Supporting Materials and Methods

Cell culture

The human hepatoma cell lines 3p, 3sp, FLC4, SNU398, SNU423, SNU449 and SNU475 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS). PLC/PRF/5, HLE, HLF and HuH7 cells were propagated in DMEM plus 10% FCS. HepG2 and SKHep1 were cultivated in EMEM with 10% FCS. HuH6 received RPMI-1640 plus 4% FCS. Human hepatic sinusoidal endothelial cells (HSECs) were cultured in endothelial cell medium (Lonza, Basel, Switzerland). All cells were kept at 37°C and 5% CO_2 and routinely screened for the absence of mycoplasma. All hepatoma cells were validated by short tandem repeat analysis.

Expression profiling using microarrays

Expression profiling of human 3p and 3sp hepatoma cells was performed by hybridization of labeled RNA on Affymetrix Gene-Chip Human gene 1.0 ST Arrays (Affymetrix, Santa Clara, USA) as described (1). After calculating the ratio of regulation, the data were analyzed by gene set enrichment analysis software (2). Complete gene expression data have been deposited in National Center for Biotechnology Information's Gene Expression Omni-bus and are accessible by GEO Series accession no. GSE26391.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

RNA was extracted, treated with DNaseI and reverse transcribed using a RNA isolation and cDNA synthesis kit (Quiagen, Hilden, Germany) as recommended by the manufacturer. Aliquots of cDNA were employed for Fast SYBR green qPCR (Applied Biosystems, Foster City, USA)

and quantified with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) as described (3). Primer sequences are shown in Supporting Table 3.

Western blot analysis

Immunoblotting was performed as described (3). The primary anti-Axl (R&D Systems, Minneapolis, USA), anti-phospho ERK (Cell Signaling Technology, Danvers, USA), anti-ERK (Cell Signaling Technology), anti-phospho Akt (Cell Signaling Technology), anti-Akt (BD Biosciences, NJ, USA), anti-phospho JNK (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-phospho Smad2 (Upstate, NY, USA), anti-Smad3L(Ser213) (Abcam, Cambridge, UK), anti-Smad2/3 (BD Biosciences, NJ, USA) and anti-actin (Sigma, St. Louis, USA) antibodies were used at dilutions of 1:1.000.

Immunofluorescence analysis

3p and 3sp cells were seeded onto collagen-coated glass slides at a confluency of 80 %. Cells were subsequently washed with phosphate-buffered saline (PBS), permeabilized with 0.05 % Triton X-100 and fixed with 4% paraformaldehyde. Following incubation with an anti-Axl antibody (R&D Systems, Minneapolis, USA) diluted 1:100 and a corresponding secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, USA), Axl was localized by confocal immunofluorescence microscopy (Zeiss, Oberkochen, Germany).

Analysis of phospho-RTKs

Human epithelial 3p and mesenchymal 3sp hepatoma cells were analyzed for the activation of RTKs by using the Proteome Profiler array ARY001 as recommended by the manufacturer (R&D

Systems, Minneapolis, USA). The phospho-RTK array contained 49 different RTK antibodies spotted in duplicates.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs for intracellular Axl (R&D Systems, Minneapolis, USA) or secreted TGF-β1 (eBioscience, San Diego, USA) were carried out according to manufacturer's protocols. Briefly, levels of intracellular Axl were assessed in whole-cell extracts from human hepatoma cells. For TGF-β1, cell supernatants were collected after cultivation in serum-free medium for 24 hours. A seven-point standard curve was generated for every plate and quantification was performed using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, USA). All samples were analyzed in triplicates.

Immunoprecipitation (IP)

IPs were performed with Dynabeads® magnetic beads (Life Technologies, Green Island, USA) according to the manufacturer's protocol. Briefly, 5 µg of anti-Axl antibody (R&D Systems, USA) was added to total protein extracted from 5 x 10^6 cells and incubated for 4 hours at 4°C. Subsequently, 50 µL of protein G- or protein A-coated beads (Life Technologies) were added and incubated for 1 hour at 4°C. The IP complexes were washed with PBS and eluted at 95°C for 5 minutes in 40 µL elution buffer containing 100 mM Tris/HCl pH 7.4, 5% SDS, 70 mM β-mercaptoethanol and 5 mM DTT.

Mass spectrometry analyses of IPs

A shotgun proteomics approach was used for protein identification consisting of gel-electrophoretic protein separation, in-gel enzymatic digestion, peptide separation by nano-HPLC

and on-line mass spectrometric peptide sequencing. In-gel protein digests were performed after SDS-PAGE separations of IPs as described (4). After tryptic digestion, dried peptides were re-solubilized and pre-concentrated on a trap column (Thermo/Dionex; C18 PepMap 100; Thermo Fisher Scientific, Bremen, Germany) of the nano-HPLC system (Ultimate 3000, Dionex; Thermo Fisher Scientific) and peptides were separated using a linear gradient. Positive ion mode and a spray voltage of 2 kV were employed. The mass spectrometer (LTQ-Orbitrap; Thermo Electron; Bremen, Germany) operated in data-dependent acquisition mode (5). Peptide mass spectra were evaluated using BioWorks 3.3.1 and UniProt database.

