2 Materials and Methods

2.1 Abbreviations

ATP	adenosine triphosphate
bp	base pair(s)
BrdU	5-bromo-2'deoxyuridine
BSA	bovine serum albumin
d	day
DAPI	4',6-Diamidin-2-phenylindoldihydrochloride
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DNase	deoxyribonuclease
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGTA	Ethylene-glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
et al.	*et altera*
EDTA	ethylene-diaminetetraacetic acid
FCS	fetal calf serum
g	gram
G418	geneticin
HEPES	4-(2-Hydroxyethyl)-piperazin-1-ethansulfonic acid
HP	partial hepatectomy
h	hour
HRP	horseradish peroxidase
HS	heat inactivated horse serum
kDa	kilodalton
kb	kilobase pairs
l	liter
LIF	leukemia inhibitory factor
M	molar

mA	milliampere
min	minute
ml	milliliter
mM	millimolar
µM	micromolar
MEM	Modified Eagle Medium
NaAc	sodium acetate
neo^R	neomycin resistance gene
ON	over night
PBS	phosphate-buffered saline
PBT	PBS containing 0.05% Tween-20
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	*potentium hydrogenii*
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TEMED	N,N,N',N'-Tetra-methylethylenediamine
Tris	Tris-(hydroxymethyl)aminoethane
U	unit (enzymatic activity)
V	Volt
Vol.	Volume
W	Watt

2.2 Materials

Chemicals, enzymes and kits for molecular biology, oligonucleotides, or antibodies were purchased from the following companies, unless indicated otherwise: Amersham-Pharmacia (Freiburg); Biotez (Berlin); Biozym (Hess. Oldendorf); Cell Signaling, Dianova (Hamburg); Gibco/BRL (Karlsruhe); Heraeus-Kulzer (Wehrheim); Invitek (Berlin); MBI Fermentas (St.Leon-Rot); Merck (Darmstadt); MWG-Biotech (Ebersberg); New England Biolabs (Frankfurt); Oncogene Pan-Biotech (Aidenbach); Promega (Mannheim); Qiagen (Hilden); Roche (Mannheim); Roth (Karlsruhe); Santa Cruz, Serva (Heidelberg); Shandon (Frankfurt); Sigma (Deisenhofen).

2.2.1 Bacterial strains

Escherichia coli XL1-Blue MRF '	(Jerpseth et al., 1992)

2.2.2 Vectors/plasmids

pBluescript II SK+	Sorge, 1988
pGEM-T	Promega, Mannheim
pTV-0 constructed by Barbara Walter	(backbone: pIC19R, (Marsh et al., 1984)
pL2-neo (contains *loxP*-neo-*loxP* cassette)	H. Gu/K. Rajewsky laboratory
pIC (Cre expression plasmid)	H. Gu/K. Rajewsky laboratory

2.2.3 ES cell line

Embryonic stem (ES) cells from the line E14.1, derived from the 129/Ola mouse strain (Hooper et al., 1987; Kühn et al., 1991) were used for introducing targeted mutations into the mouse germline**.**

2.2.4 Antibodies

a) **Primary**

Antigen	**Host animal***	**Dilution**	**Source**
Akt	rabbit	1:1000	Cell Signaling
phospho-Akt (Ser473)	rabbit	1:1000	Cell Signaling
BrdU	mouse IgG	1:200	Sigma
c-fos	rabbit	1:25	Oncogene
phospho-c-Jun (Ser63)	rabbit	1:500	Cell Signaling
caspase-3-cleaved (Asp175)	rabbit	1:500	Cell Signaling
phospho-cdk2 (Tyr15)	rabbit	1:1000	Cell Signaling
cdk2	rabbit	1:1000	Cell Signaling
cyclin A	rabbit	1:200	Santa Cruz
cyclin D	rabbit	1:200	Santa Cruz
cyclin E	rabbit	1:100	Santa Cruz
Erk1/2	rabbit	1:1500	Cell Signaling
phospho-Erk1/2 (Thr202/Tyr204)	rabbit	1:1000	Cell Signaling
HGF/SF	sheep	1:500	Ermanno Gherardi
Ki67	rat	1:40	R&D
Met	rabbit	1:100	Santa Cruz
$p19^{ARF}$	mouse	1:2000	BD Biosciences Pharmingen
p21Cip/Waf1	mouse IgG	1:50	Oncogene
p27/Kip1	rabbit	1:1000	Cell Signaling
p38	rabbit	1:1000	Cell Signaling
phospho-p38	rabbit	1:1000	Cell Signaling
p53	rabbit	1:500	Dako
PCNA	mouse	1:1000	Dako
phospho-Rb (Ser780)	rabbit	1:1000	Cell Signaling
SOCS3	rabbit	1:50	Zymed
phospho-Smad2 (Ser465/467)	rabbit	1:500	Cell Signaling

STAT3	rabbit	1:2000	Cell Signaling
phospho-STAT3 (Tyr705)	rabbit	1:1000	Cell Signaling

b) **Secondary**

The secondary antibodies coupled with fluorescent reagent or horse-radish peroxidase were purchased from Dianova (Hamburg) and dissolved in sterile 50% glycerin according to manufactures instructions.

