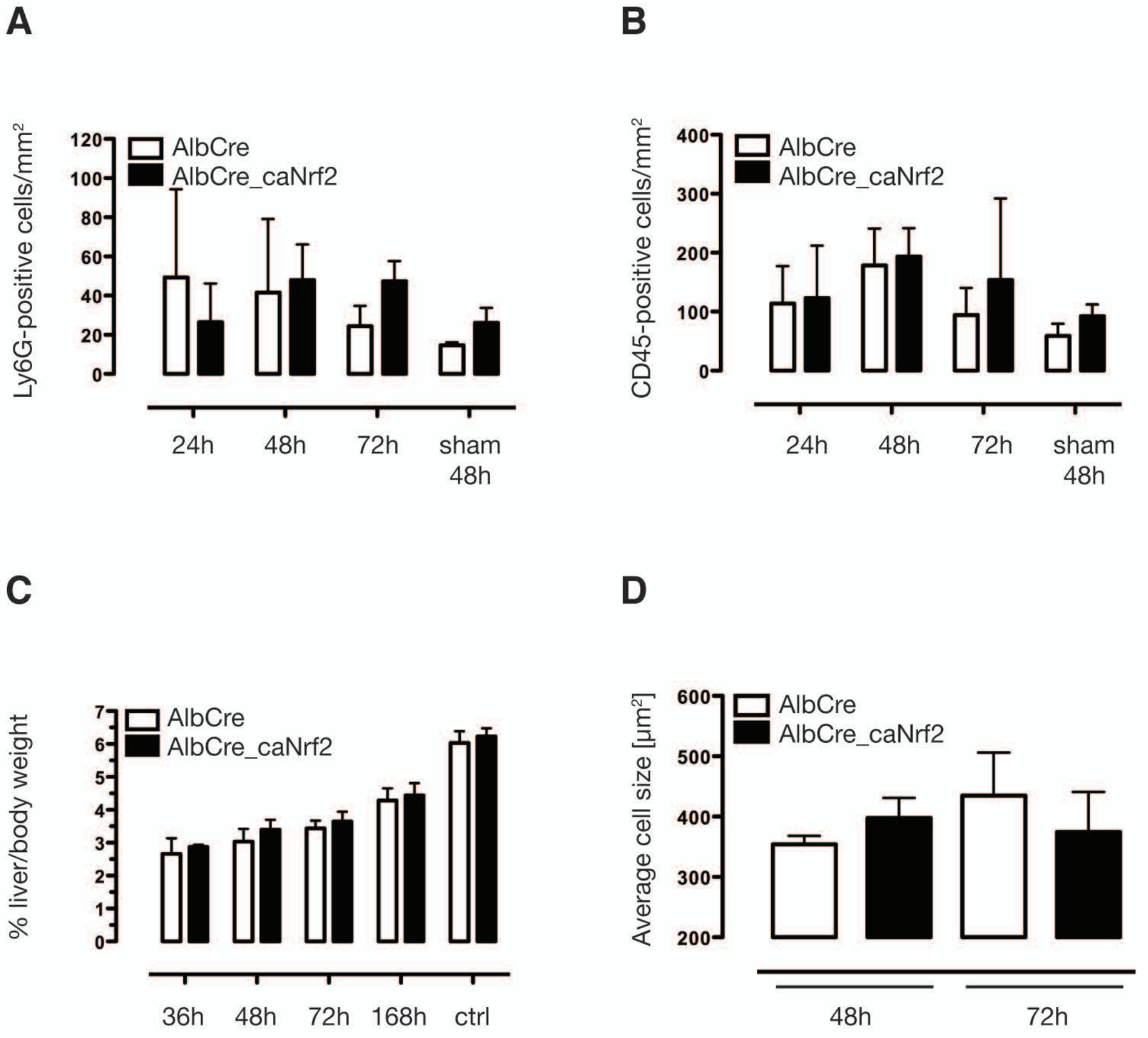

