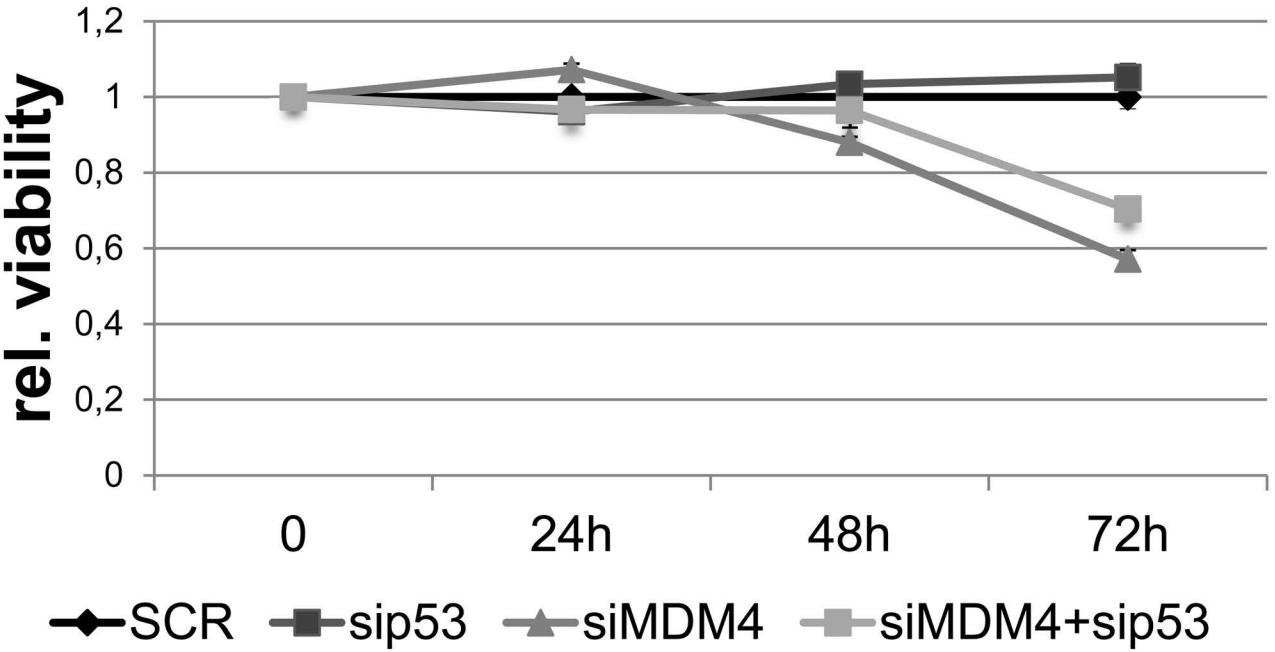
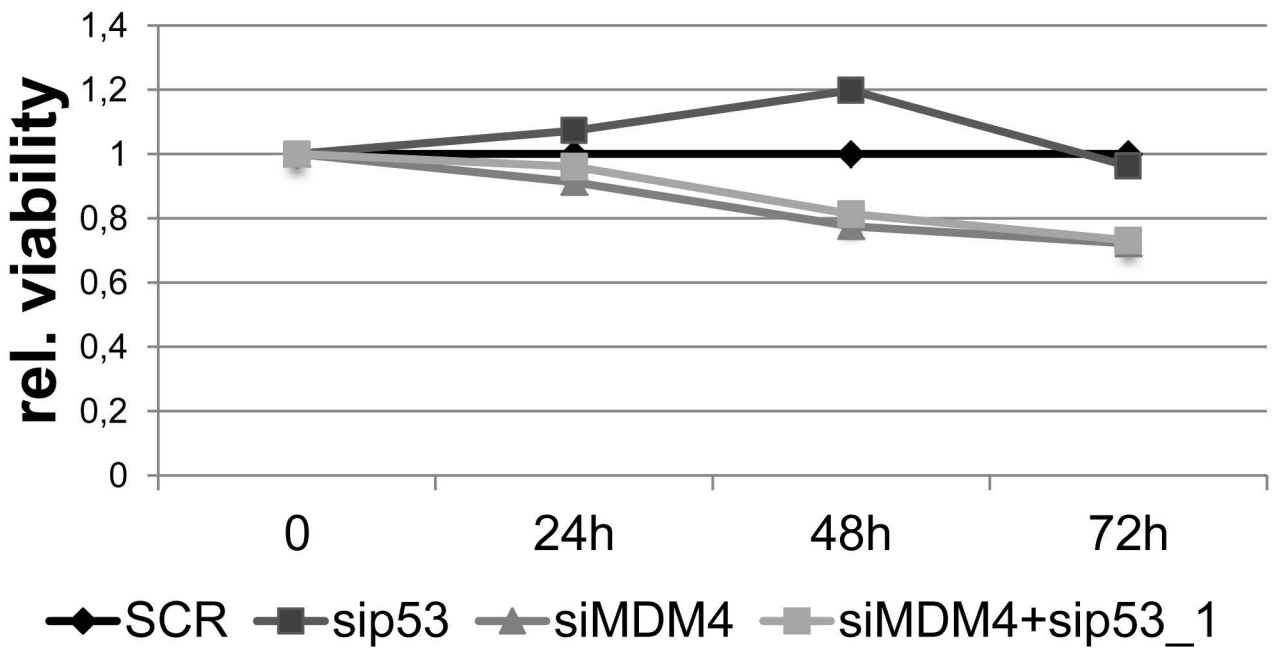
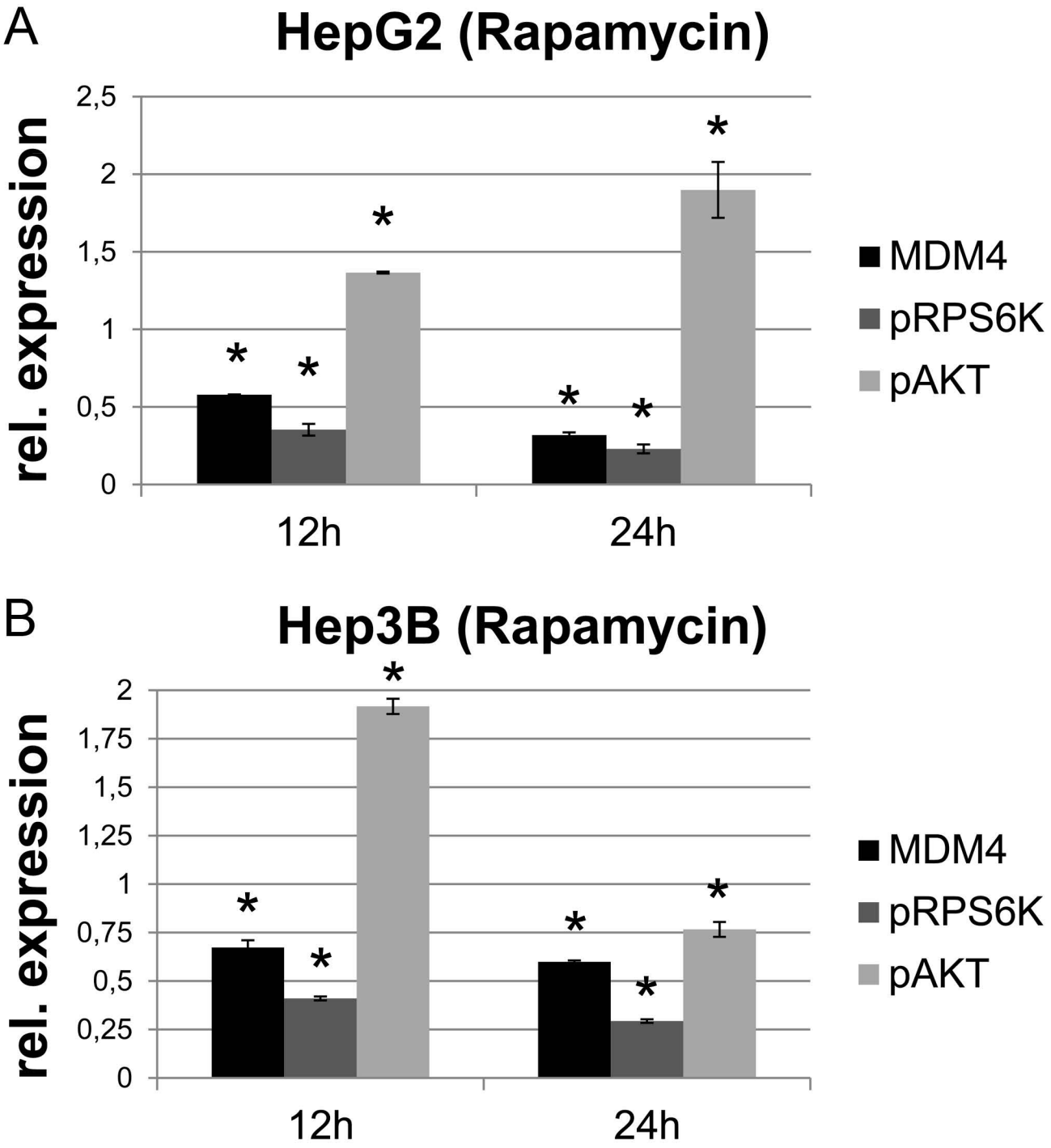
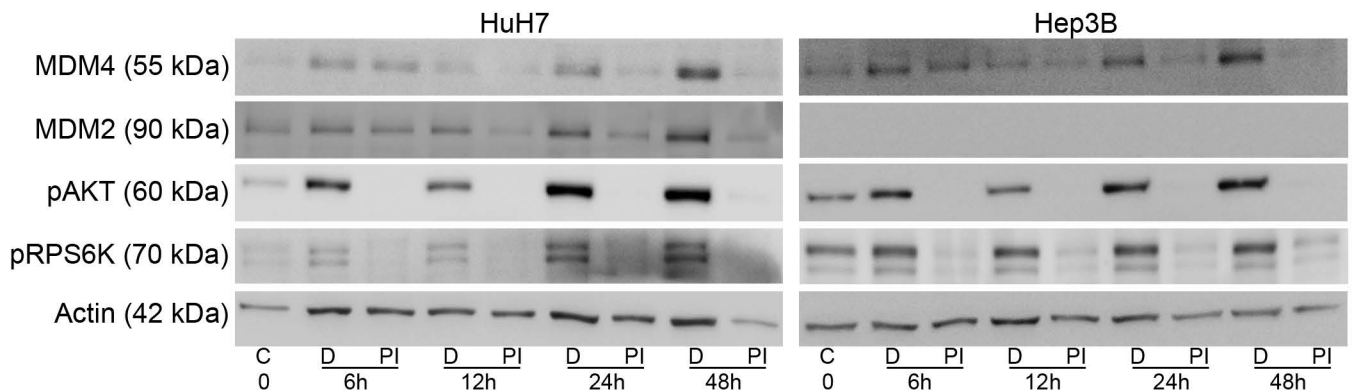
A

