The role of embryonic stem cell-expressed RAS (ERAS) in the maintenance of quiescent hepatic stellate cells

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SUMMARY
Hepatic stellate cells (HSCs) were recently identified as liver-resident mesenchymal stem cells. HSCs are activated after liver injury and involved in pivotal processes such as liver development, immunoregulation, regeneration, and also fibrogenesis. To date, several studies reported candidate pathways that regulate the plasticity of HSCs during physiological and pathophysiological processes. Here we analyzed the expression changes and activity of the RAS family GTPases and thereby investigated the signaling networks of quiescent HSCs versus activated HSCs. For the first time, we report that embryonic stem cell-expressed RAS (ERAS) is specifically expressed in quiescent HSCs and down-regulated during HSC activation via promoter DNA methylation. Notably, in quiescent HSCs, the high level of ERAS protein correlates with the activation of AKT, STAT3, mTORC2, and HIPPO signaling pathways and inactivation of FOXO1 and YAP. Our data strongly indicate that in quiescent HSCs ERAS targets AKT via two distinct pathways driven by PI3Kα/δ and mTORC2, whereas in activated HSCs RAS signaling shifts to RAF-MEK-ERK. Thus, in contrast to the reported role of ERAS in tumor cells associated with cell proliferation, our findings indicate that ERAS is important to maintain quiescence in HSCs.

INTRODUCTION
Hepatic stellate cells (HSCs; also called Ito cells, lipocytes, fat storing cells, or perisinusoidal cells) contribute 5-8% of total liver-resident cells and are located between sinusoidal endothelial cells and hepatocytes in the space of Dissé (1,2). HSCs play pivotal roles in liver development, immunoregulation, regeneration, and pathology. They exhibit a remarkable plasticity in their phenotype, gene expression profile and cellular function (3). In healthy liver, HSCs remain in a quiescent state and store vitamin A mainly as retinyl palmitate in cytoplasmic membrane-coated vesicles. Moreover, HSCs typically express neural and mesodermal markers, i.e. glial fibrillary acidic protein (GFAP) and desmin. They possess characteristics of stem cells, like the expression of Wnt and NOTCH are required for developmental fate decisions. Activated HSCs display an expression profile highly reminiscent of mesenchymal stem cells (MSCs). Due to typical functions of MSCs such as differentiation into adipocytes and osteocytes as well as support of hematopoietic stem cells, HSCs were identified as liver-resident MSCs (4).

Following liver injury, HSCs become activated and exhibit properties of myofibroblast-like cells. During activation, HSCs release vitamin A, up-regulate various genes, including α-smooth muscle actin (α-SMA) and collagen type I, and down-regulate GFAP (2). Activated HSCs are multipotent cells and recent studies revealed a new aspect of HSCs plasticity, i.e. their differentiation into liver progenitor cells during liver regeneration (5,6). Physiologically, HSCs represent well-known extracellular matrix (ECM) producing cells. In some pathophysiological conditions, sustained activation of HSCs causes the accumulation of ECM in the liver and initiates liver diseases, such as fibrosis, cirrhosis and hepatocellular carcinoma. Therefore, it is worthwhile reconsidering the impact of different signaling pathways on HSC fate decisions in order to become able to modulate them so that activated HSCs contribute to liver regeneration but not fibrosis. To date, several growth factors (PDGF, TGFβ, IGF) and signaling pathways have been described to control HSC activation through effector pathways, including Wnt, Hedgehog, NOTCH, RAS-MAPK, PI3K-AKT, JAK-STAT3, and HIPPO-YAP (7-13). However, there is a need to further identify key players that orchestrate HSC activity and to find out how they control as positive and negative regulators HSC activation in response to liver injury. Among these pathways, RAS signaling is one of the earliest which was identified to play a role in HSC activation (14) and to act as a node of intracellular signal transduction networking. Therefore, RAS dependent signaling pathways were in the focus of the present study.

Small GTPases of the RAS family are involved in a variety of cellular processes ranging from intracellular metabolisms to proliferation, migration, and differentiation, as well as embryogenesis and normal development (15-17). RAS proteins respond to extracellular signals and transform them into intracellular responses through interaction with effector proteins. The activity of RAS proteins is highly
controlled through two sets of specific regulators with opposite functions, the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs) as activators and inactivators of RAS signaling, respectively (18). In the present study, we analyzed the expression profile of different RAS isoforms in HSCs and found embryonic stem cell-expressed RAS (ERAS) specifically expressed in quiescent HSCs. To date, ERAS expression has been reported in undifferentiated embryonic stem cells and in colorectal, pancreatic, breast, gastric, and neuroblastoma cancer cell lines (19-22). Recently, we demonstrated that ERAS represents a unique member of the RAS family with remarkable characteristics. The most profound features of ERAS include its GAP-insensitivity (i.e., constitutive activity), its unique N-terminus among all RAS isoforms, its distinct effector selection properties, and the posttranslational modification site at its C-terminus (23).

Here, we investigated in detail the expression, localization, and signaling network of ERAS in quiescent and culture-activated HSCs. During ex-vivo culture-induced activation of HSCs, the expression of ERAS was significantly down-regulated at the mRNA and protein level likely due to an increase in promoter DNA methylation. We examined possible interactions and signaling of ERAS via various RAS effectors in HSCs. We found that the PI3Kα/δ-AKT, mTORC2-AKT and RASSF5-HIPPO-YAP axis can be considered as downstream targets of ERAS in quiescent HSCs. In contrast, MRAS, RRAS and RAP2A, and also the RAS/RAF/MEK/ERK cascade may control proliferation and differentiation in activated HSCs.