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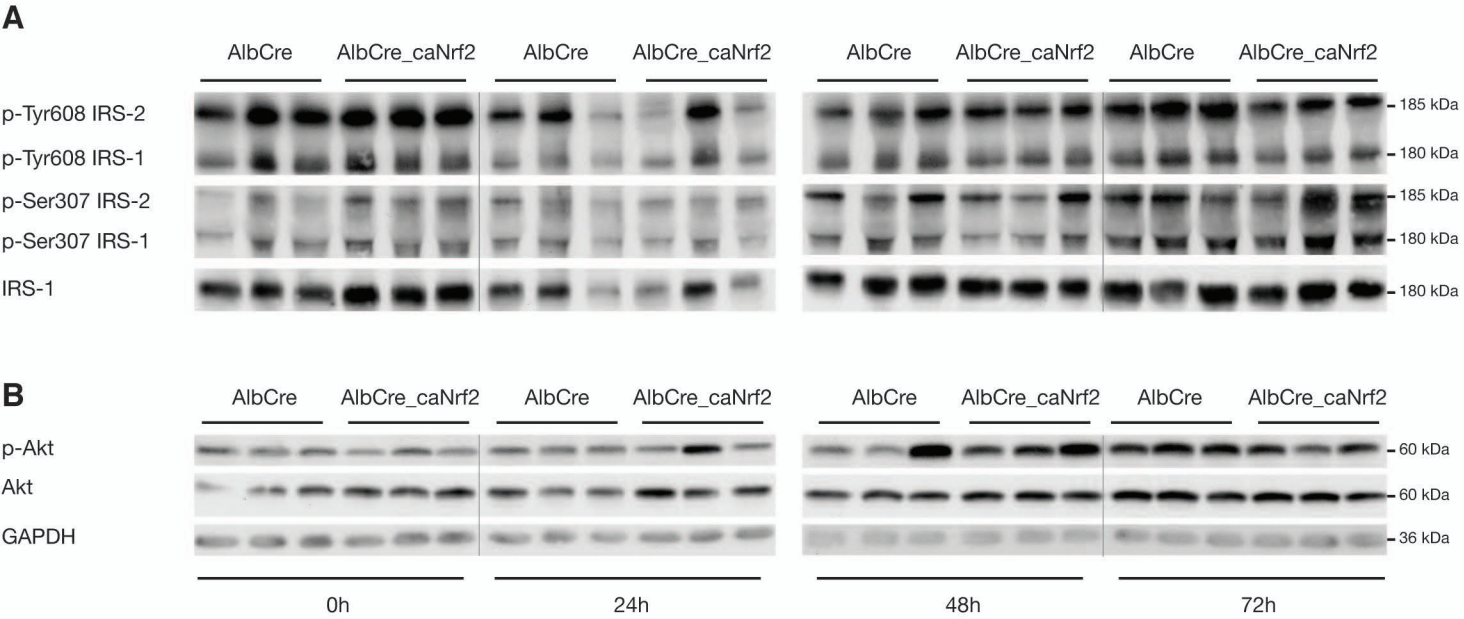