2.2.5 Mouse strains:

C57Bl/6J: Charles River (Sulzfeld)

Met^{null}: F. Bladt/ Carmen Birchmeier laboratory

Mx-cre: R. Kühn/Klaus Rajewsky laboratory

Deleter F. Schwenk/ Klaus Rajewsky laboratory

2.2.6 Cell Culture Media

Fibroblast Medium:

500ml Dulbecco's MEM with Glutamax-I, 4500mg/l Glucose, with Pyridoxin, Natriumpyruvat (Gibco BRL)

60ml FCS (Heat inactivated at 55°C for 30min, Sigma)

5.7ml 100x non-essential aminoacids (Gibco BRL)

5.7ml Penicillin/Streptomycin-solution (10000U/ml Penicillin G/10000µg/ml Streptomycin; Gibco BRL)

1.2ml 50mM ß-Mercaptoethanol (Gibco BRL)

ES-Cell-Medium:

500ml DMEM/Glutamax (see above, Gibco BRL)

90ml FCS (Heat inactivated at 55°C for 30min, Sigma)

6ml 100x non-essential aminoacids(Gibco BRL)

6ml Penicillin/Streptomycin-solution (Gibco BRL)

1.2ml ß-Mercaptoethanol (Gibco BRL)

60µl LIF (f.c. 500-1000 U/ml)

LIF-containing supernatant was routinely prepared from COS cells stably transfected with a LIF-expression plasmid (Genetics Institute Inc., Cambridge, MA, USA).

2.3 Methods

Molecular biology techniques

Apart from the techniques detailed in the following part of this section, standard procedures for molecular cloning, sequencing and targeting vector construction were carried out according to "Molecular Cloning" (Sambrook and Russell, 2001) or manufacturers' instructions in the case of kits.

2.3.1 Extraction and Purification of DNA

2.3.1.1 Extraction of Plasmid DNA

E.coli cells containing plasmid DNA were usually grown, in autoclave sterilized LB-medium (10g bacto-tryptone, 5g yeast extract, 10g NaCl in 1l H_20) with an appropriate antibiotic, ampicillin (100µg/ml) or kanamycin (30µg/ml), over-night at 37°C. Small-scale preparations (mini-preps) were performed by the alkaline lysis method (Birnboim and Doly, 1979).

Medium-25ml culture and large -100ml culture scale preparations of plasmid DNA were carried out by means of the respective Plasmid Midi- and Maxi-Kit from Qiagen (Hilden), according to the manufactures protocol.

2.3.1.2 Purification of DNA fractionated on agarose gels

DNA fragments were purified from agarose gels using "QIAEX II Gel Extraction Kit" (Qiagen, Hilden; Vogelstein und Gillespie, 1979).

2.3.1.3 Isolation of genomic DNA from ES cells

ES cells were screened for homologous recombination events by Southern-hybridization of genomic DNA. The procedure of genomic DNA isolation was adapted from Ramirez-Solis and colleagues (Ramirez-Solis et al., 1992). Briefly, the ES cells were grown in gelatinized 96 well plates up to a confluent layer. Cells were washed twice with PBS and lysed in 50µl ES cell lysis buffer/well (10mM Tris-HCl,

pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% N-Lauroylsarcosin (=„Sarcosyl“), 200µg/ml proteinase K) at 60^0C in a humid chamber overnight. The DNA was precipitated with 100µl of 100% ethanol and 1/20 volume of 3M sodium acetate pH 5.2 for 30 min at room temperature. Afterwards the DNA was washed twice with 70% ethanol, air-dried for 20 min and directly subjected to restriction digests. Digests were carried out in 50µl of restriction mix (1x restriction buffer, 100 µg/ml BSA, 50 µg/ml RNAse, 10-15 U of restriction enzyme) at 37^0C with gentle shaking for 16 h.

2.3.1.4 Isolation of genomic DNA from mouse tissue

To genotype mice by PCR, DNA was isolated from ear holes. The tissue was lysed for at least 1 hour at 55^0C in 50µl of lysis buffer containing proteinase K (1mg/ml). To inactivate the proteinase K the digests were incubated at 95^0C for 10 min. Lysates were diluted with distilled water 1:6 and directly used for PCR.

For Southern blot analysis, 200 mg of liver were snap frozen in liquid nitrogen and stored at -70^0C or directly ground using mortar and pestle to homogenize the tissue. Homogenized tissue or a piece of tail (approx. 1cm) was incubated at 55^0C in 10 volumes of lysis buffer containing 4mg/ml proteinase K until the tissue was completely dissolved. After two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and one chloroform/isoamyl alcohol (24:1) extraction the DNA was precipitated with 2 vol. of ice-cold 100% ethanol. The sample tube was centrifuged (14,000g; 10 min), and the pellet was washed once with 70% ethanol, briefly spun down and air-dried. The DNA was dissolved in TE buffer at a final concentration of 1 mg/ml. The concentration and purity of the DNA were determined by UV-spectrophotometry. $OD_{260} = 1.0$ is equal to 50 mg/ml of dsDNA. To calculate a protein contamination of a sample the equation OD_{260}/OD_{280} is used.