MATERIAL AND METHODS

Cell isolation and culture—Male Wistar rats (500-600 g) were obtained from the local animal facility of the Heinrich Heine University (Düsseldorf, Germany). The livers were used for isolation of HSCs as described previously (24). Briefly, rat livers were enzymatically digested with collagenase H (Roche, Germany) and protease E (Merck, Germany) and proceed by density gradient centrifugation to obtain primary cultures of HSCs. Purified HSCs were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum and 50 units of penicillin/streptomycin (Gibco® Life Technologies). Other liver cells, such as parenchymal cells (PCs), Kupffer cells (KCs), and sinusoidal liver endothelial cells (SECs) were isolated and cultivated as described earlier (25). MDCKII and COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum. TurboFect transfection reagent (Life Technologies) was used to transfect MDCKII and COS-7 cells according to the manufacturer's protocol.

DNA methyltransferase and histone deacetylase inhibitor treatment—Primary rat HSCs at day 3 were treated with 10 µM 5-AZA-2’-deoxycytidine (Decitabine, Sigma #A3656), a specific DNA methyltransferase inhibitor, for successive 4 days. In parallel, rat HSCs were treated with 5 µM the histone deacetylase inhibitor, SAHA (suberoylanilide hydroxamic acid, Vorinostat, Cayman Chemicals #10009929) under the same conditions. The control cells were treated with DMSO only. Cells were lysed at day 8 for RNA isolation and qPCR analysis.

Reverse transcriptase polymerase chain reaction—Cells were disrupted by QIAzol lysis reagent (QIAGEN, Germany) and total RNA was extracted via RNaseasy plus kit (Qiagen, Germany) according to the manufacturer’s protocol. The quality and quantity of isolated RNA samples were analyzed on 1% agarose gels and using a Nanodrop spectrophotometer, respectively. Possible genomic DNA contaminations were removed using the DNA-free™ DNA Removal Kit (Ambion, Life Technologies, Germany). DNase-treated RNA was transcribed into complementary DNA (cDNA) using the ImProm-II™ reverse transcription system (Promega, Germany). Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) was performed using TaqMan probes or SYBR Green reagent (Life Technologies, Germany). Probes/primers used for qPCR reaction in Taqman system, including Rn02098893_s1 for ERAS and Rn01527840_m1 for HPRT1, were purchased from Applied Biosystems (Life Technologies). Primer sequences are listed in Supplementary Table S1. The 2-△△Ct method was employed for estimating the relative mRNA expression levels and 2-△Ct for mRNA levels. HPRT1 was used for normalization.

Immunostaining—Immunostaining was performed as described previously (23). Briefly, cells were washed twice with ice-cold PBS.
containing magnesium/calcium and fixed with 4% Formaldehyde (Merck) for 20 min at room temperature. To permeabilize cell membranes, cells were incubated in 0.25% Triton X-100/PBS for 5 min. Blocking was done with 3% bovine serum albumin (BSA, Merck) and 2% goat serum diluted in PBS containing 0.25% Triton X-100 for 1 h at room temperature. Incubation with primary antibodies was performed overnight at 4°C followed by staining at room temperature for 2 h. Cells were washed 3-times for 10 min with PBS and incubated with secondary antibodies 2 h at room temperature. Slides were washed 3-times and the ProLong® Gold antifade mountant with DAPI (4’,6-diamidino-2-phenylindole) (Life Technologies) was applied to mount the coverslips. Primary antibodies included rabbit anti-FLAG (# F7425, Sigma-Aldrich), ERAS clone 6.5.2, and GFAP (# Z0334, Dako). Secondary antibodies included Alexa488-conjugated goat anti-mouse IgG and Alexa633-conjugated goat anti-rabbit IgG (Cat# A11003) (# A11008), Alexa546-conjugated goat anti-mouse IgG (Cat# A11034), Secondary antibodies included Alexa488-conjugated goat anti-mouse IgG and Alexa633-conjugated goat anti-rabbit IgG (cat# A11008), Alexa633-conjugated goat anti-rabbit IgG (A4671), and Alexa488-conjugated goat anti-mouse IgG (Cat# A11029) (all from Life Technologies). Confocal images were obtained using a LSM 510-Meta microscope (Zeiss, Jena, Germany).

**Constructs**—Rat ERAS cDNA was amplified by PCR from a cDNA library of freshly isolated rat hepatic stellate cells and subsequently cloned into pcDNA.3.1 and pEYFP-C1 vectors via the BamHI/Xho1 and EcoRI/BamHI restriction sites, respectively. Mutations of G12V in HRAS (HRAS<sup>G12V</sup>) and C220S/C222S in ERAS (ERAS<sup>G220S</sup> and ERAS<sup>G222S</sup>) were introduced by PCR-based site-directed mutagenesis as described earlier (26). To generate the N-terminal truncated ERAS variants (ERAS<sup>N</sup> and ERAS<sup>NX35</sup>), ERAS<sup>wt</sup> and ERAS<sup>G220S</sup> cDNA was PCR amplified from amino acid 39 to 227 and aa 1 to 227, respectively. Human HRAS, KRAS, NRAS, TC21, MRAS, and ERAS as well as rat ERAS were cloned in pGEX vectors and used for protein purification for *Escherichia coli* as described previously (27).

**Pull-down assay**—FLAG-tagged rat ERAS and human HRAS cDNAs were cloned into pcDNA3.1 vector and overexpressed in COS-7 cells. The RAS-binding/association domains (RBD/RA) of effector proteins, including CRAF-RBD (aa 51–131), RALGDS-RA (aa 777–872), PLCγ-RA (aa 2130–2240), p110α-RBD (aa 127–314), and RASSF5-RA (aa 200–358) were constructed as GST-fusions in pGEX-4T and transformed in *E. coli*. GST-fused proteins were isolated from total bacterial lysates using Glutathione Sepharose beads. GTP-bound RAS proteins were pulled down from total cell lysates and heated in Laemmli buffer for 10 min at 95°C.