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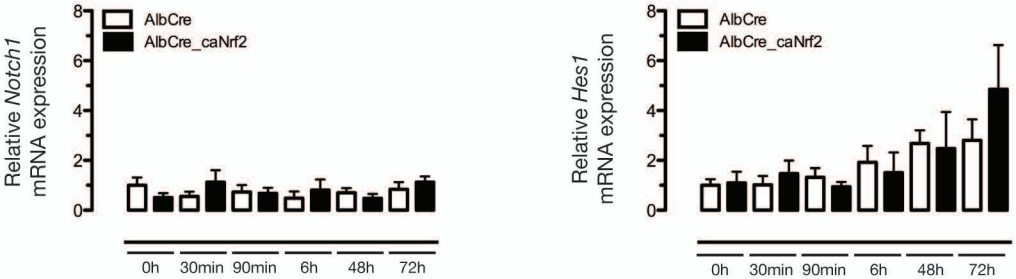
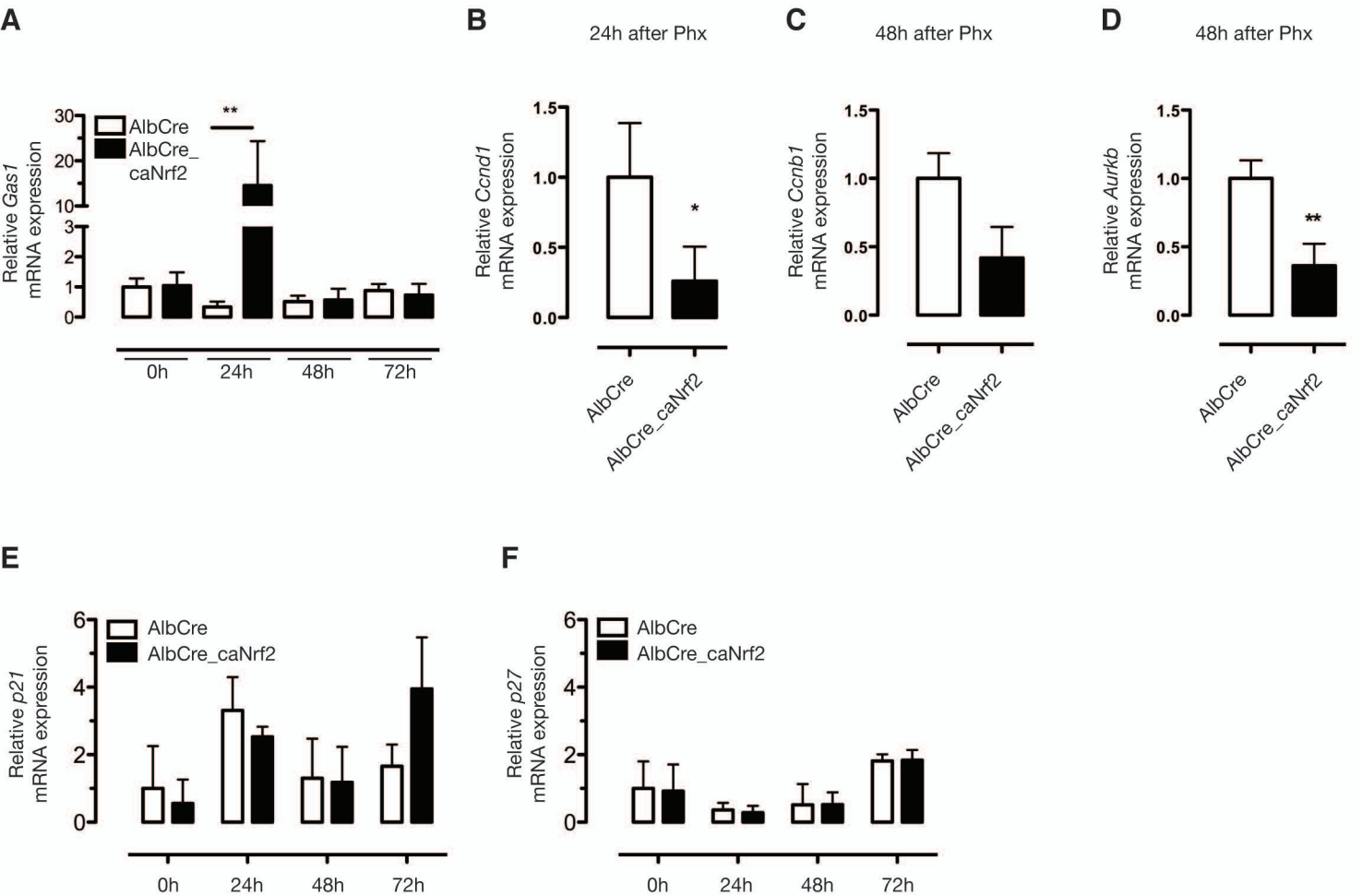
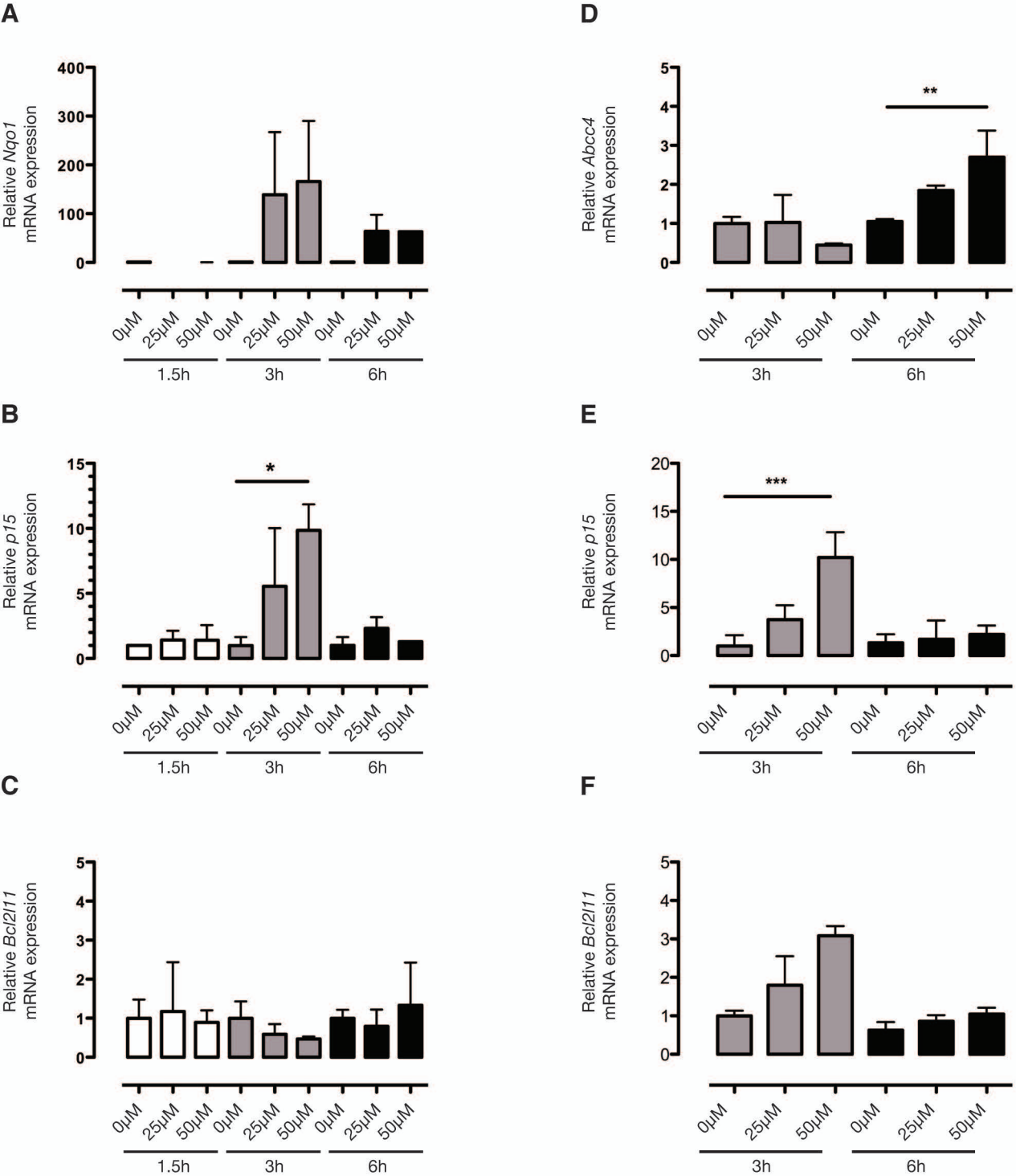