Knock-down by RNA interference and ectopic expression

Small interfering RNA (siRNA; Dharmacon, Piscataway, USA) was used at a concentration of 50 nM for the knock-down of Axl, 14-3-3ζ or JNK. siRNA was delivered via Oligofectamine (Life Technologies, Green Island, USA) according to the manufacturer's protocol. Briefly, cells were cultured at a confluency of 50% and incubated with transfection reagent in serum-free medium overnight. Experiments were conducted 48 hours post transfection and knock-down efficiency was verified by Western blotting. For stable overexpression, human Axl cDNA was cloned into the pWPI-GFP lentiviral vector and transmitted into 3sp and PLC/PRF/5 hepatoma cells. pWPI-GFP lentiviral particle expressing green fluorescent protein (GFP) were transmitted as control.

Cell migration

2.5×10^4 hepatoma cells were seeded in 100 µL serum-free cell culture medium onto 24-well cell culture inserts with a pore size of 8 µm (Corning, Tewksbury, USA). Cell culture medium supplemented with either 10% FCS, Gas6 (500 ng/mL; R&D Systems, Minneapolis, USA) or LY2109761 (10 µM; Santa Cruz, Dallas, USA) was added to bottoms of 24-well plates. After 18

hours of migration, inserts were removed and cells on membranes were fixed with 4% paraformaldehyde. Cell nuclei of migrated cells at the bottom side of the membrane were stained with Hoechst dye (Life Technologies, Green Island, USA) and counted under the fluorescence microscope (Nikon, Tokyo, Japan).

Transendothelial invasion

24-well cell culture inserts with a pore size of 8 µm (Corning, Tewksbury, USA) were coated with rat-tail collagen (50 µg/mL; BD Biosciences, NJ, USA) for 4 hours. 2 x 10^5 HSECs were seeded in 100 µL endothelial cell medium (Lonza, Basel, Switzerland) onto coated 24-well cell culture inserts and allowed to form a monolayer for 48 hours. Subsequently, endothelial cell medium was aspirated and 5 x 10^4 hepatoma cells stained with green CellTracker (Life Technologies, Green Island, USA) were seeded in 100 µL serum-free medium onto 24-well inserts. Serum-free medium containing 500 ng/mL Gas6 (R&D Systems, Minneapolis, USA) or LY2109761 (10 µM; Santa Cruz, Dallas, USA) was added to bottoms of 24-well plates. After 18 hours of incubation at 37°C and 5% CO_2, transmigrated cells were visualized and quantified as described for cell invasion.

Proliferation kinetics

1 x 10^4 cells were seeded in triplicate onto 24-well plates and cell numbers of the corresponding populations were determined after 1, 3, 5 and 7 days in a multichannel cell analyzer (CASY; Schärfe Systems, Germany).

TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

To assess cell death, TUNEL assays were conducted according to the manufacturer's protocol (Roche Diagnostics, Rotkreuz, Switzerland). Briefly, 3sp heptoma cells were seeded on collagen-coated glass slides and treated with 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, USA) for 24 hours. Cells were subsequently washed with PBS and incubated with TUNEL reaction mixture for 60 minutes at 37°C. TUNEL-positive cells were quantified by fluorescence microscopy.

Cell cycle analysis by propidium iodide (PI) staining and flow cytometry

After incubation with 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, USA) for 24 hours, 1 x 10^6 3sp or 3spAxl cells were harvested and stained with PI at 10 µg/mL for 30 min at 4°C. DNA content was subsequently determined by flow cytometry (Becton Dickinson, Franklin Lakes, USA) and cell cycle cycle analysis was performed using the ModFit LT software (Verity Software House, Topsham, USA).

Analysis of TGF-β signaling by phospho-specific antibody microarray

Replicates of TGF-β signaling arrays, each comprising 176 phospho-specific antibodies, were processed according to the manufacturer's protocol (Full Moon BioSystems, Sunnyvale, USA). Briefly, whole-cell extracts were generated from 3sp cells transfected with siRNA against Axl or 14-3-3ζ or with non-target siRNA and either left untreated or treated with 500 ng/mL Gas6 for 15 minutes. All cell extracts were biotinylated in a Biotin/N,N.Dimethylformamide solution. Proteins were then conjugated to the antibody array and labeled using Cy3-streptavidin. Detection was performed with the Agilent G2565CA high-resolution microarray scanner (Agilent Technologies, Santa Clara, USA).

Tumor formation and assessment of metastasis

1×10^7 PLC/PRF/5-GFP (PLC-GFP) or PLC/PRF/5-Axl (PLC-Axl) cells were subcutaneously injected into the flanks of immmunodeficient NSG (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice. Tumor growth was monitored by estimating the tumor volume as described (6). To assess metastatic colonization, subcutaneous tumors derived from PLC-GFP or PLC-Axl cells were both grown to a size of 750 mm^3 and resected. Pulmonary metastasis was monitored 30 days post resection. Serial sections of lung tissue were immunohistochemically analyzed for GFP expression and metastatic colonies were quantified. All experiments were performed according to the Austrian guidelines for animal care and protection.