**Immunoblotting**—Cell lysate were made with lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1% Igepal CA-630, 10% glycerol, 20 mM beta-glycerolphosphate, 1 mM Ortho-Na<sub>3</sub>VO<sub>4</sub>, EDTA-free protease inhibitor (Roche, Germany)) and protein concentrations were determined with Bradford assay (Bio-Rad). Equal amounts of cell lysates (ERAS; 120 μg, FOXY1/p-FOXO1; 50 μg; remaining proteins; 15 μg), were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting and probed with primary antibodies overnight at 4°C. All antibodies from Santa Cruz were diluted 1:200 in 5% non-fat milk (Merck, Germany)/TBST (Tris-buffered saline, 0.05% Tween 20), remaining antibodies were diluted 1:1000. The following antibodies were applied for immunoblotting: rabbit anti-FLAG (# F7425) and mouse γ-tubulin (# T5326) from Sigma-Aldrich; rabbit MEK1/2 (# 9126), rabbit ERK1/2 (# 9102), rabbit AKT (# 9272), rabbit phospho-MEK1/2 (S217/S221, # 9154), rabbit phospho-ERK1/2 (T202/T204, # 9106), rabbit phospho-AKT (S473, # 4060 and T308, #2965), rabbit p110α (# 4249), mouse STAT3 (#9139S), rabbit phospho-STAT3 (#9145S), rabbit FOXO1 (#2880), rabbit phospho-FOXY1 (#9461) all from Cell Signaling™; antibodies to rabbit p110β (# sc-602), p110γ (#sc-7177), p110δ (# sc-7176), from Santa Cruz. Mouse α-actin antibody (# MAB1510) was obtained from Millipore. Membranes were stained with horse radish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution). Signals were visualized using ECL (enhanced chemiluminescence) reagent (GE Healthcare).

**Expression and purification of GBP-nanotrap beads and co-immunoprecipitation (IP)**—For immunoprecipitation studies of overexpressed EYFP-fused HRAS and ERAS in COS-7 cells, we applied a GFP binding protein (GBP) used for Nanotrap
experiments was designed as described previously (28). Briefly, the GFP-binding V_{1\mu}H domain was cloned into pET23a-PelB vector adding C-terminal myc and histidine (His_{6}) tags and transformed in *E. coli* BL21. An overnight 50 ml *E. coli* pre-culture with the antibiotic ampicillin was used to inoculate 2,000 ml media to an OD_{600} of 0.8. The expression of recombinant genes was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside overnight at 30°C. Cells were harvested by centrifugation (2 h, 4°C, 4,000 rpm) and the supernatant was stored at -80°C. For purification, the supernatant was filtered through a 0.45 µM SFCA NALGENE®Rapid-Flow™ Bottle Top Filter (Thermo Scientific, Waltman, MA, USA) to remove cell debris. Flow through was mixed 1:1 with PP-buffer (500 mM NaCl, 50 mM Na_{2}HPO_{4}/NaH_{2}PO_{4} pH 7.4) and loaded on a pre-equilibrated Ni-NTA column (GE Healthcare) and purified. His-tagged protein was eluted by PP-Buffer containing 500 mM Imidazol. The protein was concentrated and Imidazol was removed by using Amicon® Ultra-15 10K Centrifugal Filter Devices (Merck Millipore Ltd, Tullagreen, Ireland). To perform pulldown of proteins by the GFP-nanotrap technique, 1 mg of purified protein was covalently coupled to 2 ml NHS-activated Sepharose 4 Fast Flow (GE Healthcare), according to the manufacturer’s instructions. Thereafter, beads were washed three times in ice-cold 1 mM HCl (2 min, 5,400 rpm, 4°C), added to the purified protein and mixed for 2 h at RT under constant agitation. Subsequently free binding sites of the beads were blocked by adding blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) for 2 h. Finally, beads were washed twice in 0.1 M Tris-HCl (pH 8). Beads were stored in 20% ethanol. For co-immunoprecipitation, cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl_{2}, 0.5% NP-40, 10 mM β-glycerophosphate, 0.5 mM Na_{2}VO_{4}, 10% glycerol, EDTA-free protease inhibitor). IP from total cell lysates was carried out for 2 h at 4°C with GFP-fused nanobeads. The beads were washed 5-times with IP buffer lacking NP-40, and eluted proteins were finally heated in SDS-Laemmli buffer at 95°C and analyzed by immunoblotting.

*RAS* proteins and monoclonal antibody against *ERAS*—All RAS-like proteins, including *ERAS*, were purified following the same protocol as described (29). The monoclonal anti-*ERAS* antibody was custom generated (Biogenes, Berlin) via immunization of mice with a purified N-terminal peptide of rat *ERAS* and thereafter purified from the supernatant of the respective hybridoma cell line by a protein A column (GE healthcare). The concentrated antibody solution (approximately 3 mg/ml) was supplemented with 10% glycerol and stored at -20°C.

**Subcellular fractionation of HSCs by differential centrifugation**—A differential centrifugation protocol according to Taha and coworkers (30) was used in this study to fractionate HSCs.

**DNA methylation analysis of *ERAS* promoter**—A genome-wide DNA methylation analysis from quiescent and early activated HSCs was used to analyze DNA methylation changes during HSC activation (31). The methylation data was visualized using the UCSC genome browser. Verification of DNA methylation changes was performed by direct bisulfite sequencing. DNA from freshly isolated and cultured HSCs was isolated using the DNeasy Blood & Tissue Kit (Qiagen) and subjected to bisulfite conversion by the EpiTect Bisulfite Kit (Qiagen). Bisulfite primers for *ERAS* were designed using the MethPrimer online tool (32) covering a part of the promoter region (ERAS 328bp-forward 5’-GTT GGG GGT AGG GAG TAT TTT AAT-3’, ERAS 328bp-reverse 5’-CTC AAA ATT AAA AAA AAA AAA AAA AAA AAA TAA CC-3’). Bisulfite PCR was performed using the Maxima Hot Start PCR Master Mix (Thermo Scientific) together with 20 ng bisulfite modified DNA and 0.6 µmol/l primer. After activation at 95°C a PCR protocol with denaturation at 95°C, annealing at 55°C and elongation at 72°C was used for 40 cycles. The PCR products were purified and sequenced at the DNA sequencing facility of the Heinrich-Heine-University (BMFZ). DNA methylation was quantified by the Mquant method as described (33). The height of the thymine peak at a CpG-dinucleotide was subtracted from the average signal of ten surrounding thymine peaks to quantify DNA methylation at this site. For the *ERAS* methylation analysis we calculated the mean DNA methylation of five CpG sites in the *ERAS* promoter region.
RESULTS