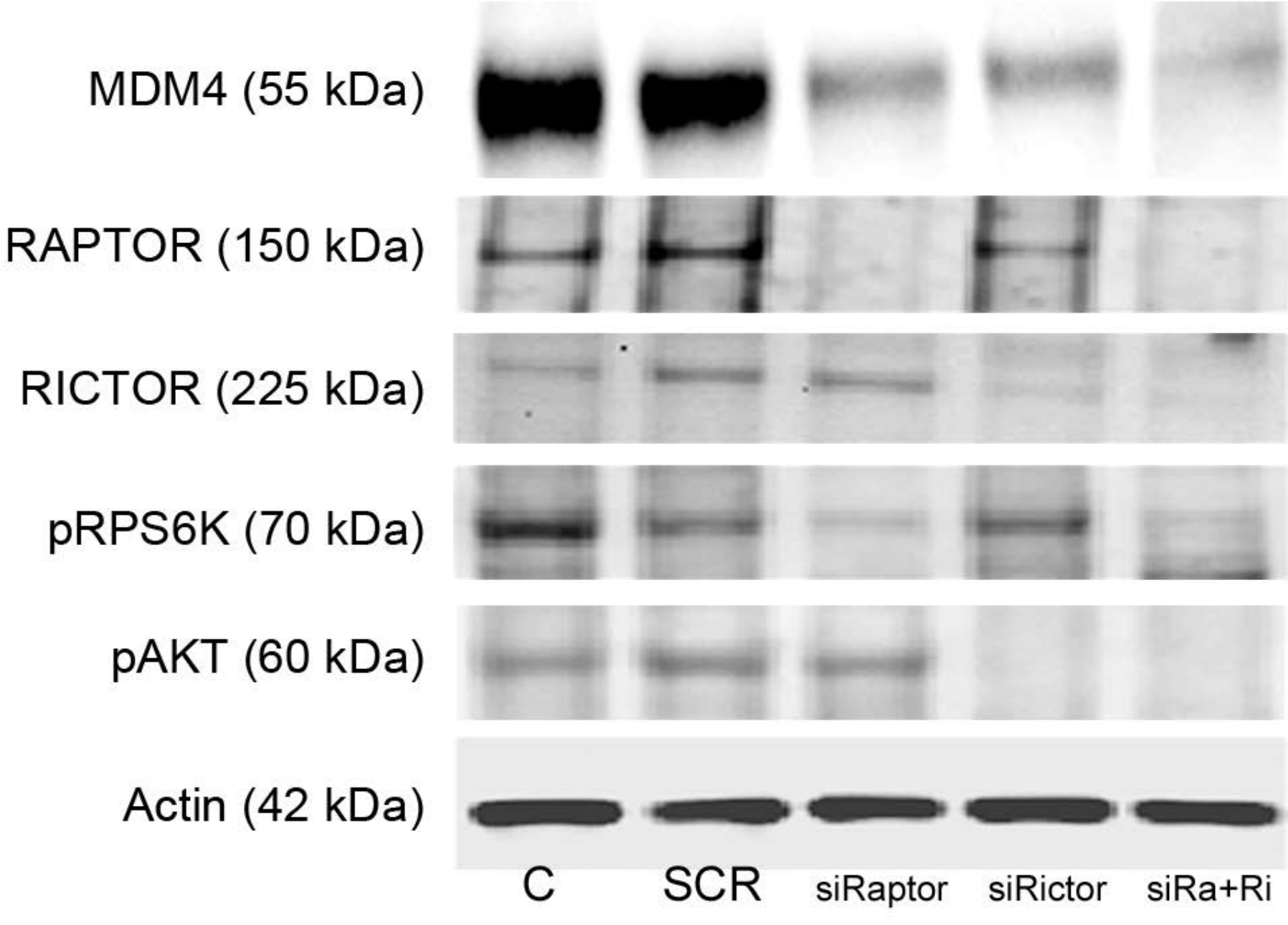

HepG2
rel. viability
1,2
1
0,8
0,6
0,4
0,2
0
0
24h
48h
72h
SCR
sip53
siMDM4
siMDM4+sip53