Statistical analysis

Data sets were compared using IBM SPSS software v20.0 (IBM Corp., Armonk, USA). Two-sided Student t-tests were used for continuous data and two-sided Fisher's exact tests or chi-squared tests for categorical data. Survival curves were compared with the Log-rank test. Univariate and multivariate survival and recurrence analyses were performed by Cox proportional hazard regression. *P values < 0.05, **P < 0.01 or ***P < 0.001 were considered statistically significant.

References to Supporting Materials and Methods

1. van Zijl F, Mall S, Machat G, Pirker C, Zeillinger R, Weinhaeusel A, Bilban M, et al. A human model of epithelial to mesenchymal transition to monitor drug efficacy in hepatocellular carcinoma progression. Mol Cancer Ther 2011;10:850-860.
2. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-15550.

3. Petz M, Them N, Huber H, Beug H, Mikulits W. La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. Nucleic Acids Res 2012;40:290-302.

4. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 2006;1:2856-2860.

5. Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, et al. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 2005;4:2010-2021.

6. Zulehner G, Mikula M, Schneller D, van Zijl F, Huber H, Sieghart W, Grasl-Kraupp B, et al. Nuclear beta-catenin induces an early liver progenitor phenotype in hepatocellular carcinoma and promotes tumor recurrence. Am J Pathol 2010;176:472-481.

Supporting Figure Legends

Supporting Fig. 1. Correlation of Axl expression as assessed by ELISA and cell migration through Transwell inserts in 14 hepatoma cell lines. R, Pearson correlation; ***, $p < 0.001$.

Supporting Fig. 2. Cell migration and transendothelial invasion as assessed by Transwell inserts. HCC cells after migration through Transwell membranes were visualized by Hoechst staining. Images were taken under UV light. (A) Cell migration. (B) Transendothelial invasion through HSECs.

Supporting Fig. 3. Proliferation of (A) 3sp and (B) Axl overexpressing 3sp cells (3spAxl) after siRNA knock-down of Axl or 14-3-3ζ. (C) Cell cycle distribution of 3sp and 3spAxl cells as determined by flow cytometry after incubation with TGF-β1 (10 ng/mL) for 24 hours. n.s., not significant; *, $p < 0.05$.

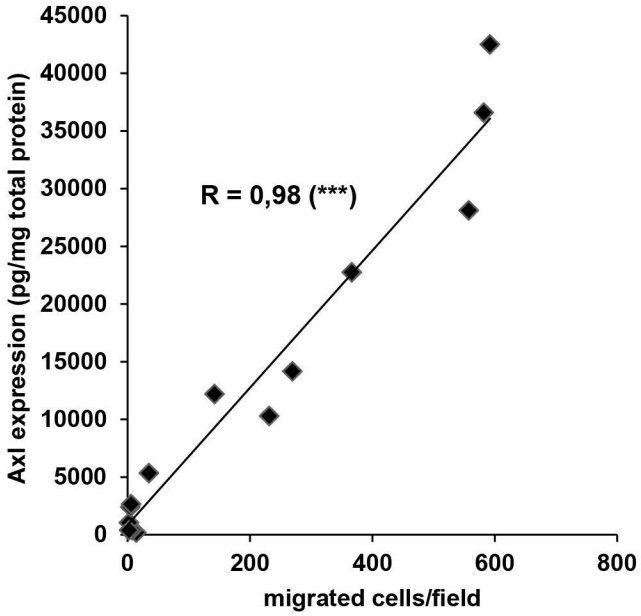
Supporting Fig. 4. (A) Cell migration and (B) transendothelial invasion after treatment of cells with the TGF-β inhibitor LY2109761 (10 μM) and stimulation with Gas6 (500 ng/mL) as assessed by Transwell inserts.

Supporting Fig. 5. Correlation of Axl expression with TGF-β1 secretion in epithelial and mesenchymal hepatoma cells. (A) 14 hepatoma cell lines analyzed for Axl expression and TGF-β1 secretion by ELISA. (B) R, Pearson correlation; ***, $p < 0.001$.