Expression of ERAS in quiescent but not activated HSCs—To investigate the impact of RAS proteins on the HSCs, we first investigated the expression profile of various members of the RAS family in quiescent vs. activated rat HSCs by qPCR. Freshly isolated primary HSCs were cultivated on plastic dishes for up to eight days, where they become activated upon ex-vivo culture and undergo myofibroblast transition (4). HSCs were analyzed at day 8 (d8) in comparison to unseeded HSCs (d0) as representative of the activated and quiescent state, respectively. Interestingly, among the different members of the RAS family, ERAS was specifically expressed in quiescent HSCs and strongly down-regulated during HSC activation (Fig. 1). In addition, we applied a probe based TaqMan real-time PCR to monitor ERAS expression at the different time points of HSC cultivation (d0, d1, d2, d4, and d8) and obtained comparable results (Supplementary Fig. S1). In contrast, Hras expression decreased only slightly in HSCs (d8). In contrast, the gene expression of Mras, Rras, Rala, and Rap2a were up-regulated in activated HSCs while other genes, including Kras and Nras, were expressed but did not significantly differ between day 0 and day 8 (Fig. 1). Collectively, these data indicates a switch from ERAS to MRAS, RRAS, RALA, and RAP2A expression during HSC activation.

Generation and validation of specific monoclonal antibodies against rat ERAS—ERAS contains an N-terminal extension upstream of its GTP-/GDP-binding (G) domain that is unique among the RAS family (23). As depicted in Fig. 2A, there is a significant difference between Homo sapiens (hs) and Rattus norvegicus (rn) ERAS proteins regarding their N-terminus (Fig. 2A). Therefore, we purified the N-terminus of rrERAS and generated antibodies against this unique ERAS region. Four clones of monoclonal antibodies (mAbs) were obtained and examined for anti-ERAS specificity. Immunoblot analysis of RAS proteins overexpressed in and purified from E. coli showed that clone mAb 6.5.2 clearly detected rat ERAS, but none of the other members of the RAS family (Fig. 2B). The selectivity of mAb 6.5.2, against hsERAS and rnERAS proteins was tested by using COS-7 and MDCKII cell lysates overexpressing hsERAS and rnERAS as EYFP-fusion proteins, respectively. As shown in Fig. 2C, mAb 6.5.2 only recognized rat ERAS (Fig. 2A). We next tested mAb 6.5.2 in confocal immunofluorescence analysis by overexpressing EYFP- and FLAG-tagged ERAS variants in MDCKII cells. As depicted in Fig. 2D, mAb 6.5.2 shows a clear specificity against full-length rat ERAS and did neither recognize hsERAS nor rnERAS lacking the N-terminal extension (rnERASΔN). Taken together, mAb 6.5.2 was validated as a rat-specific anti-ERAS antibody suitable for both immunoblotting and immunofluorescence analysis.

Among various rat liver cell types, ERAS protein is only expressed in quiescent HSCs—The monoclonal antibody (mAb) 6.5.2 was used to analyze the presence of ERAS protein in typical liver cell populations. Therefore, total cell lysates of freshly isolated HSCs, PCs, KCs, and SECs from rat liver were used for immunoblot analysis. Interestingly, ERAS was detected as a 25 kDa band in HSCs but not in other liver cell types (Fig. 3A). Consistent with the mRNA expression data (Fig. 1), the amount of ERAS protein was drastically reduced during the activation process of HSCs, thereby correlating with the loss of GFAP (Fig. 3B), which marks quiescent HSCs. In contrast, the myofibroblast marker α-SMA became detectable in cultured HSCs from day 4. Moreover, confocal imaging of HSCs revealed that ERAS was mainly cytosolic, which was, in contrast to GFAP, still detectable in cultivated HSCs, although at much lower amounts as compared to day 0 (Fig. 3C). Noteworthy, in subcellular fractions of HSCs (d0), ERAS was predominantly found in the light membrane fraction (Golgi apparatus, smooth endoplasmic reticulum, and various organelles), and to a minor extent in the heavy membrane fraction (plasma membrane and rough endoplasmic reticulum) and in the nucleus (Fig. 3D). Collectively, ERAS was detectable in quiescent HSCs and its protein levels diminished remarkably during HSC activation.

Protein-protein interaction profiling identifies PI3Ka as a specific effector of rat ERAS—Members of the RAS family GTP-binding proteins act as molecular switches which transduce extracellular signals to intracellular responses via activation of effector proteins. To gain insights into the effector binding specificity downstream of rat ERAS,
FLAG-tagged constructs of human HRAS and rat ERAS were overexpressed in COS-7 cells and total cell lysates were used for pull-down experiments. For pull-down analysis, five major RAS effector proteins were employed, i.e. CRAF-RBD, RALGDS-RA, PLCε-RA, PI3Kα-RBD, and RASSF5-RA (23), which were all produced in E. coli as GST-fusion proteins. Interestingly, we found that ERAS, in comparison to HRAS, preferentially and most strongly bound to PI3Kα, whereas only a modest interaction was observed with RASSF5 and CRAF (Fig. 4A). Unlike HRAS, no ERAS association with RALGDS and PLCε was detectable (Fig. 4A). Thus, ERAS and HRAS interact with and likely activate specifically non-overlapping set of effector proteins.