Köhler et al, Suppl. Fig. S5



Köhler et al, Suppl. Fig. S6



Köhler et al, Suppl. Table S1

Symbol	Gene name	Fold change: 0h after Phx	24h after Phx
Cbr3	Carbonyl reductase 3	123.5	101.7
Abcc4	ATP-binding cassette, sub-family C, member 4	23	61
Nqo1	NAD(P)H dehydrogenase, quinone 1	12.6	14.9
Gstm3	Glutathione S-transferase, mu 3	5.8	< 2
Gpx2	Glutathione peroxidase 2	5.7	9.7
Srxn1	Sulfiredoxin 1 homolog	4.3/3.7/3.2	4
Slc7a11	Solute carrier family 7, member 11	4	86/32.7
Ggt1	Gamma-glutamyltransferase 1	4	11.4
Cbr1	Carbonyl reductase 1	3.7	< 2
Gsta2	Glutathione S-transferase, alpha 2	3.4	9.1
Gstm4	Glutathione S-transferase, mu 4	3.3	3.1
Gstm1	Glutathione S-transferase, mu 1	2.2/2.1	< 2
Cyp2g1	Cytochrome P450, family 2, subfamily g, polypeptide 1	3.6	< 2
Mgst2	Microsomal glutathione S-transferase 2	4.8	8.4
Slc1a1	Solute carrier family 1, member 1	4.4	6.4/4.5/2
Blvrb	Biliverdin reductase B	3	4.9
Pgd	Phosphogluconate dehydrogenase	2.7/2.6/2.6	4.6/4.6/4.2
Slc1a4	Solute carrier family 1, member 4	2.4	6.6/5.4/4.4

Supplementary Materials and Methods

Harvesting of liver tissue and sample preparation. Mice were euthanized by CO_2 inhalation. Blood was collected by cardiac puncture and the liver tissue was harvested for RNA isolation, for preparation of protein lysates or for histology. For isolation of RNA or preparation of protein lysates, liver samples were immediately frozen in liquid nitrogen after removal. For histology or immunohistochemistry, liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) or in 95% ethanol/1% acetic acid and embedded in paraffin.

Cell culture und tBHQ stimulation. AML12-cells (immortalized mouse hepatocytes) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 1% Pen/Strep in 6-well plates until 75% confluency before they were treated with different concentrations of tBHQ or the vehicle DMSO only (0 µM) for 1.5, 3 or 6h. Isolation and cultivation of primary hepatocytes from adult mice was previously described.[1] Primary hepatocytes (3.5×10^5 / well) were plated in RPMI-1640 in 6-well plates. 5 hours later, they were treated with different concentrations of tBHQ or the vehicle DMSO only (0 µM) for 3 or 6h.

RNA isolation, RNase Protection Assay (RPA) and qRT-PCR. Isolation of RNA from murine liver and RPA were performed as previously described.[2,3] The RNeasy MiniKit (Qiagen, Hilden, Germany) was used to isolate RNA from cultured cells.

RPA was performed twice using RNAs pooled from different mice (samples obtained in two independent experiments). The following templates were used: nt 566-685 of the murine glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) cDNA (NM_008084); nt 988-1210 of the murine *Nrf2* cDNA (U20532).

For qRT-PCR, cDNA synthesis was performed using the iScript RT kit (Biorad, Hercules, CA) according to the manufacturer's instructions. qRT-PCR was performed as described previously.[4]

Primers used for qRT-PCR are listed below.

Name	Forward (5’ -> 3’)	Reverse (5’ -> 3’)
Gclc	AACAAGAAACATCCGGCATC	CGTAGCCTCGGTAAAATGGA
Gas1	CCATCTGCGAATCGGTCAAAG	GCTCGTCGTCATATTCTTCGTC
Gclm	TCCCATGCAGTGGAGAAGAT	AGCTGTGCAACTCCAAGGAC
Gsta2	TACTTTGATGGCAGGGGAAG	GCACTTGCTGGAACATCAGA
Nqo1	CTGGCCCATTCAGAGAAGAC	GTCTGCAGCTTCCAGCTTCT
Srxn1	CGGTGCACAACGTACCAAT	TTGATCCAGAGGACGTCGAT
p15	CCCTGCCACCCTTACCAG	GCAGATACCTCGCAATGTCAC
Notch1	TCCCACAGGCTGGCAAGGTCAAAC	AGCGGTAGCTGCCATTGGTGTTCTG
Hes1	TACCCAGTGCCTTTGAGAAG	AACCCCAAACTCCGATAGTC
Bcl2l11	GAAGACCACCCTCAAATGGTTAT	TTCTCCATACCAGACGGAAGAT
Ccnd1	GCGTACCCTGACACCAATCTC	ACTTGAAGTAAGATACGGAGGGC
Cccnb1	GCGTGTGCCTGTGACAGTTA	CCTAGCGTTTTTGCTTCCCTT
Aurkb	CAGAAGGAGAACGCCTACCC	GAGAGCAAGCGCAGATGTC
Abcc4	GGCACTCCGGTTAAGTAACTC	TGTCACTTGGTCGAATTTGTTCA
Cbr3	CATCGGCTTTGCGATCACG	GACCAGCACGTTAAGTCCCC
Gstm3	GCGGACTGACTCACTCCATC	CCCCATGACATATCTCTTCTCCT
18s rRNA	GATCCATTGGAGAAGTCT	CCAAGATCCAACTACGAGCTTTTT