2.3.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (Saiki et al., 1985) was used to genotype littermates and each specific PCR was established according to general rules (Innis et al., 1989). A list all reaction conditions used for each genotyping PCR is presented below.

a) Met^{flox}, 2 mM $MgCl_2$

Primers: mfs1: 5´-AGCCTAGTGGAATTCTCTGTAAG -3´
mfas2: 5´-CCAAGTGTCTGACGGCTGTG -3´

Cycling conditions:

94°C	2min		
94°C	45sec	┐	
62°C	45sec	├	32x
72°C	45sec	┘	
4°C	∞		

Amplicon length: wild-type 330 bp;
Met^{flox}: 372 bp

b) Met

- Wild-type-PCR, 3 mM $MgCl_2$

Primers: Wmet8S: 5'-CTTTTTCAATAGGGCATTTTGGCTGTG-3'
Wmet10: 5'-GTACACTGGCTTGTACAATGTACAGTTG-3'

- Mutant-PCR, 4 mM $MgCl_2$

Primers: Wmet5: 5'-CACTGAGCCCAGAAGAGCTAGTGG-3'
neo1L: 5'-CCTGCGTGCAATCCATCTTGTTCAATG-3'

Cycling conditions:

94°C	45s	┐	
70°C	30s	├	40x (wild-type PCR)
72°C	30s +1s/cycle	┘	35x (mutant PCR)
4°C	∞		

Amplicon length: wild-type: 520 bp;
mutant: 310 bp

c) Cre, 1.5 mM $MgCl_2$

Primers: crenew1: 5'-GAACGCACTGATTTCGACCA-3'
crenew2: 5'-AACCAGCGTTTTCGTTCTGC-3'

Cycling conditions:

94°C	1min	
96°C	20s	┐
58°C	25s	├ 34x
72°C	30s	┘
4°C	∞	

Amplicon length 200 bp

d) Deleter, 2 mM $MgCl_2$

Primers: Deleter1: 5'-CGCCATCCACGCTGTTTTGACC-3'

Deleter2: 5'-CAGCCCGGACCGACGATGAAG-3'

Cycling conditions:

94°C	2min	
94°C	45s	┐
60°C	30s	├ 36x
72°C	30s	┘
4°C	∞	

Amplicon length 371 bp

e) Mx-cre, 2 mM $MgCl_2$

Primers: mx1: 5'-TTCACGGTTTCAATTCTCCTCTGG-3'

mx2: 5'-CACCGGCATCAACGTTTTCTTTC-3'

Cycling conditions:

94°C	2min	
94°C	45s	┐
63°C	30s	├ 35x
72°C	45s	┘
4°C	∞	

Amplicon length 440 bp

2.3.3 DNA sequencing

DNA sequences were determined using the dideoxy-chain-termination reaction protocol

(Sanger et al., 1977) modified by Tabor and colleagues (Tabor and Richardson, 1987) and using the non-radioactive "Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing"-Kit (Amersham). Fluorescently labeled primers were obtained from MWG-Biotech. Cycle sequencing was performed using the following cycling parameters.

95°C	3min	
95°C	35s	30x
53°C	35s	
70°C	1min	
95°C	3min	

Reaction products were resolved on 6% Sequagel XR polyacrylamide gels (Biozym) in 1xTBE running buffer and analyzed using Li-Cor-Sequencing device (Model 4000L or 4200, MWG-Biotech).

2.3.4 Southern blotting

The Southern blot analysis (Sambrook and Russell, 2001; Southern, 1975) was used to screen ES cell clones for homologous recombination events, as well as during the establishment of the F1 and F2 generations of Met^{flox} mice (to confirm germline transmission) and to verify efficiency of cre-mediated recombination.

Between 5 and 10 µg of genomic DNA were digested overnight with 20 units of restriction enzyme. The digested DNA was resolved on a 0.8% agarose gel containing 0.5 µg per ml ethidium bromide. To confirm complete digestion of the genomic DNA, gel was exposed to UV-light and photographed. The gel was depurinated in 0.25 M HCl solution for 10 to 15 min. Then the gel was rinsed in distilled water and denatured by two 30 min incubations with gentle shaking in a solution of 1.5 M NaCl and 0.5 M NaOH. Finally the gel was then rinsed in 10X SSC and blotted overnight using 20X SSC, so as to transfer the DNA onto a nylon membrane (Hybond N_+, Amersham-Pharmacia) as described by (Southern, 1975; Sambrook und Russell, 2001). On the next day, after transfer, the membrane was air-dried and the DNA was cross-linked to the membrane using UV-light at 120 mJ/cm2. Subsequently the membrane was hybridized

with specific radioactive probes. DNA probes (20-50 ng) were radioactively labeled with 50 µCi γ^{32}P-dCTP (Amersham-Pharmacia) using the 'Prime-It RmT Random-Primed Labeling Kit' (Stratagene). The labeled probes were purified over Sephadex-G50 spin columns (Probe Quant G50, Amersham-Pharmacia). Before hybridization probes were denatured by boiling for 5 min.