Similar to HRAS and NRAS, ERAS contains conserved C-terminal motifs for posttranslational modifications, a farnesylation and palmitoylation like HRAS (23). ERAS has an N-terminal extension with various motifs and shows a critical amino acid deviation, a serine at position 50 (S50) instead of a glycine (G12 in HRAS), which makes ERAS GAP-insensitive (23). These properties may influence physical interaction of ERAS with PI3K and its downstream signaling. Therefore, we generated and analyzed different ERAS variants, either lacking the N-terminus (ERAS<sup>ΔN</sup>), or conserved cysteines for palmitoylation (ERAS<sup>SS</sup>) or both (ERAS<sup>AN/SS</sup>) (Fig. 4B). First, we investigated binding of ERAS variants to the catalytic subunit of PI3Kα. The obtained data revealed that all ERAS variants were able to associate with PI3Kα-RBD (Fig. 4C, upper panel). This suggests that the N-terminus of ERAS and its C-terminal modification by palmitoylation are not essential for the association of PI3Kα-RBD with the G domain of ERAS.

To examine the signaling activity of ERAS variants towards AKT via PI3K and mTORC2 pathways, we next monitored the phosphorylation states of AKT using specific anti-phospho-AKT (threonine 308 and serine 473) antibodies. Noteworthy, ERAS strongly activated AKT and induced its phosphorylation at two distinct sites, i.e. at T308 by PI3K-PDK1 (p-AKT<sup>T308</sup>) and at S473 by mTORC2 (p-AKT<sup>S473</sup>; Fig. 4C, lower panel). Interestingly, in comparison to ERAS-wild type (WT), the ERAS variants, most notably the truncated N-terminus (ERAS<sup>AN</sup>), the palmitoylation deficient variants with two cysteines 220 and 222 replaced to serines (ERAS<sup>SS</sup>), and a combination of both variants (ERAS<sup>AN/SS</sup>), elicited a significantly reduced AKT phosphorylation, especially of p-AKT<sup>S473</sup>, which is indicative of mTORC2 activity. These data indicate that both the ERAS N-terminus and its plasma membrane anchorage via palmitoylation are essential and critical for AKT activation via the PI3K and mTORC2 axis, although the formation of the GTP-bound state and the interaction with PI3K were not affected.

ERAS-PI3Kα/δ-AKT and mTORC2-AKT axis are highly activated in quiescent HSCs—Our findings suggest that the catalytic subunit of PI3K is a candidate effector downstream of ERAS. There are four isoforms of the p110 catalytic subunit of PI3K, p110α, p110β, p110γ, and p110δ, raising the question about the p110 isoform specificity in ERAS-PI3K interaction in HSCs. mRNA expression analysis data revealed that the α isoform of PI3K did not change remarkably between quiescent and activated HSCs, whereas the mRNA levels of the β and δ isoforms increased in the course of the HSC activation (Fig. 4D). At the protein level, however, α and γ isoforms were found at clearly higher levels in quiescent HSCs as compared to the β isoform (Fig. 4E). Upon HSC activation the protein levels of β, and to a certain extent also δ isoforms increased, whereas a decrease in α and γ isoforms was observed (Fig. 4E). Next, we investigated the interaction of ERAS with the four PI3K isoforms in co-immunoprecipitation experiments using ERAS overexpression in COS-7 cells. Wild-type and a constitutive active variant of HRAS (HRAS<sup>WT</sup> and HRAS<sup>V12</sup>) were used as controls. Data shown in Figure 4F demonstrated that not only PI3Kα, but also the δ isoform co-immunoprecipitated with ERAS. Notable, PI3Kδ appeared to strongly bind HRAS<sup>V12</sup> (Fig. 4F). Thus, cell-based investigations confirmed the interaction between ERAS and PI3Kα, which is consistent with our data obtained under cell-free conditions (Fig. 4A).

In the next step, we monitored the AKT phosphorylation states and found that quiescent HSCs at day 0 and to a certain extent at day 1, as compared to activated HSCs, exhibited much higher p-AKT<sup>S473</sup> and p-AKT<sup>T308</sup> levels, representing mTORC2 and PI3K-PDK1 activity, respectively (Fig. 4G). In addition, we also analyzed the phosphorylation states of
FOXO1 and STAT3, two other signaling molecules that have been suggested to be downstream of ERAS (34). Interestingly, in ERAS-expressing quiescent HSCs we observed high levels of STAT3 phosphorylation at T705 and of FOXO1 phosphorylation at S256 (Fig. 4G). Thus, it is obvious that ERAS signaling towards PI3K-PDK1 and mTORC2 pathways activates AKT and maybe also STAT3, but inactivates FOXO1 in order to maintain HSCs in their quiescent state.

ERAS does not actively impact on the MAPK pathway—In the next step, we investigated the interaction of ERAS with CRAF-RBD and the MAPK pathway in quiescent vs. activated HSCs. Both wild-type ERAS and its palmitoylation deficient variant (ERASS/S) strongly bound to CRAF-RBD, although with considerably lower affinity as compared to the constitutive active HRASV12 variant (Fig. 5A). This binding was, however, weaker for ERAS<sup>ΔN</sup> and ERAS<sup>ΔN/S/S</sup>, both lacking the N-terminal extension. It is important to note that the latter variants are efficiently expressed and also exist in GTP-bound forms (Fig. 4C). The same is true for HRAS<sup>WT</sup>, which was expressed to a similar level as HRAS V12 (Fig. 4C). However, its GTP-bound level was much lower due to its ability to hydrolyze GTP normally, therefore resulting in low amounts of HRAS<sup>WT</sup> in the CRAF-RBD pull-down experiment (Fig. 5A). Most remarkably, expression of ERAS<sup>WT</sup> in COS-7 cells clearly led to a strong reduction of p-MEK1/2 and p-ERK1/2 levels that were far below those obtained with vector control and the HRAS variants (Fig. 5A). Notably, similar effects were observed for all ERAS variants analyzed (ERAS<sup>ΔN</sup>, ERAS<sup>SS</sup> and ERAS<sup>ΔN/S/S</sup>).