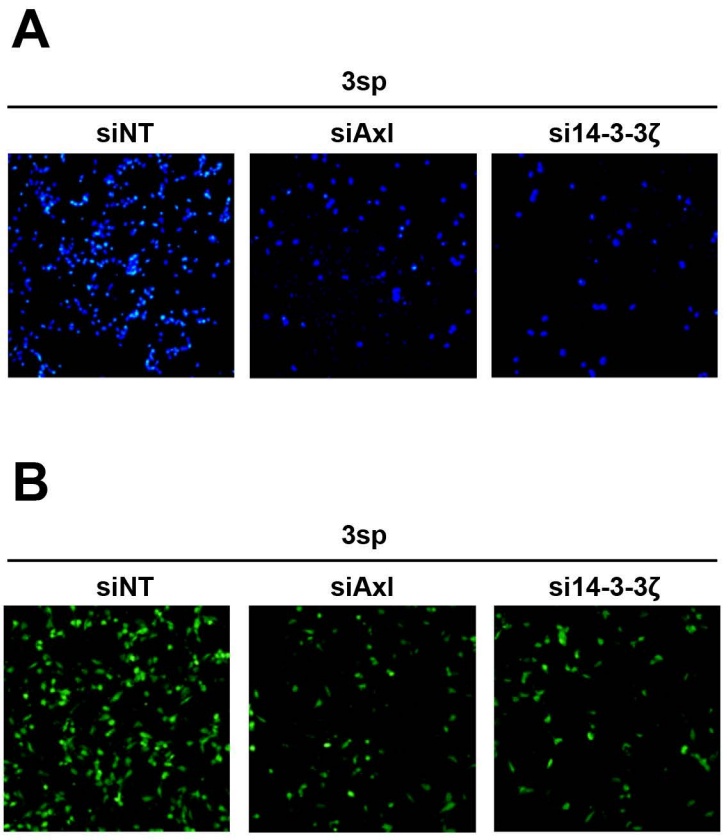
Supporting Fig. 6. Representative photomicrographs of HCC patient tissue samples immunohistochemically stained for (A) Axl, (B) 14-3-3ζ, (C) phospho-Smad3L or (D) TGF-β1.

Supporting Fig. 7. (A) Kaplan-Meier survival analysis of HCC patients expressing low, medium or high phospho-Smad3L (pSmad3L). Patients exhibiting no phosphorylation of Smad3L were omitted due to the low number (n = 8). Numbers in parentheses indicate median survival in months. (B) Distribution of TGF-β1 expression among HCC patients expressing no, low, medium or high Axl.