Primers used for ChIP experiments:

Name	Forward (5’ -> 3’)	Reverse (5’ -> 3’)
rNqo1ARE	AGCAGAACGCAGCACGAAT	CACTCAGCCGTGGGAAGT
rNqo1NS	TACGCTGTAGTGGTGGTGGA	TCTGGGGACTTGGGTATCTG
mBcl2l11ARE1-set1	GGAACCAATGGCTTCTTTGA	AGAACAAGTGTTGCGACCTG
mBcl2l11ARE1-set2	CACAGACCTTGGTCAGGACA	TGTACCAGGGATAGGCTTCG
mBcl2l11NS	GGAGAAAAATCCTGCGGTAA	TGTACCAGGGATAGGCTTCG

Microarray analysis. RNA from the liver of AlbCre_caNrf2 and AlbCre mice that was removed during surgery and from the remaining liver 24h after PH was analyzed using Affymetrix GeneChip Mouse Genome 430 2.0 microarrays. Raw data were analyzed using the Affymetrix GCOS 1.4 software. Probability scores and fold changes were calculated using the Rosetta Resolver software (Rosetta Biosoftware, Seattle, WA).

Preparation of protein lysates and Westernblot. Liver samples were homogenized in lysis buffer (T-PER™ tissue protein extraction reagent (Pierce, Rockford, IL) supplemented with 0.1U/ml aprotinin, 1% leupeptin, 1% pepstatin, 1mM AEBSF, 10mM sodium pyrophosphate, 1mM sodium vanadate and 50mM sodium fluoride. Protein lysates were cleared by sonication and centrifugation. The BCA™ Protein Assay Kit (Pierce) was used to determine protein concentrations. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Unspecific binding sites were blocked in 5% milk in TBS-T (10mM Tris/HCl pH7.5, 150mM NaCl, 0.05% Tween 20). Antibodies were applied in blocking solution or in 5% BSA/TBS-T at 4°C overnight. The following primary antibodies were used:

a-GAPDH (HyTest, Finland, #5G4)

a-Nrf2 (Dr. Dennis Roop, Colorado, USA)

a-pTyr612 IRS-1/2 (mouse: Tyr608) (Santa Cruz Biotechnology, CA, sc-17195-R)

a-Ser307 IRS-1/2 (Millipore, Billerica, MA, #07-247)

a-total IRS-1 (Cell Signaling, Danvers, MA, #2382)

a-phospho Akt (Santa Cruz Biotechnology, CA, sc-7985-R)

a-total Akt (Santa Cruz Biotechnology, CA, sc-1619)

a-p15 (Abcam, Cambridge, UK, ab53034)

a-Bcl2l11 (Cell Signaling, #2933)

Alkaline phosphatase - or horseradish peroxidase-coupled secondary antibodies were purchased from Promega (Madison, WI) (anti-mouse IgG, anti-rat IgG and anti-rabbit IgG) or from Calbiochem (Billerica, MA) (anti-goat IgG).

Histology and histomorphometry. Liver samples were fixed in 4% PFA in PBS overnight and embedded in paraffin. Sections (3.5 µm) were stained with hematoxylin/eosin (H/E), Sirius Red (Sigma) or Phalloidin-FITC, photographed (4 pictures/animal), and necrotic and fibrotic areas as well as cell size were determined using histomorphometry.