In addition, we analyzed the binding property of rat ERAS to cellular RAF isoforms (ARAF, BRAF and CRAF) by overexpressing and immunoprecipitating EYFP-tagged ERAS from COS-7 total cell lysates. As controls, we used HRAS<sup>WT</sup> and HRAS<sup>V12</sup>. Figure 5B shows that ERAS, compared to HRAS<sup>V12</sup>, bound weakly only to ARAF and CRAF, which is consistent with the data obtained with CRAF-RBD in pull-down experiments (Fig. 5A). Thus, we ERAS can be excluded as an activator of RAF proteins and thus of the MAPK pathway.

The MAPK pathway is highly dynamic in activated HSCs—Our data described above showed that ERAS is endogenously expressed in quiescent HSCs and does not seem to be an activator of the MAPK pathway under overexpression conditions in COS-7 cells. Therefore, we analyzed the activity of the MAPK pathway in HSCs following their activation. First, we analyzed the expression of RAF, MEK and ERK isoforms in quiescent vs. activated HSCs by qPCR. As indicated in Figure 5C, the overall mRNA levels were very similar except for the low expression of BRAF in both quiescent and activated HSCs (Fig. 5C). To further examine the role of the MAPK pathway in HSC activation, we looked at the protein levels of phosphorylated (i.e., activated) vs. total MEK1/2 and ERK1/2. As shown in Figure 5D, expression of MEK1/2 and p-ERK1/2, especially p-ERK2, suggested increased activation of the MAPK pathway in activated HSCs (Fig. 5D). In contrast, the amounts of the RAF isoforms and total RAS were the highest in quiescent HSCs (day 0) and decreased during HSCs activation (Fig. 5D). Taken together, it seems that HSCs reciprocally utilize distinct pathways downstream of ERAS to maintain their fate, i.e. PI3K-PDK1 and mTORC2 pathways could be activated by ERAS in quiescent HSCs and the MAPK pathway by RAS in activated HSCs.

ERAS contributes to repression of YAP activity and thus may counteract activation of quiescent HSCs—In vitro protein-protein interaction studies revealed that ERAS, like HRAS, directly interacts with RASSF5 (Figs. 4A and 6A). It has been reported that RASSF5 enables the HIPPO pathway (via MST2/STK3) to respond to and integrate diverse cellular signals by acting as a positive regulator of MST2/STK3 (35). A recent study revealed a role of YAP, the central effector of the HIPPO pathway during HSC activation (13), thus, we analyzed if ERAS activates the HIPPO pathway, which may lead to phosphorylation and proteolytic degradation of YAP (Supplementary Figs. S2 and S3A). We further investigated, if YAP and its target genes are...
expressed in activated rat HSCs. To address the first question we used COS-7 cells, which normally contain significant amounts of YAP and its phosphorylated form (p-YAP; Fig. 6B; see vector control). Interestingly, p-YAP and YAP levels were considerably reduced when rat ERAS was overexpressed (Fig. 6B and S2), strongly indicating that ERAS activated the HIPPO pathway in COS-7 cells. Similar results were obtained with the HRAS variants (Fig. 6B). Importantly, we next probed YAP and p-YAP in HSC lysates and detected them in activated HSCs (day 8) but not in quiescent HSCs (Fig. 6C). Consistently, mRNA analysis further revealed that MST1/2 (mammalian orthologues of HIPPO) isoforms were expressed in both states but with more elevated levels of MST1 as compared to MST2. YAP and its target genes, CTGF (connective tissue growth factor) and NOTCH2 exhibited a distinct increase in their expression levels after HSC activation (Fig. 6D). Moreover, the effector binding domain (switch regions) of ERAS differs considerably from those of HRAS in critical residues, which may determine the specificity of ERAS binding to its effectors (23) (Supplementary Fig. S3B). Interestingly, we found that mutation of two surface exposed residues (H70Y/Q75E) in the effector binding region of ERAS (ERAS SW1) abolishes the binding affinity for RASSF5 as compared to wild-type ERAS (Supplementary Fig. S3C). These findings indicate that ERAS needs specific residues which are not conserved within HRAS to interact with RASSF5. To monitor the activity of the ERAS-RASSF5-MST1/2-LATS1/2-YAP cascade downstream of mutated ERAS we next analyzed the levels of YAP protein in total cell lysates. Consistently, we detected larger amounts of YAP under conditions when the RASSF5 binding-deficient ERAS mutant (ERAS SW1) was overexpressed (Figs. S3D and E). These data further support the idea that ERAS is upstream of the HIPPO-YAP pathway. Collectively, activation of the HIPPO pathway appears to keep HSCs in their quiescent state, whereas YAP clearly may play a role in the activation and eventually further development of HSCs. YAP is obviously repressed in quiescent HSCs potentially mediated through ERAS-RASSF5 signaling.