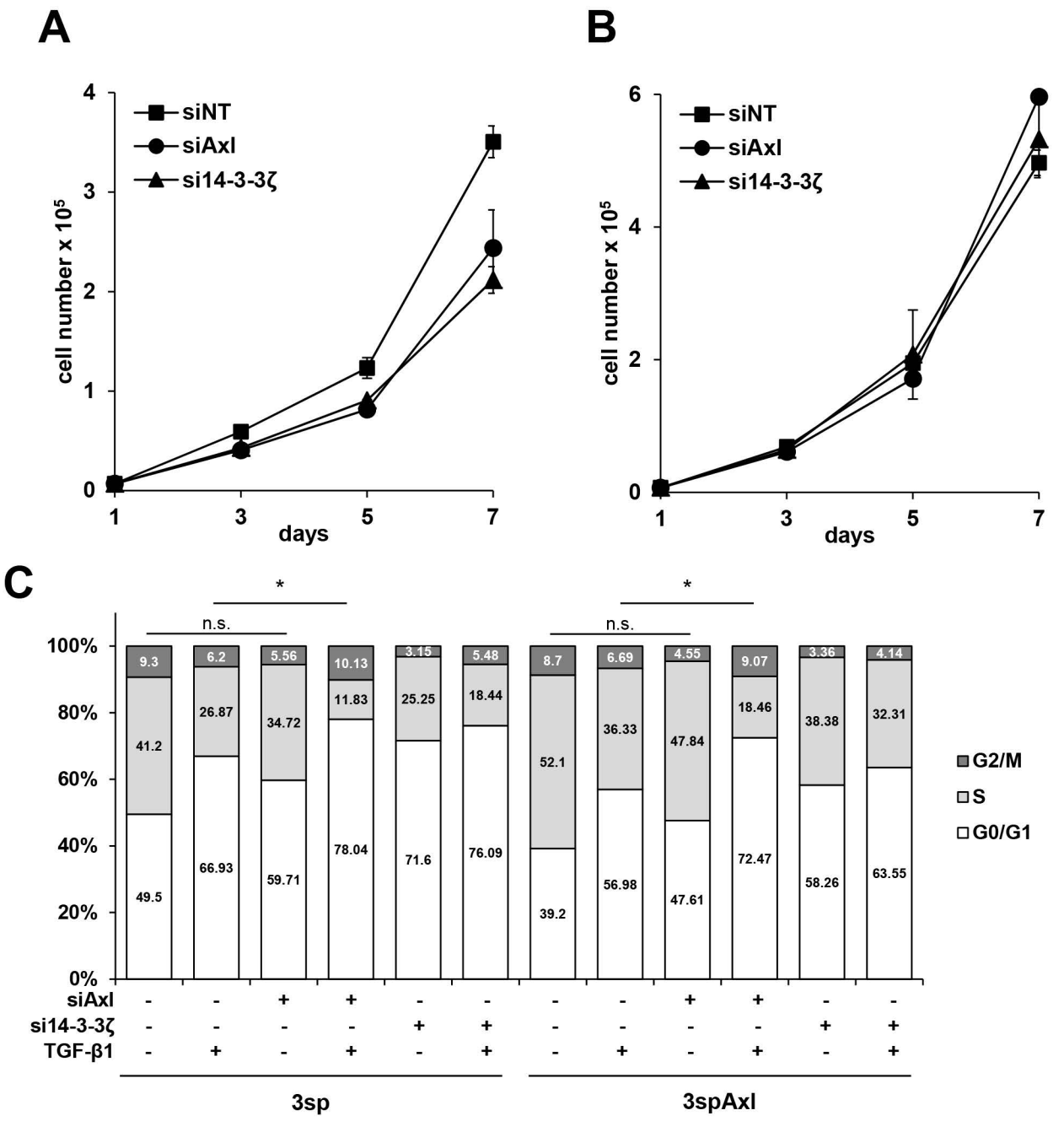
Reichl et al, Supporting Fig. 1



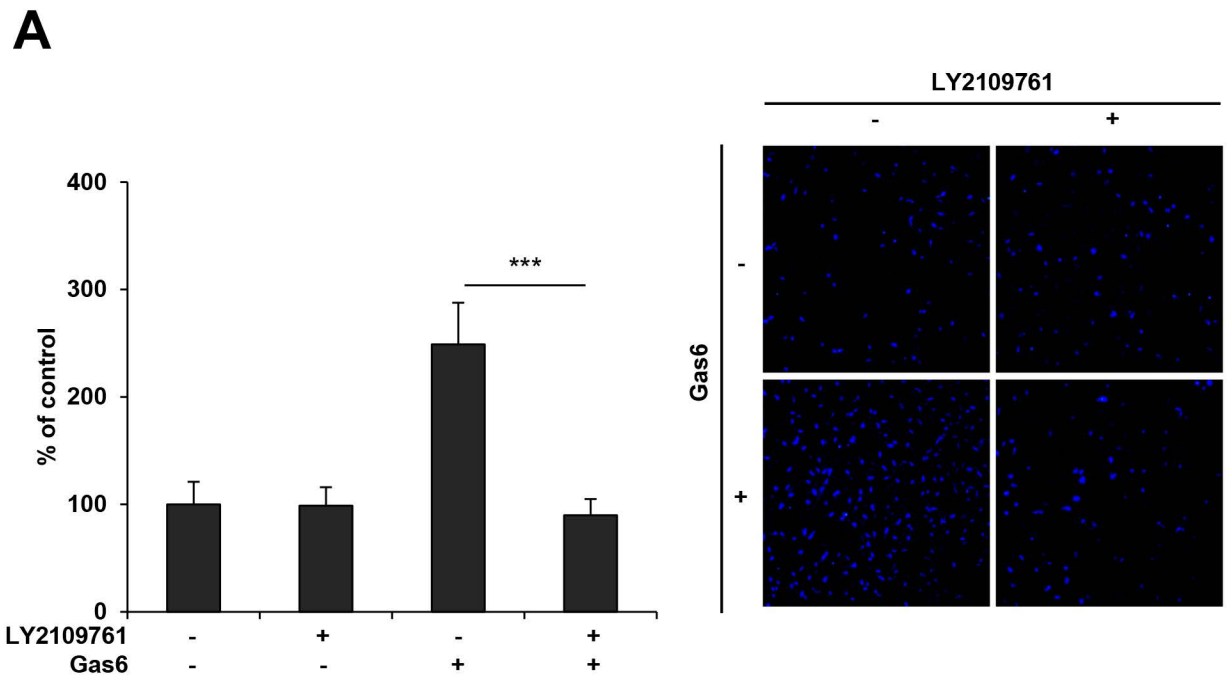
Reichl et al, Supporting Fig. 2

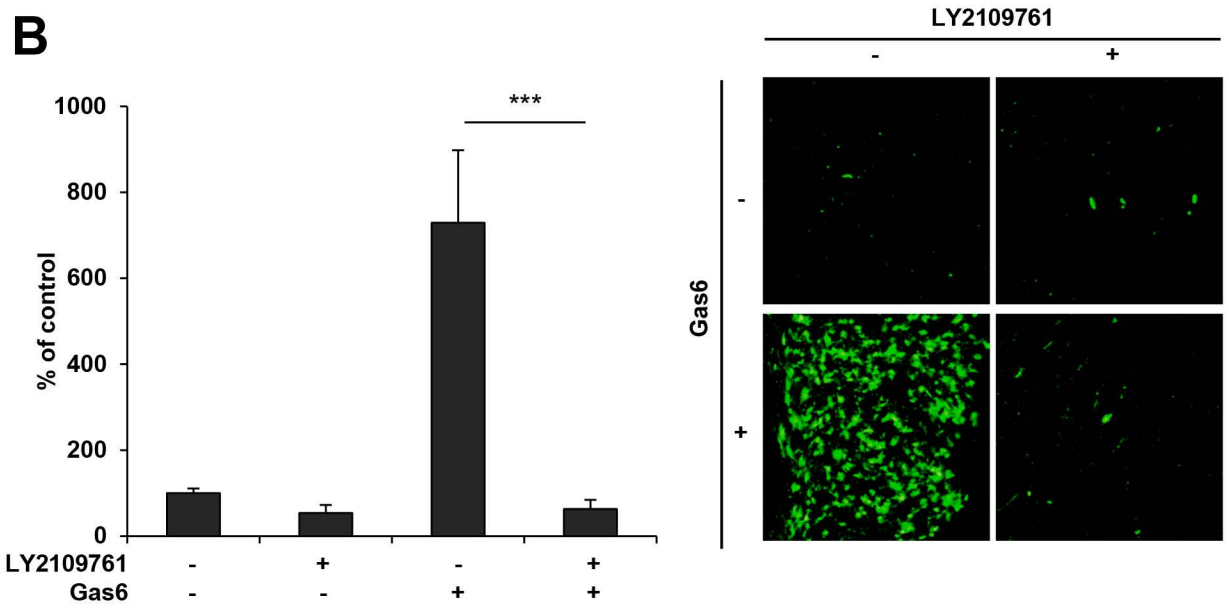


Reichl et al, Supporting Fig. 3