Prehybridization was carried out according to Denhardt (1966) with modifications. The membranes were saturated in 20-25 ml hybridization solution (6x SSC, 5x Denhardt's solution, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA) at 65^0C for at least 2 hours in the hybridization oven (Biometra). The denatured probes were then added to the tubes incubating the membranes in prehybridization buffer. Hybridization was carried out at 65^0C for 16-24 hours. In order to remove the non-specifically bound probe, the following washing steps were carried out in a shaking water bath at 65°C: 2x 15 min in 2x SSC, 0.1% SDS, 1x 30 min in 0.1x SSC, 0.1% SDS. The membranes were then sealed in plastic bags and exposed to a Biomax MS autoradiographic films (Kodak) at –80°C for overnight or exposed to a Phosphoimager (Fujix, BAS 2000) for several hours.

If a membrane should be reused for hybridization with a different probe, the old probe was stripped by boiling the membrane in 1% SDS for 30 min. After rinsing with 2x SSC the membrane was stored at -20°C or was reprobed directly.

2.3.5 Cell culture

2.3.5.1 Preparation and culture of embryonic fibroblast

All cell culture procedures were based on protocols according to "Gene Targeting: A Practical Approach" (Joyner, 1999). To maintain the pluripotency, ES cells were cultured in the presence of leukemia-inhibitory factor (LIF) on a layer of growth-arrested feeder cells derived from embryonic fibroblasts.

Embryos (E13.5-E16.5) obtained from mating wild-type mice with homozygous transgenic strains, which contain a neomycin resistance cassette (*neo*^{R}), were used for fibroblasts preparation. *neo*^{R} feeder cells survive during positive selection of ES cells with G418. Embryos were dissected in sterile conditions and head as well as internal

organs were removed. Such carcasses were washed in large volume of PBS^{++} (1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.68 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2) to remove blood. Then carcasses were minced into small cubes and pressed though the screen into a flask that contains 20 ml of glass beads. The suspension of cells was incubated at 37°C in 50 ml 0.05% Trypsin/0.02% EDTA solution together with 200 ml of DNase I (10 mg/ml), for 30 min with stirring. Then an additional 50 ml of trypsin/EDTA was added, stirred for another 30 min and the trypsinisation procedure was repeated. After decantation of glass beads, the cells were centrifuged at 1500g for 5min. The pellet was washed twice in PBS^{++} and resuspended in 5ml PBS (1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.2). Afterwards, feeder cells (5 x 10^6) were plated onto 150 mm tissue culture dish with 10 ml of fibroblast medium. In 3-4 days, when the cells reached confluence (ca. 4-5 x 10^6 cells/plate), the feeders were split 1:3 or 4 for additional expansion or frozen for storage. For splitting, the cell were twice washed with PBS, each plate was incubated with 3 ml of 1x trypsin/EDTA at 37°C for 5 min. Once the cells detached, they were gently pipetted up and down to break aggregates. Trypsinized cells were added to 6 ml of feeder medium in a conical tube and centrifuged at 3,000g for 2 min. The supernatant was discarded, the cell pellet re-suspended in feeder medium and plated. Generally, embryonic fibroblasts were not passed more than 3 times, because they are not anymore suitable for ES-cell culture. The feeders can be stored for several days at --70°C or for longer time in liquid nitrogen. For freezing trypsinized cells have to be re-suspended in cold freezing medium A (ES-cells-Medium/50% FCS) and then an equal volume of cold freezing medium B (ES-cells-Medium/20% DMSO) is slowly added. The suspension was then transferred to cryo-tubes (Nalgene) and freeze down first at -20°C and later at -70°C.

For thawing vials with feeders were warmed-up at 37°C for short time and then the cells were transferred to 10 ml of warm fibroblasts medium. After centrifugation (1100g, RT) cells were re-suspended in fresh medium and platted.

2.3.5.2 Growth-arrest of Embryonic Fibroblast by mitomycin C treatment

A confluent plate of embryonic fibroblasts was washed with PBS and incubated for 2 h with 100 µl of mitomycin C stock solution (1 mg/ml in PBS, 5% DMSO, Sigma) in

10 ml of feeder medium. Then the cells were washed two times with PBS, incubated with 3 ml of 1x trypsin/EDTA at 37°C for 5 min, re-suspended and centrifuged in feeder medium. The cell pellet was brought to a concentration 2-3x10^5 cells/ml of feeder medium and plated on gelatinized plates.

2.3.5.3 ES cell culture, electroporation and neomycin-resistance selection

Frozen ES were thawed rapidly and DMSO-containing medium was immediately replaced with warm (37°C) ES medium. As a standard procedure, 10^7 ES cells were electroporated with 20 µg of linearised targeting vector in 0.8 ml PBS (240 V, 500 µF, BioRad Gene Pulser). The transfected cells were then plated out on growth-arrested neomycin resistant mouse embryonic primary fibroblasts (see 2.3.5.1 and 2.3.5.2) at a density of 2.5x10^6 cells per 10-cm dish and cultured in ES cell medium. Selection with 400 µg/ml G418 (Geneticin) was started 48 hours later. Fresh selection media was added daily to the ES cells. After further 5-7 days culture with selective medium single, undifferentiated ES cell colonies were picked and cultured for additional 1-2 days in 96 well plates with layer of feeder cells. Then ES cell colonies were trypsinised and split into two 96 well plates; one plate without feeder cells for screening and one plate with feeder cells for freezing. For freezing down, 1 volume of ice cold 2X freezing medium (ES medium plus 13.3% DMSO) was added to confluent, trypsinised 96 well plates that had 1volume of trypsin in them. The plates were carefully wrapped in paraffin and gradually frozen down to –80^0C in styrofoam boxes. To screen the cells for homologous recombination by Southern analysis, a replica plate was made of each 96-well plate. Replica plate was coated with 0.1% gelatin (Sigma) before seeding of ES cells. These plates were grown to confluence and used to extract DNA to screen for targeted clones as described earlier.