Increased DNA methylation of the ERAS locus is associated with ERAS gene silencing in activated HSCs—To characterize possible mechanisms responsible for the down-regulation of ERAS expression in activated HSCs, epigenetic analysis of the promoter region of the rat ERAS gene was conducted. Evaluation of a previously performed genome-wide DNA methylation analysis showed an increase of CpG methylation at the ERAS promoter of approximately 18% during early HSC activation (Fig. 7A). More detailed bisulfite-sequencing analysis during in vitro HSC activation revealed a significant increase in promoter DNA methylation, which correlates with the drastic decrease in ERAS expression in HSCs during their activation (Figs. 1, 7B and S1). Of note, the overall degree of promoter DNA methylation increased from 65.5% to approximately 80% at day 7 of HSC culture. To investigate the functional impact of ERAS promoter methylation, we examined whether a DNA methyltransferase inhibitor 5-AZA-2′-deoxycytidine (5-AZA) could restore ERAS expression in activated HSC. Therefore, we cultivated primary rat HSC for three days such that the levels of ERAS mRNA were downregulated (Fig. S1). At day 8 of HSC activation (and four days of 5-AZA treatment), we analyzed ERAS expression. As indicated in Fig. 7C, 5-AZA treatment restored ERAS expression by approximately 4-fold in activated HSC. To test whether ERAS expression is also regulated via histone modifications, such as histone acetylation, we treated HSCs with 5µM SAHA (histone deacetylase inhibitor). As indicated in Fig. 7C, SAHA treatment alone was not sufficient to rescue ERAS expression. Taken together, our data indicate that the profound decrease of ERAS expression, but not other RAS-related genes such as RRAS, RAP2A and NRAS (data not shown) during HSC activation may be caused by epigenetic gene silencing.

DISCUSSION

In this study, we found embryonic stem cell-expressed RAS (ERAS) specifically expressed in one type of liver resident cells, hepatic stellate cells (HSC). The presence of ERAS mRNA was detected in quiescent HSCs but not in activated HSCs. In contrast, other RAS-related genes, such as RRAS, MRAS, RALA and RAP2A, were upregulated during HSC activation. ERAS protein was detected in quiescent HSCs but not in other liver cell types, and ERAS was considerably down-regulated
during HSC activation (d4 and d8). To elucidate the functions of ERAS in quiescent HSCs, we sought for ERAS specific effectors and the corresponding downstream pathways. Interaction analyses with a set of RAS effectors showed that ERAS preferentially interacts with PI3Kα and activates the PI3K-PDK1-AKT axis. The prominent AKT phosphorylation by mTORC2 in quiescent HSCs suggests that mTORC2-AKT acts as a candidate pathway mediates signaling downstream of ERAS. Interestingly, in quiescent HSCs, ERAS does not show any activity towards the MAPK cascade, which is the opposite in activated HSCs. The MST1/2-LATS1/2-YAP (HIPPO pathway) results in inactivation and proteosomal degradation of YAP if activated for example by RAS and RASSFs. The fact that YAP was hardly detectable in quiescent HSCs and also in COS-7 cells expressing ERAS, as well as the interaction between ERAS and RASSF5 suggests that ERAS may act as an activator of the HIPPO pathway in quiescent HSCs. Consistently, we detected both YAP protein and its up-regulated target genes in activated HSCs.

Role of the PI3K-AKT-mTORC1 axis in quiescent HSCs—Transient expression of ERAS in COS-7 cells and endogenous ERAS expression in quiescent HSCs strongly correlates with high levels of AKT phosphorylated at T308 and S473 through PDK1 and mTORC2, respectively. Protein interaction and immunoprecipitation analysis further revealed that ERAS physically interacts with PI3Kα and also PI3Kδ (Figs. 4C and 4F). Thus, in quiescent HSCs, we propose ERAS as a regulator of the PI3K-PDK1-AKT-mTORC1 axis. This axis is involved in various processes including cell cycle progression, autophagy, apoptosis, lipid synthesis, and translation (36-40). The latter is controlled by mTORC1-S6 kinase, which in turn phosphorylates different substrates, such as ribosomal protein S6, mTOR itself at S2448, and mSIN1 at T86, an upstream component of mTORC2 (Fig. 8) (41-43). Previous studies have shown that quiescent HSCs produce and secrete a significant amount of HGF (44,45), which is known to regulate hepatocyte survival (46). HGF production and secretion is modulated by the mTORC1-S6 kinase pathway (47). Apart from the retinoid transport from hepatocyte to HSCs, the mTORC1 activity may influence de novo lipid synthesis in HSCs. mTORC1 might promote lipid synthesis in HSCs through sterol regulatory element-binding protein (SREBP) and peroxisome proliferative-activator receptor-γ (PPARγ) (48). In this regard, it has been shown that curcumin inhibits SREBP expression in cultured HSCs by modulating the activities of PPARγ and the specificity protein-1 (SP1) thereby repressing LDLr expression, which blocks a proposed LDL-induced HSC activation (49). Thus, the AKT-mTORC1-SREBP/PPARγ pathway appears to play a critical role in lipid metabolism that is obviously required together with other pathways to regulate HSC fate.

Recently, Kwon and colleagues have shown that in mouse embryonic stem cells overexpression of ERAS induces SP1 activation through the JNK pathways. However, it remains to be addressed whether JNK-SP1 signaling is also a downstream target of endogenous ERAS in HSC (50).

Activity of the mTORC2-AKT-FOXO1 axis in quiescent HSCs. In comparison with mTORC1 the regulation of mTORC2 is less understood (51). For example, the TSC1-TSC2 complex can physically associate with mTORC2, but not with mTORC1, which has been suggested to promote mTORC2 activity (52). Our findings indicate that ERAS may act as an activator of the mTORC2 pathway. Exogenous ERAS has been shown to promote phosphorylation of both AKT (S473) and FOXO1 (S256) in induced pluripotent stem cells (iPSCs) generated from mouse embryonic fibroblasts (34). Thus, ERAS-AKT-FOXO1 signaling may be important for somatic cell reprogramming. We detected high levels of p-AKT (S473) and p-FOXO1 (S256) in quiescent HSCs endogenously expressing ERAS (Fig. 4G). Phosphorylated FOXO1, sequestrated in the cytoplasm, cannot translocate to the nucleus where it binds to gene promoters and induces apoptosis (53). Interestingly, a possible link between ERAS and mTORC2 may be mSIN1, which appears to be an upstream component and modulator of mTORC2 activity (54). It has been reported that mSIN1 contains a RAS-binding domain with some homology to that of CRAF (55). Taken together, the ERAS-mTORC2-AKT-FOXO1 axis may insure the survival of HSCs in the space of Dissé by interfering with programmed cell death (Fig. 8).