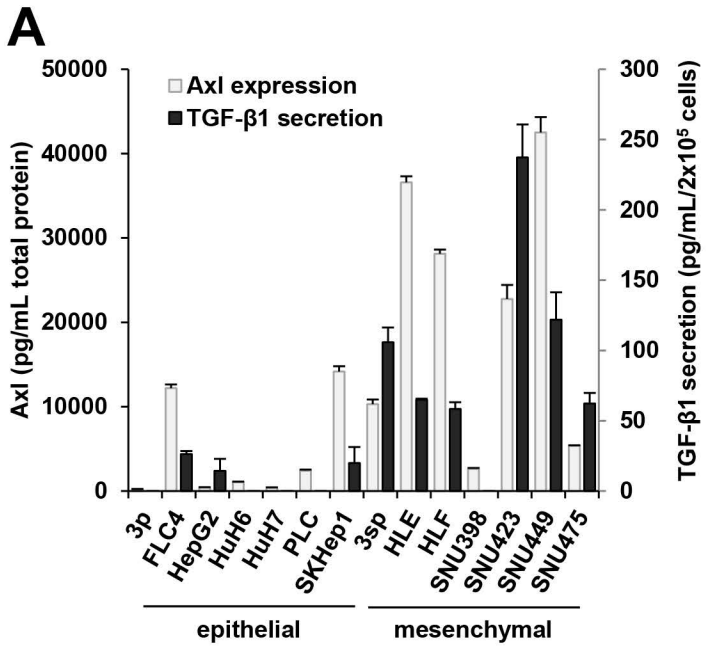


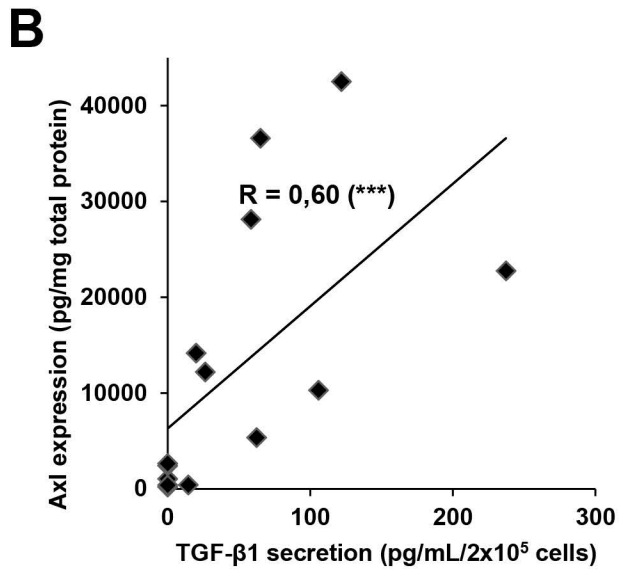
Reichl et al, Supporting Fig. 4



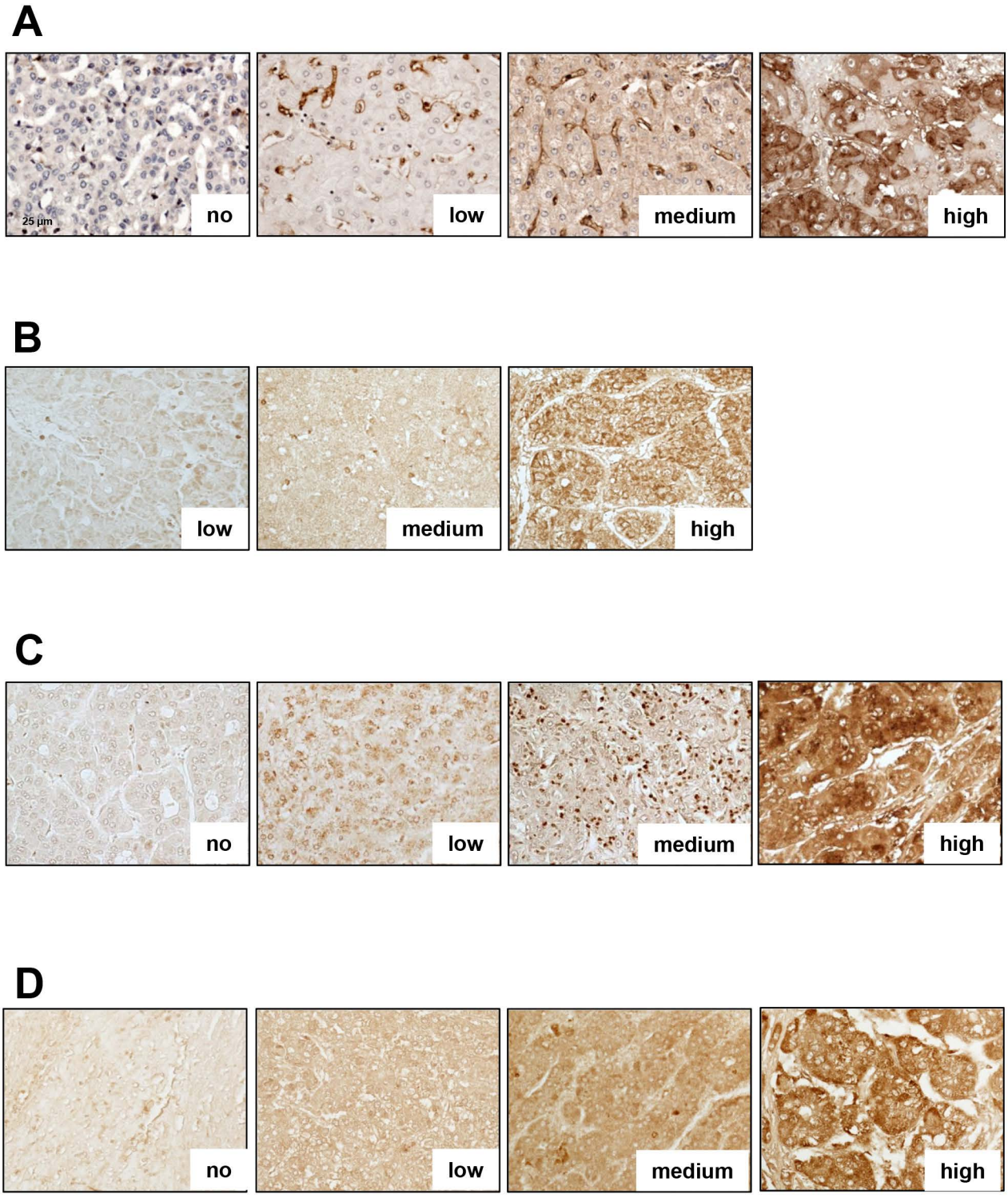


Reichl et al, Supporting Fig. 5