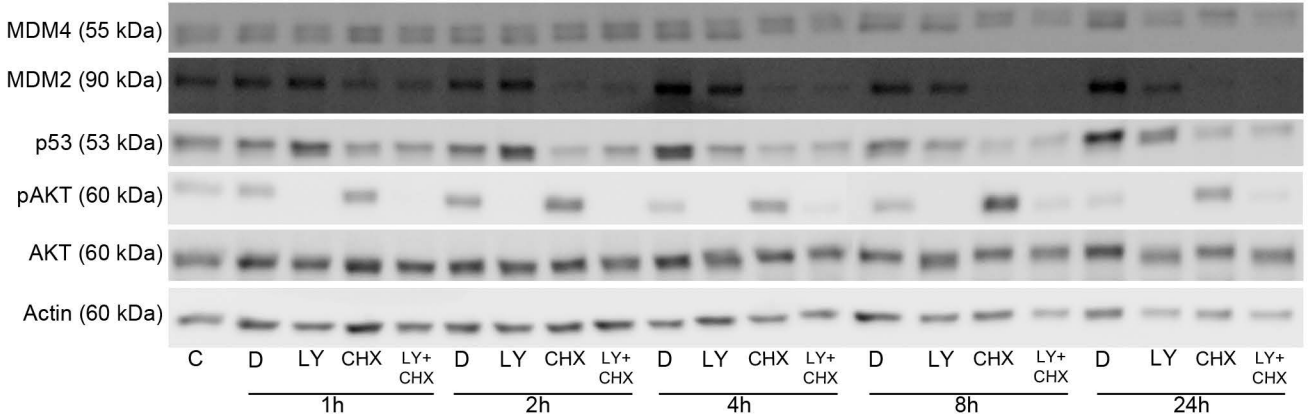
B

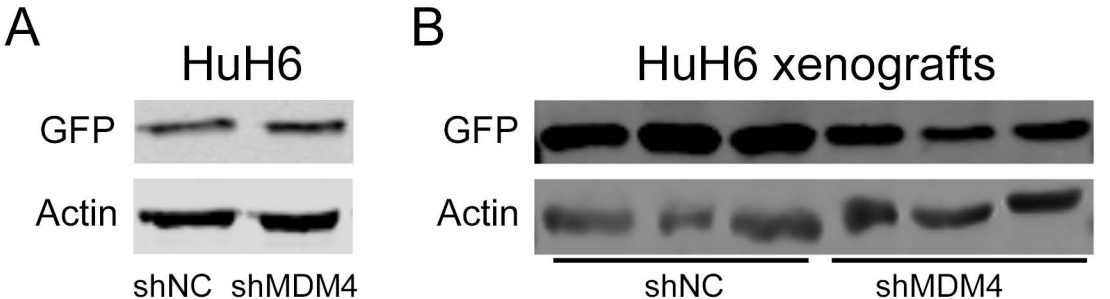

HuH7
rel. viability
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
24h
48h
72h
SCR
sip53
siMDM4
siMDM4+sip53_1

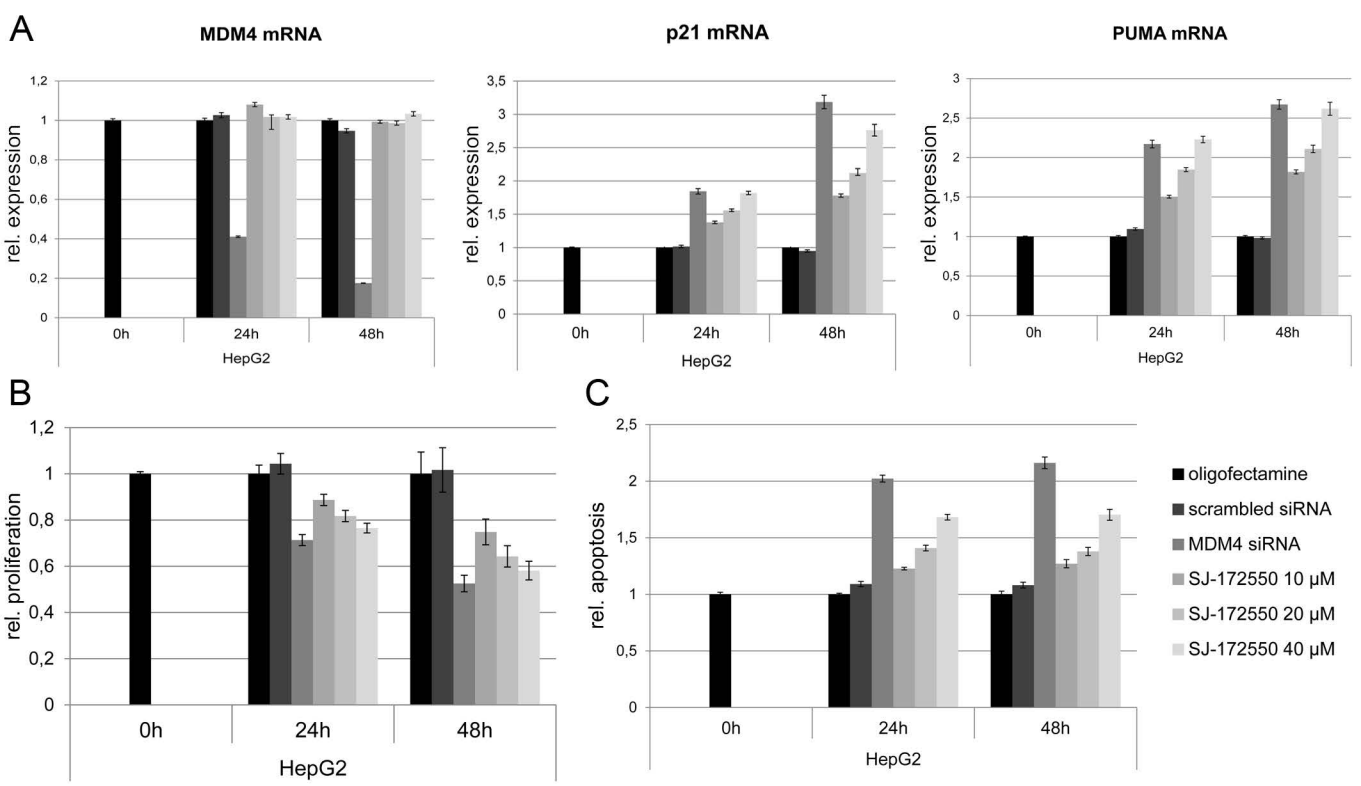

A
HepG2 (Rapamycin)
rel. expression
2,5
2
1,5
1
0,5
0
12h
24h
MDM4
pRPS6K
pAKT
B
Hep3B (Rapamycin)
rel. expression
2
1,75
1,5
1,25
1
0,75
0,5
0,25
0
12h
24h
MDM4
pRPS6K
pAKT