The *neo*^{R} gene was removed afterwards by transient expression of Cre recombinase in ES cell (Gu et al., 1994; Kuhn and Torres, 2002). The ES cell were electroporated with pIC-cre vector as described above except that 10 µg of vector was used. The ES cell suspension was diluted with ES cell medium, and up to 1 x 10^3 cells were placed in each 100 mm cell culture dish. Colonies were picked after 8-9 days in culture and processed as above. ES clones were screened by Southern analysis as described above.

2.3.6 Generation of conditional knockout mice

Two independent heterozygous ES cell clones were used to generate chimeras by injecting 10-15 ES cells into blastocysts obtained from C57BL/6 super-ovulated females (Hogan et al., 1994). Females (20-23 days old) were injected interperitoneally with 100 μl 50U/ml PMS in PBS („Pregnant Mare's Serum“ = Intergonan, Intervet GmbH, Tönisvorst). This serum contains Follicle-Stimulating hormone (FSH). Two days later females were injected with 100μl 50U/ml hCG in PBS (human Chorionic-Gonadotropin, Ovogest, Intervet GmbH, Tönisvorst) und then mated with C57Bl/6J males. Blastocysts were recovered on day 3.5 post-coitum by flushing the uterus with blastocyst medium (Fibroblasts-medium with 30 mM HEPES pH 7.2). Round ES cells (12-15) were injected into the blastocoelic cavity and approximately 16 of such injected blastocysts were implanted into the uterine horns of time-matched pseudo-pregnant CB6F1 foster mice (day 2.5 post-coitum). Chimeras were identified by coat color and backcrossed to C57BL/6 mice to achieve germ-line transmission. The offspring with brown coat color was analyzed for heterozygosity by genotype PCR and by Southern blot analysis.

Methods: Analysis of phenotype

2.3.7 Partial hepatectomy

The liver is composed of four lobes. The median and left lateral lobes comprise about 70% of the liver and their removal is recognized as partial hepatectomy. The third lobe is the right lateral lobe, which overlaps the median lobe on the right side. The smallest lobe is the caudate lobe and its two segments surround the abdominal part of the esophagus. The lobes of the liver are bound together by a fold of pertitoneum.

Eight to ten weeks old mice were anaesthetized by ketamine/xylazine combination (90 mg/kg of ketamine and 10 mg/kg of xylazine) administered by intraperitoneal injections. The animals were placed on a heating pad to avoid the decrease of body temperature. A midline ventral abdominal skin and abdominal muscles incision was made, starting at just above the xiphoid cartilage and extending for approximately 2 cm. The suspensory ligament attaching the liver to the diaphragm was cut with blunt ended

scissors. Each individual lobe was raised vertically and ligated at the base of lobe. Every lobe was separately cut down as near as possible to blood vessels using the blunt-ended scissors. Afterwards, the muscle and skin incision were closed. No special therapy is required other than a standard postoperative care.

2.3.8 Histology and staining procedures

2.3.8.1 Preparation of paraffin sections

Animals were anaesthetized and heart perfusion was performed in order to flush the blood form the liver and other organs. Livers were dissected in PBS and fixed with 4% PFA at 4°C overnight. After extensive washing with cold PBS, the organs were dehydrated at room temperature in an ethanol series: 50%, 80%, 96%, 100% for three hours each step After dehydration, it is necessary to replace the ethanol with an agent miscible with paraffin, therefore livers were incubated in xylene two times for one hour. Then the livers were incubated in paraffin (Roti-Plast, Roth, Karlsruhe) for several hours at 56^0C; this step was repeated two times with fresh batches of paraffin. The organs were then embedded into cassettes at room temperature and the resulting solid molds were used for sectioning. The 5-7 µM sections were spread out onto glass slides and left at 40^0C for 6 hours. The paraffin sections were then stained with hematoxylin and eosin (H&E), as well as used for Ki67 antibody and TUNEL staining.

2.3.8.2 Preparation of methacrylate sections

For histological analysis dehydrated livers were also embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim), which is a cold-polymerizing resin. Therefore, livers (5 mm pieces) were fixed for 16 h in 4%PFA/PBS, washed in PBS and dehydrated in series of graded alcohols. Livers were incubated in Technovit 7100/100% Ethanol (1:1) for 4-6 h at RT followed by overnight pre-infiltration in Technovit 7100. Afterwards, the tissue was incubated in infiltration solution (1g of hardener I/100ml Technovit 7100) for 2 h up to 2 days at 4^0C. Livers were embedded in infiltration solution/hardener II (15:1), which was degassed shortly in vacuum chamber. After

overnight polymerization, the blocks were mounted with Technovit 3040 and stored at RT until sectioning. 4-5 µm semi- thin sections were cut using Microm HM360 (Walldorf), dropped into a warm water-bath for spreading and collected onto slides (Roth, Karlsruhe). After drying sections were stained with hematoxilin/eosin as described below.