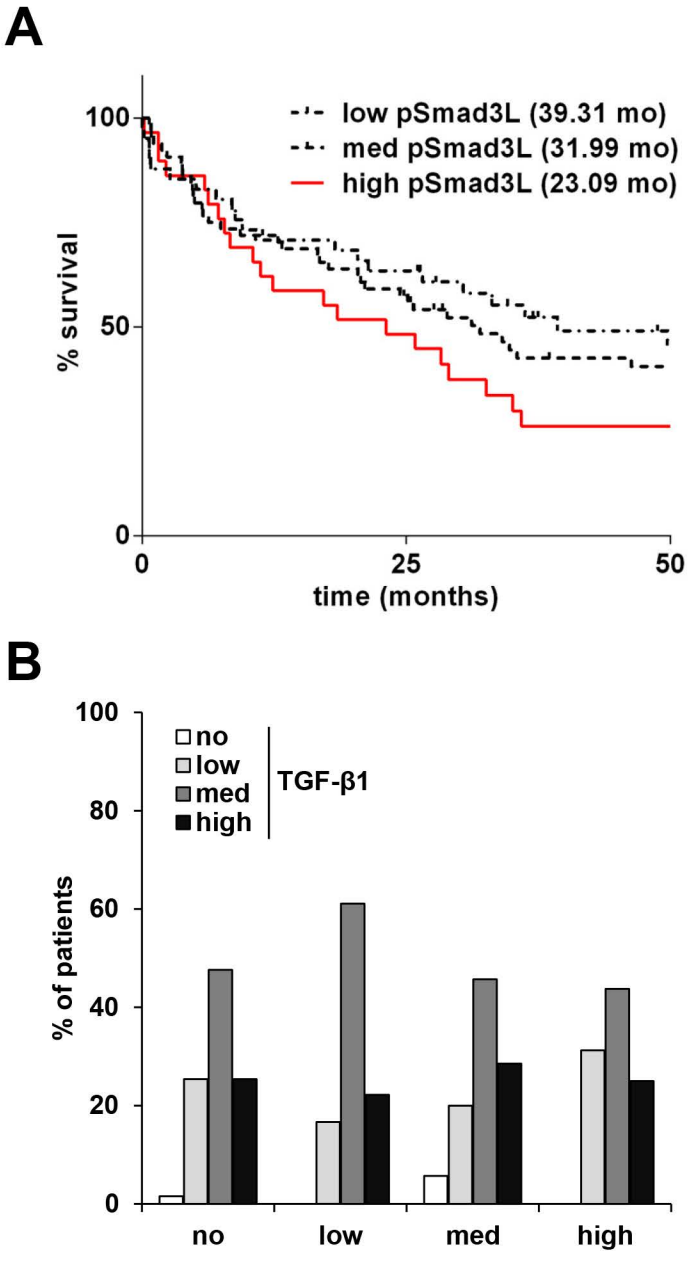




Reichl et al, Supporting Fig. 6



Reichl et al, Supporting Fig. 7



Reichl et al, Supporting Table 1

Supporting Table 1. Correlation of Axl expression with clinicopathological parameters