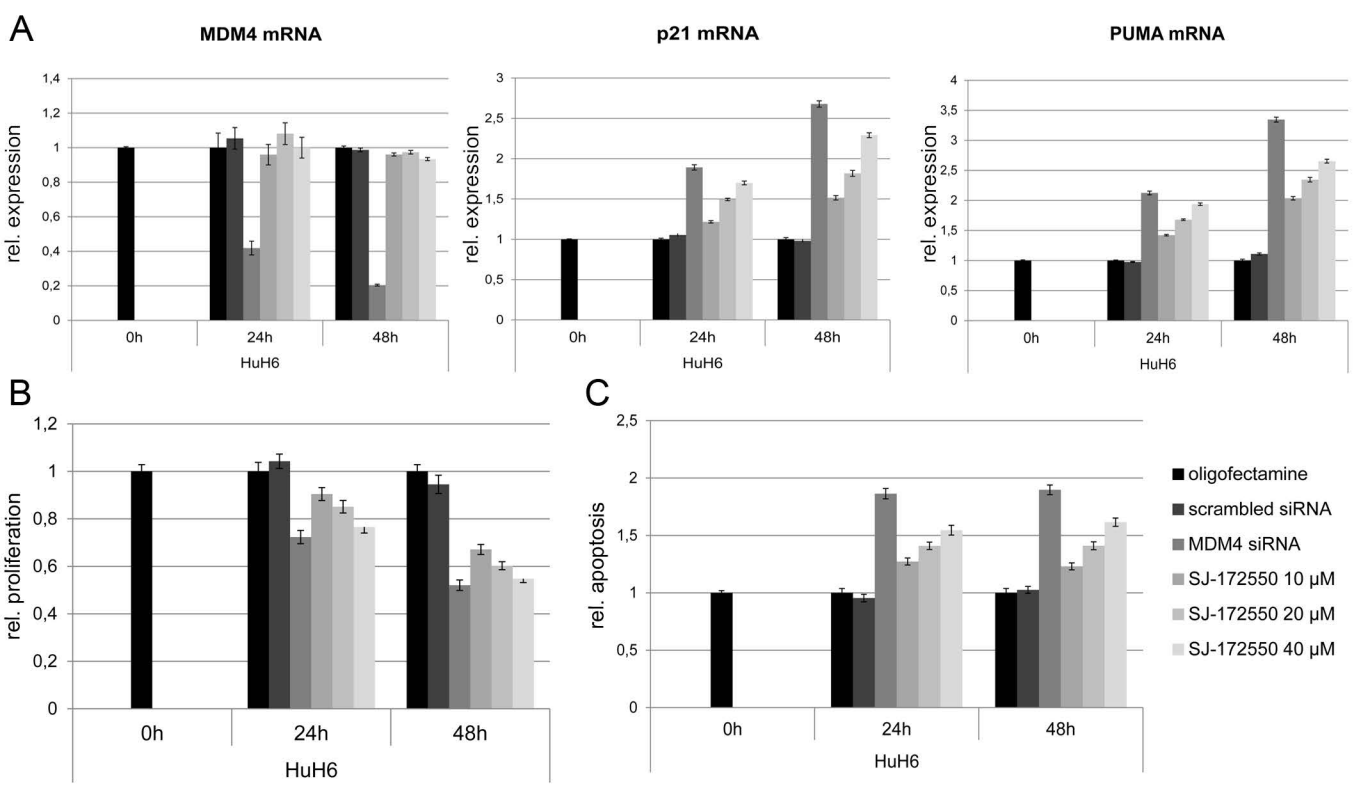

HuH7
Hep3B
MDM4 (55 kDa)
MDM2 (90 kDa)
pAKT (60 kDa)
pRPS6K (70 kDa)
Actin (42 kDa)
C D PI D PI D PI D PI
0 6h 12h 24h 48h
C D PI D PI D PI D PI
0 6h 12h 24h 48h

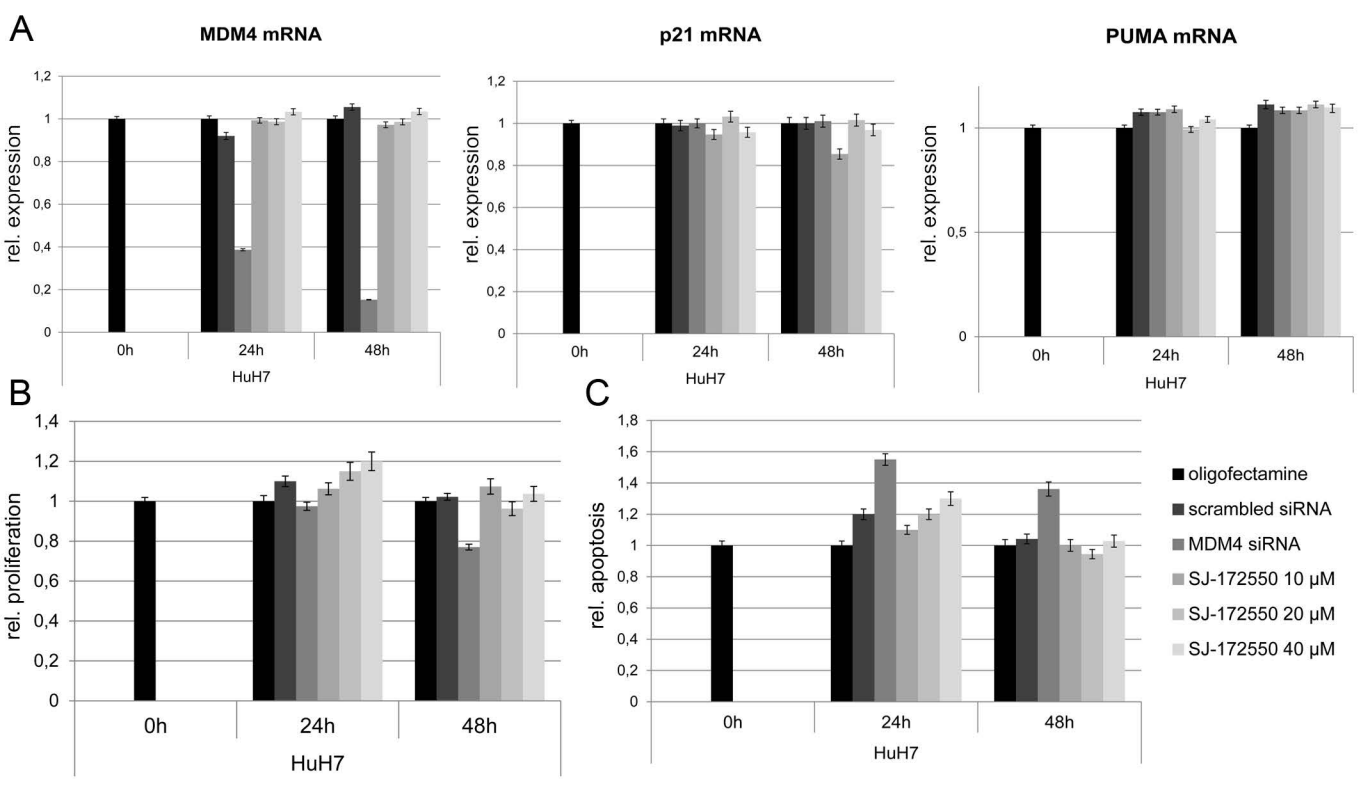

MDM4 (55 kDa)
RAPTOR (150 kDa)
RICTOR (225 kDa)
pRPS6K (70 kDa)
pAKT (60 kDa)
Actin (42 kDa)
C
SCR
siRaptor
siRictor
siRa+Ri