2.3.8.3 Hematoxylin/eosin (H&E) staining on paraffin sections

Delafield's hematoxilin solution (Prudden, 1885; from Clark, „Staining Procedures", Williams & Wilkins, Baltimore, MD, USA, 1981) was prepared by dissolving 4 g of hematoxylin in 25 ml 95% ethyl alcohol and 40g/400ml $NH_4Al(SO_4)_2 \cdot 12\ H_2O$. After one-week exposition to air and light, the solution was filtered and mixed with 100 ml of glycerin and 100 ml of methyl alcohol. Then the solution was exposed to light until it becomes dark (6-8 weeks). Directly before use, the hematoylin solution was diluted with an equal volume of distilled water.

For H&E staining slides were dewaxed by three incubations in xylene for 10 min. Afterwards, slides were hydrated by 5 min washes in a series of ethanol (100%, 95%, 70%, 50%, 30%) and washed for 2 min in distilled water. Such prepared paraffin section as well as methacrylate sections were stained with hematoxylin for 10-15 min and washed in tap water for 10 min to allow differentiation. Afterwards, the slides were washed in 0.1% $NaHCO_3$ for 1-2 min and stained with freshly prepared eosin (0.25% eosin Y, 0.1 M acetic acid) for 5-8 min. After washing (first in tap water and then in distilled water), sections were rapidly dehydrated in ethanol series. Finally the sections were washed three times for 5 min in xylene, mounted in "Entellan" (Merck, Darmstadt) and covered with cover slips.

2.3.8.4 Preparation of frozen sections

Immunohistochemical analysis and lipid-specific staining were performed on frozen sections. Livers were cut into 5 mm cubes and fixed with freshly prepared 4% PFA/PBS for 2 h at room temperature. Afterwards, the tissue was washed extensively at 4^0C with PBS for several hours and cryo-protected by incubation in 20% sucrose/PBS overnight. At the next day pieces of liver were embedded in "TissueTek" (= „OCT-Compound";

Sakura, Zoeterwoude, Nederland) and 12 µm sections were cut on a cryostat (Microm HM560, Walldorf). Sections were collected onto slides (Histobond, Marienfeld) and dried at 37^0C for 2 hours. Slides as well as "TissueTek" blocks can be stored up to 6 month at -80^0C.

2.3.8.5 Lipid specific staining

Staining with oil-soluble dyes is based on the greater solubility of the dye, Oil Red O (Sigma), in the lipoid substances than in the usual hydroalcoholic dye solvents. Oil Red O was dissolved in propylene glycol to obtain 0.7% a concentration. The solution was heated to 100^0C, for a few minutes with constant stirring. After cooling down to room temperature, Oil Red O solution was filtered through Whatman #2 paper filter and stored at 60^0C. Slides were incubated in 85% propylene glycol two times for 5 min and then sections were stained in Oil Red O solution 7-10 min. Non-specific staining was removed by washing with distilled water and sections were mount with aqueous mounting media, like glycerin jelly. Glycerin jelly was prepared by dissolving with heat 10 g of gelatin in 60 ml distilled water. Subsequently the solution was mixed with 70 ml of glycerin and 1 ml of phenol.

2.3.8.6 Immunohistochemistry

Tissue sections were thawed at room temperature for 2 h and then washed shortly with PBS to remove the "TissueTek". Unspecific binding of antibodies was blocked by incubation with 10% inactivated horse serum/PBT (HS/PBT) for 1-2 h at RT. Afterwards, slides were incubated with the primary antibody diluted in 10% HS/PBT O/N at 4°C with rocking or alternatively the incubation was performed at 37^0C for 1 h. The sections were washed 4 times with PBT for 10 min to remove unbound antibodies, and then the sections were incubated with Cy2 or Cy3-conjugated secondary antibodies (diluted in 10% HS/PBT) for 1 h at room temperature. The same washing procedure as after primary antibodies was performed or if necessary washes were repeated more than 4 times. Nuclei were visualized by addition of the DNA specific dye DAPI to the secondary antibodies solution. Finally, slides were covered with "Immunomount" (Shandon, Frankfurt).

2.3.8.7 Detection of cell proliferation and apoptosis

To detect hepatocyte proliferation animals were injected intraperitoneally with 75 µg of BrdU (5-Bromo-2'-deoxy-uridine) per gram of body weight at the indicated time after partial hepatectomy. BrdU is a thymidine analog and is incorporated into DNA only in mitotically active cells and can be detected using anti-BrdU antibodies. After 1 hour of chasing time, livers were perfused with PBS and processed as described for preparation of frozen sections. Sections were postfixed in 4% PFA for 15 min at room temperature and then washed with PBS three times for 10 min. DNA was denaturated by incubation in 2.4 M HCl for 30 min at 37^0C. Afterwards sections were washed as above and incubated with 20 mg/ml proteinase K (Roche, Mannheim) in PBS at room temperature for 10 min to ensure good penetration of the antibody. After extensive washing, sections were blocked and immunohistochemistry was performed as described above.