Variable	Number of cases	Axl		OR	CI 95 %	*p*
		Med/High	No/Low			
Age (years)						
< 56	76	35 (46.1 %)	41 (53.9 %)	0.514	0.252-1.051	0.077
≥ 56	59	18 (30.5 %)	41 (69.5 %)			
Gender						
Male	117	48 (41.0 %)	69 (59.0 %)	1.809	0.605-5.408	0.315
Female	18	5 (27.8 %)	13 (72.2 %)			
HBV status						
Negative	120	43 (35.8 %)	77 (64.2 %)	3.581	1.149-11.159	0.027*
Positive	15	10 (66.7 %)	5 (33.3 %)			
HCV status						
Negative	92	36 (39.1 %)	56 (60.9 %)	1.017	0.485-2.134	1.000
Positive	43	17 (39.5 %)	26 (60.5 %)			
Cirrhosis						
No	16	7 (43.8 %)	9 (56.2 %)	0.810	0.282-2.325	0.787
Yes	119	46 (38.7 %)	73 (61.3 %)			
Tumor stage > III						
No	63	17 (27.0 %)	46 (73.0 %)	2.706	1.313-5.576	0.008**
Yes	72	36 (50.0 %)	36 (50.0 %)			
Vascular invasion						
No	94	28 (29.8 %)	66 (70.2 %)	3.683	1.709-7.935	0.001***
Yes	41	25 (61.0 %)	16 (39.0 %)			
Lymph node metastasis						
No	129	51 (39.5 %)	78 (60.5 %)	0.765	0.135-4.329	1.000
Yes	6	2 (33.3 %)	4 (66.7 %)			

CI, confidence interval. OR, odds ratio. HBV, hepatitis B virus. HCV, hepatitis C virus.
***, $p < 0.001$. **, $p < 0.01$; *, $p < 0.05$,

Reichl et al, Supporting Table 2

Supporting Table 2. Correlation of 14-3-3ζ expression with clinicopathological parameters

Variable	Number of cases	14-3-3ζ		OR	CI 95 %	*p*
		High	Low/Med			
Age (years)						
< 56	77	42 (54.5 %)	35 (45.5 %)	0.877	0.467-1.649	0.748
≥ 56	78	40 (51.3 %)	38 (48.7 %)			
Gender						
Male	134	72 (53.7 %)	62 (46.3 %)	1.277	0.508-3.209	0.644
Female	21	10 (47.6 %)	11 (52.4 %)			
HBV status						
Negative	138	75 (54.3 %)	63 (45.7 %)	0.588	0.212-1.634	0.318
Positive	17	7 (41.2 %)	10 (58.8 %)			
HCV status						
Negative	110	56 (50.9 %)	54 (49.1 %)	1.320	0.655-2.657	0.481
Positive	45	26 (57.8 %)	19 (42.2 %)			
Cirrhosis						
No	19	11 (57.9 %)	8 (42.1 %)	0.794	0.301-2.097	0.807
Yes	136	71 (52.2 %)	65 (47.8 %)			
Tumor stage > III						
No	69	38 (55.1 %)	31 (44.9 %)	0.855	0.453-1.613	0.746
Yes	86	44 (51.2 %)	42 (48.8 %)			
Vascular invasion						
No	108	59 (54.6 %)	49 (45.4 %)	0.738	0.379-1.439	0.399
Yes	51	24 (47.1 %)	27 (52.9 %)			
Lymph node metastasis						
No	148	78 (52.7 %)	70 (47.3 %)	1.197	0.259-5.533	1.000
Yes	7	4 (57.1 %)	3 (42.9 %)			

CI, confidence interval. OR, odds ratio. HBV, hepatitis B virus. HCV, hepatitis C virus.

Reichl et al, Supporting Table 3

Supporting Table 1. qRT-PCR primer sequences

mRNA	Primer sequence	Product length (bp)
Axl		
F	5‘-CCGGCTGGCGTATCAAGGCC-3‘	199
R	5‘-TGGCTGTGCTTGCCCTGGG-3‘	
TGF-β1		
F	5‘-GCAGTACAGCAAGGTCCTG-3‘	107
R	5‘-CGTAGTACACGATGGGCAG-3‘	
PAI1		
F	5‘-TAGAAGCCTAATCAGCCCAC-3‘	116
R	5‘-GGATCCTGTACATGGCTGAC-3‘	
MMP9		
F	5‘-CTTCCAGTACCGAGAGAAAGC-3‘	83
R	5‘-CCACCTGGTTCAACTCACT-3‘	
SNAI		
F	5‘-GTAATGGCTGTCACTTGTCG-3‘	112
R	5‘-TGTAAACATCTTCCTCCCAGG-3‘	

F, forward. R, reverse