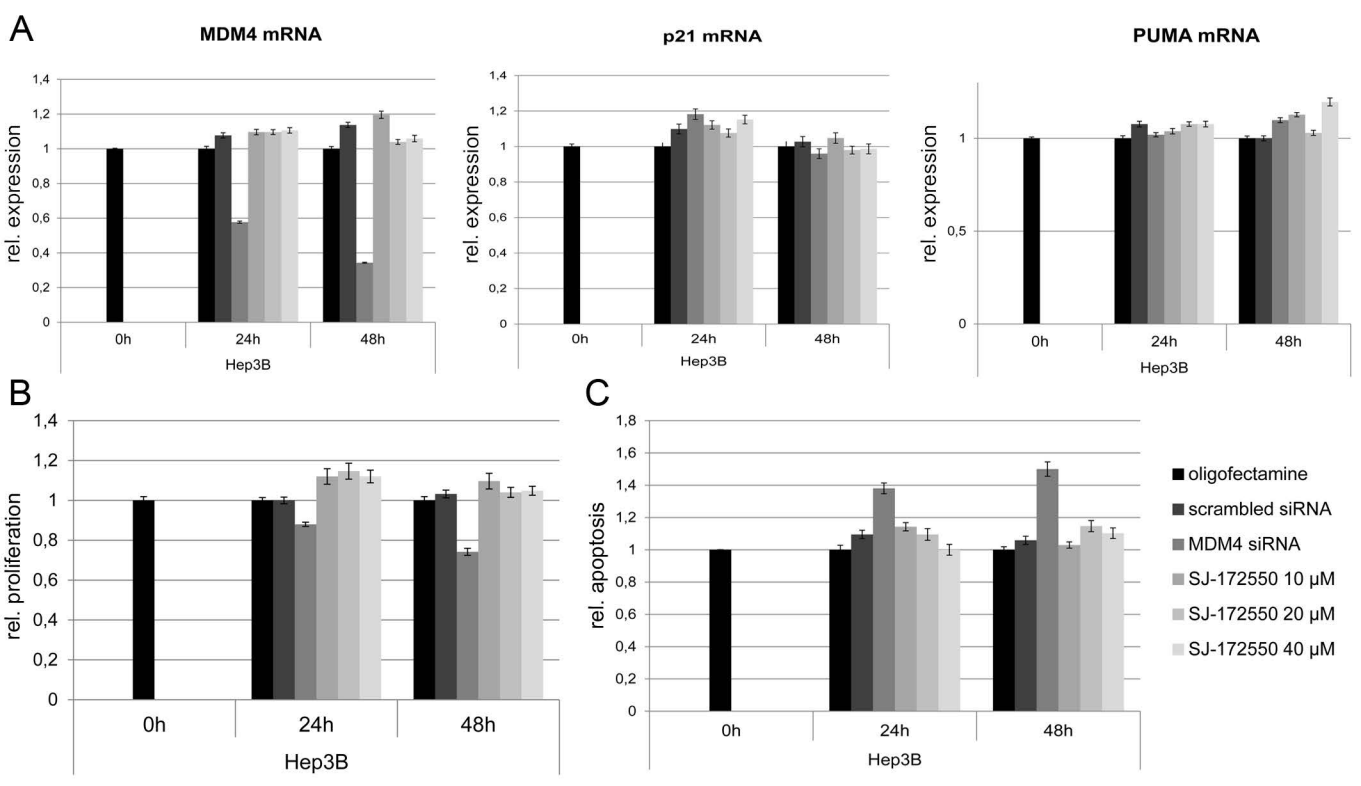

MDM4 (55 kDa)
MDM2 (90 kDa)
p53 (53 kDa)
pAKT (60 kDa)
AKT (60 kDa)
Actin (60 kDa)
C
D
LY
CHX
LY+ CHX
1h
2h
4h
8h
24h