Extensive DNA degradation occurs very often during early stages of apoptosis. Therefore, apoptosis was detected by terminal deoxynucleotidyle transferase nick-end labeling (TUNEL; Gavrieli et al., 1992). During the TUNEL assay blunt ends of double stranded DNA breaks are enzymatically labeled with flourescin. The 3-end labeling of DNA breaks was performed using an '*In situ* Cell Death Detection Kit, Flourescein' (Roche, Mannheim) with minor modifications. Before the procedure, the specimens were heated at 60°C for 1 hour. After deparaffinization in xylene and rehydration through graded ethanol series, the sections were incubated with 20 mg/ml proteinase K (Roche, Mannheim) in PBS at room temperature for 20 min. Then the slides were processed according to manufacturer instructions.

2.3.9 Protein biochemistry

2.3.9.1 Extraction of total protein

Proteins were extracted from liver of control and mutant mice killed at the indicated time after partial hepatectomy. The right lobe (1-2mg) of the liver was homogenized with a dounce homogenizator in ice-cold 2x RIPA buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, 1% Na-deoxycholate, 2% NP-40, 2 mM sodium orthovanadate and 2 mM NaF) in the presences of protease inhibitors cocktail (Roche

Diagnostic, Mannheim). Lysates were clarified by ultra-centrifugation for 45 min at 60,000 rpm and supernatants containing proteins were aliquoted, snap-frozen in liquid nitrogen and stored at -80^0C. All steps were carried out at 4^0C temperature.

2.3.9.2 Quantification of proteins by the Bradford Assay

The Bradford method is a colorimetric method based on the binding of Coomassie brilliant blue to unknown protein. The measured absorbance of the unknown protein is then compared with a bovine serum albumin (BSA) standard also stained with Coomasie brilliant blue. The absorbance maximum of the Coomassie Brilliant Blue G-250 dye shifts when it binds to protein (Bradford, 1976). The quantification of proteins in extracts was performed using the Bio-Rad reagents according to manufactures instruction. Briefly, different concentrations of BSA (0.3 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml and 4 mg/ml) were used as standards. Protein samples (1ml) were mixed with 1 ml of diluted dye reagent and incubated at room temperature for 2 min and then absorbance was measured at 595 nm. The protein concentration of a sample is deduced from the standard BSA curve.

2.3.9.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein electrophoresis was carried out in polyacrylamide gels under conditions that ensure dissociation of proteins. During electrophoresis the proteins first enter into a short stacking gel to sharpen protein bands into thin zones before proteins enter the separating gel. The concentration of the separating gel depends on size of the protein of interest. The electrophoresis was carried out in a discontinuous buffer containing the nonionic detergent, SDS.

Separating Gel:

	Final acrylamide concentration		
	8%	10%	12.5%
30% acrylamide/0,8% bis-acrylamide	1.6 ml	2 ml	2.5 ml
glycerol	0.5g	0.5g	0.5g
2x resolving buffer	3 ml	3 ml	3 ml
H_2O	0.9 ml	0.5 ml	null

10% ammonium persulfate	60 µl	60 µl	60 µl
TEMED	4 µl	4 µl	4 µl

Resolving buffer was composed from 0.2% SDS, 4 mM Na_4EDTA, and 0.75 M Tris-HCl pH 8.9

Stacking gel:
In a flask 0.4 ml of 30% acrylamide/0,8% bisacrylamide, 1.5 ml of 2x Stacking buffer (0.25 M Tris-HCl, pH 6.7, 4 mM EDTA, 0.2% SDS) and 1.05 ml of H_2O was mixed. Prior to pouring 30 µl of 10% ammonium persulphate and 2 µl of TEMED were added.

Prior to loading the protein samples (40-60 µg) were diluted 1:2 with 2x Laemmli SDS sample buffer (2% 2-mercaptoethanol, 0.2 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.004% Bromophenol Blue) and heated 5 min at 100°C.
The electrophoresis was performed in 1x running buffer (made up from a 4x stock of 0.2 M Tris-HCl, 1.52 M glycine, 0.4% SDS, 8 mM EDTA) for 4-5 hours at 30 mA constant current.

2.3.9.4 Staining SDS-Polyacrylamide Gels with Coomassie Blue

After electrophoresis, Coomassie Blue staining was used to detect proteins. The gel was immersed in 5 volumes of Coomassie Blue staining solution (0.25% Coomassie blue brilliant R250, 50% methanol, 10% acetic acid) and incubated at room temperature for 4 h with gentle agitation. To remove the excessive stain a destaining solution (30% methanol. 10% acetic acid) was used. The gel was soaked in this solution for 4-8 hours while slowly rocking.