Immunohistochemistry and immunofluorescence. Paraffin sections from tissue that had been fixed in 95% ethanol/1% acetic acid overnight or non-fixed frozen sections were incubated for 45 min in 12% bovine serum albumin (BSA) in PBS/0.025% NP40 to block unspecific binding sites. The primary antibody was diluted in blocking buffer and incubated overnight at 4°C. After washing in PBS, sections were stained using the ABC Vectastain Peroxidase Kit and the AEC Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) as described by the manufacturer. Sections were counterstained with hematoxylin and mounted with Mowiol. Alternatively, Cy2- or Cy3-confugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, CA) were used for detection of stained cells by fluorescence microscopy. Sections were photographed using a Zeiss Imager.A1 microscope equipped with an Axiocam MRm camera and EC Plan-Neofluar objectives (10x/0.3, 20x/0.5). For data acquisition we used the Axiovision 4.6 software (all from Carl Zeiss, Inc., Oberkochen, Germany). The following primary antibodies were used:

a-CD68 (Serotec, Düsseldorf, Germany, #MCA1957B)

a-CD45 (BD Biosciences, San Jose, CA, #553076)

a-Ly6G (BD Biosciences, A#553124)

a-BrdU (Roche, Rotkreuz, Switzerland, #11585860001)

Antibodies used for ChIP analysis:

a-Nrf2 (kindly provided by Dr. Dennis Roop, Aurora, CA)

a-histone H3 (Abcam, Cambridge, UK)

a-histone H3K4me2 (Active Motif Inc., Carlsbad, CA),

normal rabbit IgG (Santa Cruz)

normal sheep IgG (Millipore)

References

1. Beyer TA, Xu W, Teupser D, auf dem Keller U, Bugnon P, Hildt E, et al. Impaired liver regeneration in Nrf2 knockout mice: Role of ROS-mediated insulin/IGF-1 resistance. EMBO J 2008;27:212-223.
2. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
3. Werner S, Weinberg W, Liao X, Peters KG, Blessing M, Yuspa SH, et al. Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. Embo J 1993;12:2635-2643.
4. Koegel H, Werner S. Loss of serum response factor in keratinocytes results in hyperproliferative skin disease in mice. J Clin Invest 2009;119:899-910.

Legends to Supplementary Figures

Fig S1: caNrf2 expression does not affect liver homeostasis. (A) The liver to body weight-ratio was determined in AlbCre and AlbCre_caNrf2 mice (N = 5-7 per genotype). (B) Representative H/E-stained liver sections from AlbCre and AlbCre_caNrf2 mice are shown. Bars represent 100 µm. (C) Cell proliferation in the liver was assessed by BrdU incorporation. Representative sections from mice of both genotypes are shown. Note the lack of proliferating cells in mice of both genotypes. Bars represent 50 µm. (D) TUNEL staining was performed to determine apoptotic cells in the liver of untreated mice of both genotypes (N = 6 per genotype). (E) Serum LDH, AST and ALT activities were determined (N = 3 per genotype). Error bars represent mean ± s.d.

Fig S2: Repair of CCl_4-induced liver damage and fibrosis is not altered in AlbCre_caNrf2 mice. (A, B) AlbCre_caNrf2 and AlbCre control mice were injected once