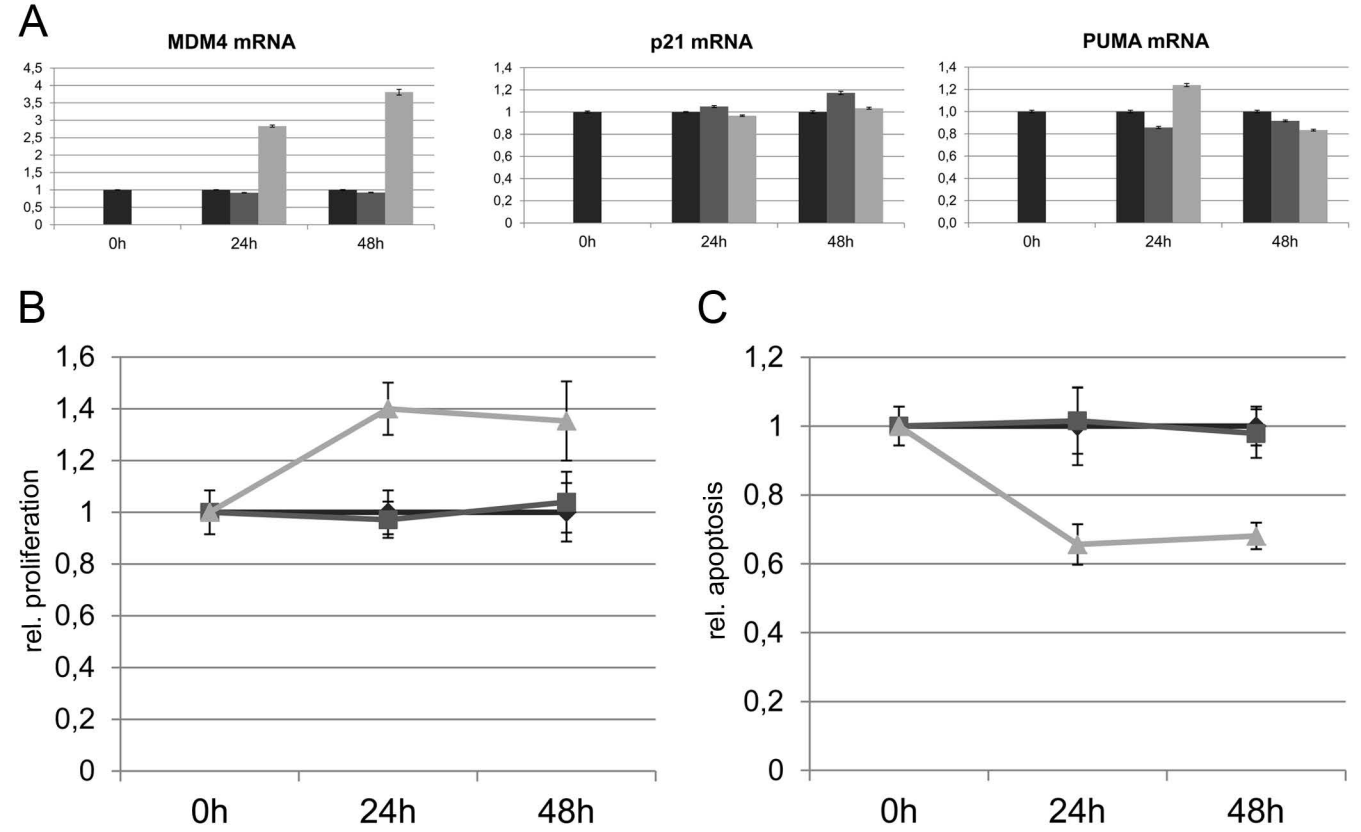

A
HuH6
GFP
Actin
shNC
shMDM4
B
HuH6 xenografts
GFP
Actin
shNC
shMDM4

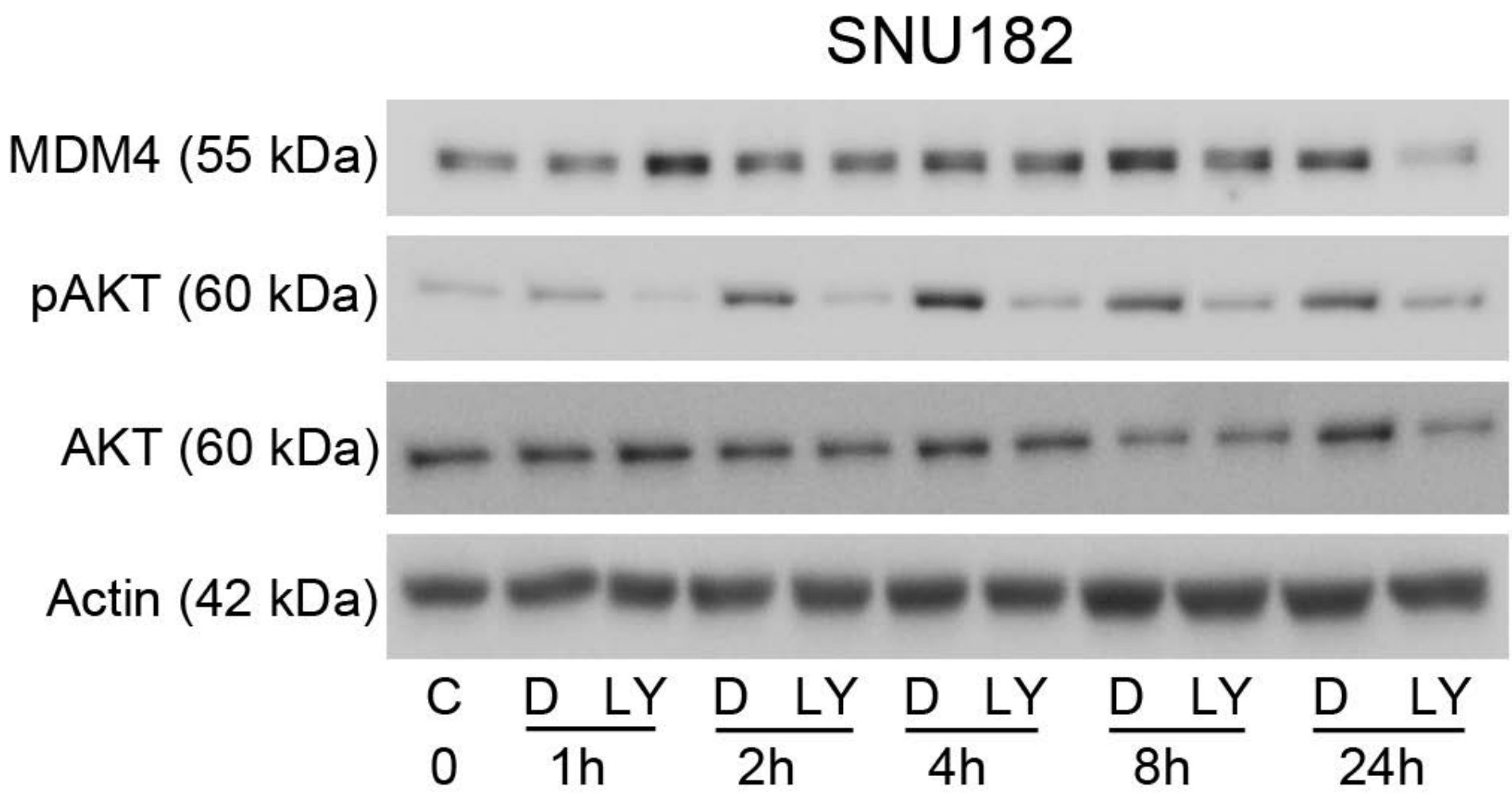

A
MDM4 mRNA
p21 mRNA
PUMA mRNA
rel. expression
1,2
1
0,8
0,6
0,4
0,2
0
3,5
3
2,5
2
1,5
0,5
0h
24h
48h
HepG2
B
rel. proliferation
C
rel. apoptosis
oligofectamine
scrambled siRNA
MDM4 siRNA
SJ-172550 10 µM
SJ-172550 20 µM
SJ-172550 40 µM

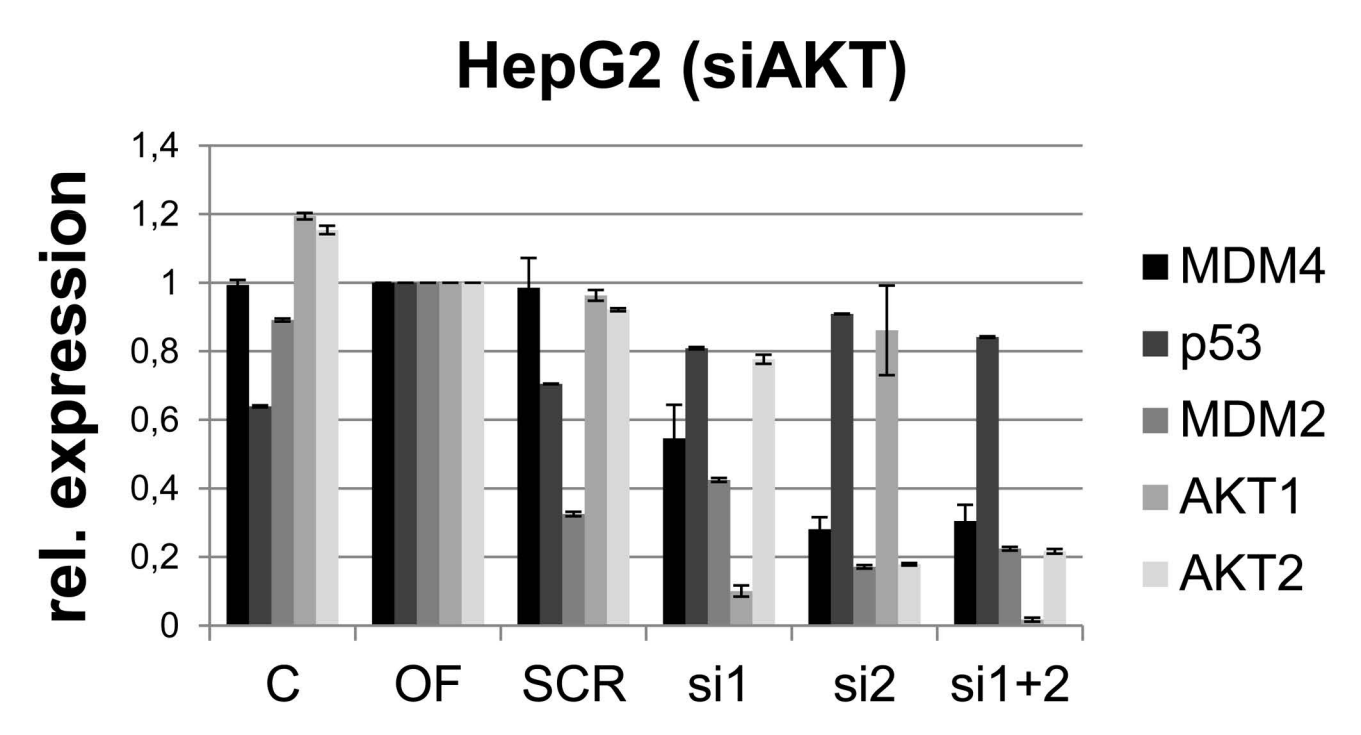

A
MDM4 mRNA
p21 mRNA
PUMA mRNA
rel. expression
0h
24h
48h
HuH6
B
rel. proliferation
C
rel. apoptosis
oligofectamine
scrambled siRNA
MDM4 siRNA
SJ-172550 10 µM
SJ-172550 20 µM
SJ-172550 40 µM

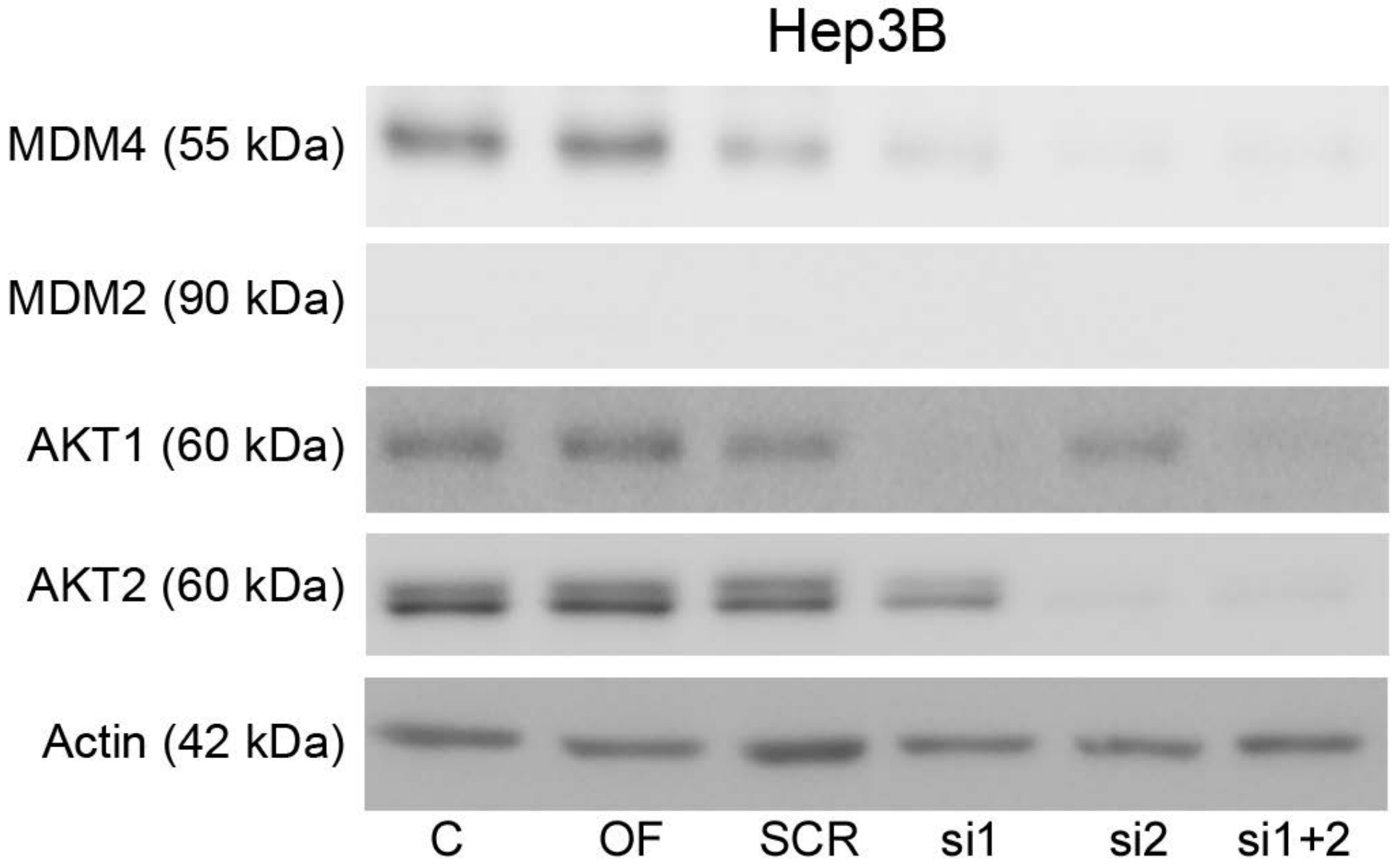

A
MDM4 mRNA
p21 mRNA
PUMA mRNA
rel. expression
1,2
1
0,8
0,6
0,4
0,2
0
0,5
0h
24h
48h
HuH7
B
rel. proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
C
rel. apoptosis
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
oligofectamine
scrambled siRNA
MDM4 siRNA
SJ-172550 10 µM
SJ-172550 20 µM
SJ-172550 40 µM