2.3.9.5 Western blotting

Proteins were transferred from the gel onto membranes (nitrocellulose or nylon) by a wet-transfer method. After separation of the proteins, the gel and the nitrocellulose or nylon PVDF membrane was prewetted in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.1%SDS) for 5 min. In case of PVDF membranes, they were first activated for 15 sec in 100% methanol. Then the gel was placed between the prewetted

membrane with two prewetted Whatman papers underneath and two further Whatman papers were placed underneath the memebrane. The transfer was performed at 200 mA constant current for 2 hours in cold room (Biorad, Model 200/2.0). Afterwards, the membranes were washed three times with water and the transfer efficiency was determined by Ponceau staining (2% Ponceau, 1% acetic acid in distilled water) for 2 min at room temperature with constant shaking. For further processing, the membranes were destained with water for 20 min with agitation.

2.3.9.6 Immunodetection and ECL

Non-specific binding sites on membranes were blocked for 1 h at room temperature in blocking solution (5% (w/v) skimmed milk powder in PBS, 0.05% Tween-20). The primary antibodies were diluted in blocking solution and incubated with the membrane for 2-3 hours at RT or overnight at 40C. After washing in PBT (1x PBS, 0,05% Tween 20) four times for 10 min, the horseradish peroxidase-conjugated secondary antibodies diluted in blocking solution were applied for 45 min. Then the membranes were washed 4 times for 10 min in PBT.

For visualization of immuno-reactive bands the chemiluminescent detecting ECL reagent was used according to manufacturer's instructions (Amersham Biosciences, Freiburg). Briefly, detection solution was applied to the membrane to cover it evenly. After one-minute incubation, excess solution was drained from the membrane, and the membrane was placed on a flat sheet of Saran Wrap. The edges of the wrap were folded over the backside of the membrane to seal it. The membrane was then exposed to a Kodak X-ray film for varying lengths of time.

2.3.9.7 Auto-kinase assay

The activity of Met protein was measured by in vitro auto-kinase assay. For this the Met protein was immuno-precipitated and incubated with radioactively labeled ATP. The phosphorylated Met was then identified by SDS-PAGE and autoradiography.

Liver tissue (approx. 5 mg) was homogenized with 5-10 strokes of dounce in ice-cold lysis buffer (50 mM Hepes, pH 7.5, 50 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% (w/v) TritonX-100 including freshly added proteinase and phosphatase inhibitors like in RIPA buffer). Lysates were incubated for 5 min on ice and then

cleared by centrifugation at 13,000g at 40C for 20-30 min. The supernatant was transferred to a new tube and incubated with the primary antibody (rabbit anti-mouse Met) overnight at 40C with gentle agitation. Afterwards, protein G-Sepharose was added (75 µl per 1000 µl of sample) and the mixture was incubated by rotary mixing for 60-90 min at 4^0C. Protein-G-Sepharose immuno-complexes were seperated by centrifugation for 10 sec at 13,000g and were washed three times with cold wash buffer (50 mM Hepes, pH 7.5 and 50 mM NaCl). The pellets were resuspended in 15 ml kinase-buffer (50 mM Hepes, pH 7.5, 5 mM MnCl2, 0.1% TritonX-100) together with 2 µl of ^{32}P-γ-ATP for 30 min at room temperature. The reaction was stopped by boiling in Laemmli-buffer and standard SDS-PAGE electrophoresis was performed. Afterwards, the gel was dried and exposed to autoradiographic film (Kodak).

2.3.9.8 Enzyme Linked Immunosorbent Assay (ELISA)

The concentration of growth factors and cytokines in the blood stream was measured by an ELISA assay. IL-6 and TNF-a ELISA's were performed using commercial kits (PharMingen) according to the manufacturers instructions. For HGF/SF ELISA a mouse recombinant HGF/SF protein and polyclonal anti-HGF/SF antibodies were used, those were a kind gift from Ermanno Gherardi. Blood was collected from the portal vein of anesthetized animals at indicated time points. After blood clotting the serum was collected and stored frozen.

The ELISA was performed in 96-well plates (Nunc), which were coated with specific antibodies (4mg/ml in 0.1 M sodium carbonate, pH 9.5) for 2-3 h at room temperature. The plates were then washed three times with PBS/0.05% Tween20 and after the last wash the residual buffer was carefully removed. Wells were then blocked with 10% fetal calf serum (heat inactivated) in PBS for 1 h at room temperature and afterwards, wells were washed as described above. Standards and samples were diluted in blocking solution and incubated in wells for 2 h at room temperature. The plates were washed five times with 300 µl of PBS/0.05% Tween20 per well. Then the biotinylated HGF/SF antibodies (2 mg/ml) were added to the wells and were incubated for 1 h at room temperature. Biotinylation of the antibodies was performed using kit according to the manufactures instruction (Amersham Biosciences, Freiburg). Unbound antibodies were aspirated and the wells were washed ten times for 2 min each with PBS/0.05%

Tween20 and a dilution 1:1000 of avidin-horse-raddish-peroxidase conjugate (Sigma) in blocking buffer was added to each well and incubated for 30 min. After extensive washing, the color was developed using tetramethylbenzidine substrate (TMB). Reaction was stopped after 30 min by addition of 50 µl of 2 M H_2SO_4 to each well and absorbance was measured at 450 nm. The assay was repeated at least three times for each sample and a mean± standard deviation was calculated.