with CCl_4. Necrotic areas were identified by staining of liver sections with H/E. A representative liver section from an AlbCre_caNrf2 mouse at 24h after CCl_4 injection is shown in (A). Necrotic areas are encircled. Bar represents 100mm. The area of necrotic tissue was determined morphometrically in 4 independent sections per mouse at different time point after injection of CCl_4. N = 3-5 animals per time point and genotype. (B) Activity of ALT in the serum was determined in mice of both genotypes at different time points after CCl_4 injection. Serum activities in mice 24h after vehicle (oil) injection were analyzed for comparison. N = 3 animals per time point and genotype. Differences between the two genotypes were determined using the Mann-Whitney U test. Error bars represent mean ± s.d. (C, D) AlbCre_caNrf2 and AlbCre mice were injected with CCl_4 every third day for the duration of 45 days. Fibrotic tissue was identified by Sirius Red staining. A representative liver section from an AlbCre_caNrf2 mouse at 72h after the last CCl_4 injection is shown in (C). The area of fibrotic tissue was determined morphometrically in 4 independent sections per mouse. Bar represents 100 µm. N = 7 animals per genotype (N = 3 for vehicle only). D) Activity of ALT in the serum was determined in mice of both genotypes 72h after the last CCl_4 injection. Serum activities in mice injected with the vehicle (oil) only were analyzed for comparison (N = 3-5 animals per time point and genotype). Comparisons between multiple groups in (C) and (D) were assessed by one-way ANOVA including Bonferroni correction. Error bars represent mean ± s.d.

Fig S3: caNrf2 does not affect the immune cell infiltrate and the liver-to-body weight ratio after PH. (A,B) Numbers of Ly6G- and CD45-positive cells in liver sections from AlbCre and AlbCre_caNrf2 mice at different time points after PH were determined by immunohistochemistry (N = 3-4 per genotype and time point). (C) Liver and body weight were measured at different time points after PH. The liver-to-body weight ratio of unchallenged adult mice served as a control. (N = 4-8 per genotype and time point). (D) The average cell size was determined by histomorphometric analysis of pictures from Phalloidin/Hoechst stained liver sections at 40x magnification (N = 4-5 per genotype and time point). Error bars represent mean ± s.d.

Fig S4: Insulin/IGFR-1 and *Notch* signaling are not obviously affected in the regenerating liver of AlbCre_caNrf2 mice. (A,B) Total liver lysates (30 µg protein) from AlbCre and AlbCre_caNrf2 mice at different time points after PH were analyzed by immunoblotting for the levels of (A) Tyr608- and Ser307- phosphorylated IRS-1/2 and total IRS-1, and (B) phosphorylated and total Akt. Immunoblotting for GAPDH served as a loading control. Representative blots from 2 or 3 independent experiments with lysates from different PH experiments are shown. (C) qPCR analysis for *Notch1* and *Hes1*. N = 5 mice per genotype were analyzed for each gene. Expression was normalized to the expression of *18s rRNA*. Error bars represent mean ± s.d.

Fig S5: Differential expression of genes/proteins involved in cell cycle regulation in AlbCre_caNrf2 mice after PH. qPCR analysis for (A) *Gas1*, (B) *Cyclin D1*, (C) *Cyclin B1*, (D) *Aurora B kinase*, (E) *p21* and (F) *p27*. N = 5 mice per genotype were analyzed for each gene. Expression was normalized to the expression of *18s rRNA*. Error bars represent mean ± s.d.

Fig S6: *p15* expression is induced by chemical activation of endogenous Nrf2 *in vitro*. (A-C) RNA from AML12-cells treated with different concentrations of tert-butylhydroquinone (tBHQ) or DMSO vehicle for 1.5, 3 or 6h was analyzed for the expression of (A) *Nqo1*, (B) *p15* and (C) *Bcl2l11*. N = 3. (D-F) RNA from primary hepatocytes (isolated from AlbCre mice) treated with different concentrations of tBHQ for 3 or 6h was analyzed for the expression of (D) *Abcc4*, (E) *p15* and (F) *Bcl2l11*. N = 2-3. Expression levels were normalized to *18s rRNA*. 0 µM tBHQ refers to treatment with the vehicle DMSO only. Comparisons between multiple groups were assessed by one-way ANOVA including Bonferroni correction. Error bars represent mean ± s.d.

Table S1. Differentially expressed Nrf2 target genes in the normal and injured liver of AlbCre_caNrf2 mice. RNA microarray results showing previously identified Nrf2 target genes with a cytoprotective function that are differentially expressed in the liver of AlbCre_caNrf2 and AlbCre mice that was removed during PH (0h) and in the remaining liver 24h after PH. Genes with a fold change > 2 and a p-value < 0.05 are listed. Multiple

values indicate that a gene was identified by different probes spotted onto the microarray.