A
MDM4 mRNA
p21 mRNA
PUMA mRNA
rel. expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0,5
0h
24h
48h
Hep3B
B
rel. proliferation
C
rel. apoptosis
1,8
1,6
oligofectamine
scrambled siRNA
MDM4 siRNA
SJ-172550 10 µM
SJ-172550 20 µM
SJ-172550 40 µM


A
MDM4 mRNA
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0h
24h
48h
p21 mRNA
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0h
24h
48h
PUMA mRNA
1,4
1,2
1
0,8
0,6
0,4
0,2
0,0
0h
24h
48h
B
rel. proliferation
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0h
24h
48h
C
rel. apoptosis
1,2
1
0,8
0,6
0,4
0,2
0
0h
24h
48h


SNU182
MDM4 (55 kDa)
pAKT (60 kDa)
AKT (60 kDa)
Actin (42 kDa)
C
D LY
D LY
D LY
D LY
D LY
0
1h
2h
4h
8h
24h


HepG2 (siAKT)
rel. expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
C
OF
SCR
si1
si2
si1+2
MDM4
p53
MDM2
AKT1
AKT2


Hep3B
MDM4 (55 kDa)
MDM2 (90 kDa)
AKT1 (60 kDa)
AKT2 (60 kDa)
Actin (42 kDa)
C
OF
SCR
si1
si2
si1+2

Supplementary Materials

Cell lines, transfection and treatments

HuH6, HepG2 cell lines (harboring wild-type p53), and HuH7, HLE, Hep3B, Focus, SNU182, and SNU423 cell lines (harboring mutated or deleted p53) were cultured either in DMEM or RPMI medium, supplemented with 10% fetal bovine serum (PAA, Pasching, Austria) and 1% penicillin–streptomycin (10 mg/ml, PAA) at 37°C (5% CO_2) and passaged every 3-4 days. Transient transfection experiments of HLE cells with either *MDM4 or EEF1A2* cDNA in pCMV6-XL5 vector (OriGene Technologies, Rockville, MD) was performed following the manufacturer's protocol using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for transfection procedures. For stable transfection of the *AKT1* gene (in a pCMV6-XL5 plasmid; Origene Technologies) in the HLE cell line, stable transfectants were selected with cloning cylinders after 3–4 weeks in medium containing Geneticin (600 µg/ml) and were obtained from a previous study(1). For the treatment with chemical inhibitors, HCC cells were plated at a density of 1.5×10^5 cells/well in 6 cm plates and were incubated with the following drugs after 24 hours, as indicated: Rapamycin (mTORC1 inhibitor; 10 nmol/L; Enzo Life Sciences, Lörrach, Germany), LY29004 (PI3K inhibitor; 50 µmol/L; Enzo Life Sciences), PI-103 (PI3K, mTORC1 and mTORC2 inhibitor; 2 µmol/L; Enzo Life Sciences), SJ-172550 (blocker of the MDM-p53 interaction; 10-40 µM; Santa Cruz Biotechnology), AKT1/2 (AKT1/2 inhibitor; 10 µM; Sigma-Aldrich, St. Louis, MO), cycloheximide (protein synthesis inhibitor; Merck, Darmstadt, Germany), and/or MG132 (proteosomal inhibitor, 450 µmol/L; Enzo Life Sciences). All siRNA transfections were performed using oligofectamine (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The sequences of the small interfering RNAs and the final concentrations used (siRNA, Eurofins MWG Operon, Ebersberg, Germany), are listed in Supplementary Table 2. siRNAs against Raptor and Rictor (Santa Cruz Biotechnology, Santa Cruz, CA) were used according to the manufacturer's recommendations. For functional assays HCC cell lines were seeded at a density of 6 x 10^3 cells in 96 well plates and were transfected with siRNA or cDNA after 24 hours. Combined PI103 treatment was performed 48 hours after transfection of a p53-specific siRNA. Cell viability (MTT-assay) and apoptosis were determined using the Cell Proliferation Kit and Cell Death Detection Elisa Plus Kit (Roche Molecular Biochemicals, Mannheim, Germany), as described previously (2, 3). For all cell based assays, results were confirmed in three independent experiments.

Murine xenograft model

For analysis of tumorigenicity, Hep3B, HuH6, HepG2 and HuH7 cells were stably transfected with a specific GFP-tagged miR-30–based shRNAs targeting MDM4 (shMDM4) and Renilla luciferase (shNC), as control (see Supplementary Table 2 for sequences), cloned in a LMP vector, as previously described (4). Briefly, 5 x 10^6 cells (Hep3B, HuH6, HuH7) were subcutaneously injected into both

flanks of 5- to 6-week-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice as described previously (5). Tumor volumes were monitored over a period of 6-8 weeks using calipers. Tumors were isolated and analyzed by histology and Western blot analysis.

Western Blot Analysis and Immunoprecipitation

Tissue and cell samples were homogenized in lysis buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 10% glycerol, and 2 mM EDTA] containing the Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals) and sonicated. Protein concentrations were determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. For Western blotting, aliquots of 100 μg were denatured by boiling in Tris-Glycine SDS Sample Buffer (Invitrogen), separated by SDS-PAGE, and blotted onto nitrocellulose membranes (Invitrogen). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and probed with specific antibodies listed in Supplementary Table 1. Each incubation with a primary antibody was followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h (1:5000; Cell Signaling) and visualized using the Super Signal West Pico (Pierce Chemical, New York, NY). Protein densities were calculated by ImageQuaNT 5.1 software (GE Healthcare, Piscataway, NJ) and normalized to β-actin to determine the relative expression levels. For immunoprecipitation (IP), 600 μg of HCC cell protein lysate were immunoprecipitated with 6 μg of MDM4 mouse monoclonal antibody. After immunoblotting, the membranes were incubated with a goat polyclonal anti-USP2a antibody (Santa Cruz Biotechnology) or anti-AKT1 antibody (Santa Cruz Biotechnology). As negative control for IP, primary antibodies were neutralized, prior to IP, by a preincubation of 2 h at room temperature with the respective immunogen peptide (I :20 w/w), which resulted in the inhibition of the IP. Dephosphorylation of the protein lysate prior to IP was employed using the λ protein phosphatase (Cell Signaling Technology), following the manufacturer's protocol.

Tissue microarrays and immunohistochemistry

A tissue microarray (TMA) containing tissue from normal livers (n=20), non-tumorous liver tissue of HCC patients (n=66), and HCCs (n=76; Supplementary Table 3) was constructed as previously described (6), and immunohistochemistry was performed on 5 mm sections. Primary antibodies used for incubation are listed in Supplementary Table 1. Antigens were retrieved using citrate buffer (pH 6.1; Dako, Glostrup, Denmark). For detection, the EnVision method (Dako) was used. Counterstaining was performed using hemalum. Staining was assessed using the immunoreactive score as described previously (2): 0, absent; 1-4, weak; 5-8, moderate; 9-12, strong expression.

Statistical Analysis

The correlation between gene expression and clinico-pathological parameters was tested by Wilcoxon-signed-rank tests and measured by Spearman's rank correlations. $P<.05$ was considered statistically significant. Survival was defined as the time interval between diagnosis and death. Cut-off values for the investigated biomarkers were defined based on the median expression levels in the investigated sample (about 24 patients with a decreased, and about 24 patients with an increased expression). Univariate survival analysis was based on the Kaplan-Meier method and multivariate survival analyses relied on a Cox regression model. Statistical analyses were conducted using SPSS 20.0 (SPSS, Chicago, IL, USA), SAS version 9.2 and Kaplan-Meier curves were plotted using R version 2.11.1 (The R Project for Statistical Computing, http://www.r-project.org/).

References

1. Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, Destefanis G, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology 2011;140:1071-1083.
2. Schlaeger C, Longerich T, Schiller C, Bewerunge P, Mehrabi A, Toedt G, Kleeff J, et al. Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. Hepatology 2008;47:511-520.
3. Neumann O, Kesselmeier M, Geffers R, Pellegrino R, Radlwimmer B, Hoffmann K, Ehemann V, et al. Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors. Hepatology 2012.
4. Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, Lowe SW. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet 2005;37:1289-1295.
5. Rabenhorst U, Beinoraviciute-Kellner R, Brezniceanu ML, Joos S, Devens F, Lichter P, Rieker RJ, et al. Overexpression of the far upstream element binding protein 1 in hepatocellular carcinoma is required for tumor growth. Hepatology 2009;50:1121-1129.
6. Longerich T, Breuhahn K, Odenthal M, Petmecky K, Schirmacher P. Factors of transforming growth factor beta signalling are co-regulated in human hepatocellular carcinoma. Virchows Arch 2004;445:589-596.

Supplementary Table 1. List of the primary antibodies used.

Protein	Antibody	Epitope mapping	Company
AKT	Rabbit polyclonal	COOH terminus	Cell Signaling Technology (Danvers, MA)
AKT1	Goat polyclonal	COOH terminus	Santa Cruz Biotechnology (Santa Cruz, CA)
cleaved Caspase3	Rabbit polyclonal	Human Active Caspase-3 Fragment	BD Biosciences (Franklin Lakes, NJ)
EEF1A2	Rabbit polyclonal	NH_2 terminus	Epitomics (Burlingame, CA)
HA-Tag	Mouse monoclonal	HA-Tag	Cell Signaling Technology (Danvers, MA)
MDM2	Mouse monoclonal	region between residues 154-167	Santa Cruz Biotechnology (Santa Cruz, CA)
MDM4 (WB/IHC)	Rabbit polyclonal	region between residues 125 and 175	Bethyl Laboratories, (Montgomery, TX)
$P21^{WAF1}$ (WB)	Mouse monoclonal	Full length	Santa Cruz Biotechnology (Santa Cruz, CA)
$P21^{WAF1}$ (IHC)	Mouse monoclonal	region between residues 145-164	BD Biosciences (Franklin Lakes, NJ)
P53 (WB)	Mouse monoclonal	Full length	BD Biosciences (Franklin Lakes, NJ)
P53 (IHC)	Mouse monoclonal	NH_2 terminus	Dako (Glostrup, Denmark)
pAKT	Rabbit monoclonal	Serine 473[a]	Cell Signaling Technology (Danvers, MA)
pRPS6	Rabbit monoclonal	Residues surrounding Serine 235 and 236	Cell Signaling Technology (Danvers, MA)
pp706S6K	Rabbit monoclonal	serine 371	Cell Signaling Technology (Danvers, MA)
PARP	Rabbit polyclonal	Full length	Cell Signaling Technology (Danvers, MA)
PI3K (p110a)	Rabbit polyclonal	region between residues 189-390	Santa Cruz Biotechnology (Santa Cruz, CA)
PI4Kb	Mouse monoclonal	NH_2 terminus	Santa Cruz Biotechnology (Santa Cruz, CA)
Raptor	Mouse monoclonal	Full length	Santa Cruz Biotechnology (Santa Cruz, CA)
Rictor	Rabbit polyclonal	NH_2 terminus	Santa Cruz Biotechnology (Santa Cruz, CA)
USP2a	Rabbit polyclonal	COOH terminus	Abgent (San Diego, CA)
β-ACTIN	Rabbit polyclonal	COOH terminus	Santa Cruz Biotechnology (Santa Cruz, CA)

Supplementary Table 2. Primer, shRNA and siRNA sequences

BAX-fw	5'-TGGAGCTGCAGAGGATGATTG-3'
BAX-rev	5'-AAACATGTCAGCTGCCACTCG-3´
Human MDM2-fw	5'-TCT GTG AGT GAG AAC AGG TGT CAC-3'
Human MDM2-rev	5'-ACA CAC AGA GCC AGG CTT TC-3´
Human MDM4-fw	5'-CAG CAG GTG CGC AAG GTG AA-3'
Human MDM4-rev	5'-CTG TGC GAG AGC GAG AGT CTG-3´
P21-fw	5'-CACCGAGACACCACTGGAGG-3'
P21-rev	5'-GAGAAGATCAGCCGGCGTTT-3'
Puma-fw	5'-CCTGGAGGGTCCTGTACAATCT-3'
Puma-rev	5'-GCACCTAATTGGGTCCATCT-3'
18s-fw	5'-AAACGGCTACCACATCCAAG-3'
18s-rev	5'-CCTCCAATGGATCCTCGTTA-3'
EEF1A2-fw	5'-AGGACCATTGAGAAGTTCG-3'
EEF1A2-rev	5'-AGATGTCGATGGTGATGC-3'
siAKT1 (40 nM)	5´-CAG GCU UGG UCC CGA GGC CAA-dTdT3´
siAKT2 (40 nM)	5´-AAC AAC UUC UCC GUA GCA GAA-dTdT3´
siEEF1A2 (40 nM)	5´-UCA AGA AGA UCG GCU ACA A-dTdT3´
siMDM4 (40 nM)	5´-AGC AAC UAU ACA CCU AGA A-dTdT3´
siP53 (5 nM)	5´-UGU UCC GAG AGC UGA AUG A-dTdT3´
siUSP2a (40 nM)	5´-CUC GUC CAU ACU CCA AGA A-dTdT3´
scrambled siRNA (SCR; 40 nM)	5´-UUC UCC GAA CGU GUC ACG U-dTdT3´
shMDM4 (MDM4.3084)	5´-UGCUGUUGACAGUGAGCGACCCUAGAAUUGUCAAACUUAAUAG UGAAGCCACAGAUGUAUUAAGUUUGACAAUUCUAGGGCUGCCUAC UGCCUCGGA-3´
shNC (neutral control shRNA, Ren.713; effectively targeting Renilla Luciferase)	5´-CUCGAGAAGGUAUAUUGCUGUUGACAGUGAGCGCAGGAAUUAU AAUGCUUAUCUAUAGUGAAGCCACAGAUGUAUAGAUAAGCAUUAU AAUUCCUAUGCCUACUGCCUCGGAAUUC-3´

Supplementary Table 3: Patient´s characteristics of TMA cohort

Gender	
male	59 (78%)
female	17 (22%)
Median age (range)	58 (17-78)
Etiology	
HBV	16 (21.1%)
HCV	25 (32.9%)
co-infection	4 (5.3%)
alcohol	13 (17.1%)
cryptogenic	19 (25.0%)
genetic hemochromatosis	3 (3.9%)
Grading	
well differentiated HCC	13 (17.1%)
moderately differentiated HCC	55 (72.4%)
poorly differentiated HCC	8 (10.5%)
Tumor size	
< 2.0 cm	7 (9.2%)
2 .0 – 5.0 cm	39 (51.3%)
> 5.0 cm	30 (39.5%)
UICC stage	
I	35 (46.1%)
II	24 (31.6%)
III	13 (17.1%)
IV	4 (5.3%)
Vascular invasion	
present	27 (35.5%)
none	49 (64.5%)
Liver cirrhosis	
present	46 (60.5%)