Role of the HGF-JAK-STAT3 axis in...
Quiescent HSCs—Ectopic expression of ERAS stimulates phosphorylation of STAT3 likely downstream of leukemia inhibitory factor (LIF) (34). ERAS may compensate for lack of LIF to support the iPSC generation (34). Moreover, the LIF-STAT3 axis is essential for keeping mouse stem cells undifferentiated in cultures and regulates self-renewal and pluripotency of embryonic stem cells (56). Phosphorylated STAT3 (p-STAT3) has been shown to directly interact with FOXO1/3 transcription factors and regulates their translocation into the nucleus (57). Consistently, we detected high levels of p-STAT3 and p-FOXO1 in quiescent HSCs (Fig. 4G), which may control survival, self-renewal, and multipotency of quiescent HSCs. In addition, stimulation of the HGF receptor (c-MET), which is expressed in HSCs, results in JAK activation and phosphorylation of STAT3 (1,58). Interestingly, HGF is a target gene of IL6-STAT3 signaling (59,60). Therefore, an autocrine HGF-JAK-STAT3 signaling may also account for STAT3 phosphorylation in quiescent HSCs (Fig. 8). However, the presence and activity of a LIF-STAT3 axis in HSCs needs further investigations.

Quiescent HSCs display a locked RAS-MAPK signaling pathway—In quiescent HSCs only basal levels of activated (phosphorylated) MEK and ERK could be observed although all components of the RAS-RAF-MEK-ERK axis were expressed (Figs. 1, 5C and 5D). There are several explanations for the strongly reduced activity of RAS-MAPK signaling in quiescent HSCs (Fig. 8): (i) Absence of external stimuli, such as PDGFA and TGFβ1 in healthy liver. These growth factors are strong activators of the MAPK pathway in activated HSCs (7,8). (ii) Presence of an intracellular inhibitor, like special AT-rich binding protein 1 (SATB1), which is specifically expressed in quiescent HSCs and down-regulated during HSC activation (61). Interestingly, SATB1 has been shown to be a strong inhibitor of RAS-MAPK pathway that may block this signaling in quiescent HSCs (61). (iii) MicroRNAs (miRNAs), especially miRNA-21, may play a role in the reciprocal regulation of the RAS-MAPK pathway in quiescent vs. activated HSCs. Uregulated miRNA-21 in activated HSCs results in MAPK activation, which is based on depletion of sprouty homolog 1 (SPRY1), a target gene of miRNA-21 (62) and a negative regulator of the RAS-MAPK pathway (63).

Biological functions of PI3K/AKT pathway regarding different p110 isoforms—The catalytic PI3K isoforms p110α and β are reported to be ubiquitously expressed, whereas the presence of p110γ and δ is restricted mainly to hematopoietic cell types (64-67). We identified ERAS as an activator of AKT by interacting with p110α and moderately also with p110δ (Fig. 4F). Our RNA and protein analyses indicated high levels of p110α/γ in quiescent HSCs and elevated levels of p110β/δ in activated HSCs (Figs. 4D and 4E). Wetzker and colleagues reported that retinoic acid treatment can stimulate expression of p110γ, but not p110β/δ, in U937 cells, a myelomonocytic cell line (68). Quiescent HSCs store high levels of retinoid acids as retinol esters in their lipid droplets which may elicit the same function in HSCs by upregulation of p110γ. Khadem et al. have shown that HSCs also expresses the p110δ isoform and that p110δ deficiency in HSCs prevents their activation and their supportive roles in Treg expansion in mice infected with visceral leishmaniasis (69). Therefore, high level of the p110δ isoform in activated HSCs may correlate with its immunoregulatory functions.

Epigenetic regulation of ERAS expression in HSCs—Unlike other RAS proteins, ERAS is GAP insensitive and refractory to inactivation by RASGAP proteins (21,23). This raises the question about potential mode(s) of ERAS regulation. Since ERAS is not ubiquitously expressed and seems to be limited to a few cell types, we proposed that ERAS is mainly regulated at the transcriptional level as described before for gastric cancers (70). Our epigenetic studies of the ERAS promoter revealed that its DNA methylation increases (up to 18%) during HSC activation (Fig. 7A and B). Moreover, treatment with DNA methyltransferase inhibitor induced re-expression of ERAS in culture-activated HSCs (Fig. 7C). Consistently, ERAS expression was also induced in certain gastric cell lines by the DNA methyltransferase inhibitor (70). Collectively, our findings clearly indicate that DNA methylation is one of the mechanisms suppressing expression of ERAS during activation of HSCs. Conceivably, ERAS-specific microRNAs may also control mRNA degradation and translation of ERAS when HSC activation is induced.
Cellular signaling signature of activated HSCs—In vitro culturing of hepatic stellate cells changes their gene expression profile and cellular properties thereby stimulating the activation of HSCs (1,31,71,72). HSCs typically lose their lipid droplets, expression of GFAP, and elicit the synthesis of collagens, matrix-metalloproteinases (MMP2, 9 and 13), and α-SMA as important differentiation markers (2,11). Collectively, during this process HSCs alter their quiescent characteristics and develop into myofibroblast-like cells, which are recognized as proliferative, multipotent, and migratory cells (6,73,74). Comprehensive mRNA analysis of various RAS family members revealed that RRAS, MRAS, RALA and RAP2A were upregulated during HSC activation (Fig. 1). These genes may also play a role in the coordination of cellular processes, which are required for activation and differentiation of HSCs, such as polarity, motility, adhesion and migration. Interestingly, RRAS has been implicated in integrin-dependent cell adhesion (75). Of note, in endothelial cells the RRAS-RIN2-RAB5 axis stimulates endocytosis of β1 integrin in a RAC1-dependent manner (76). On the other hand, the muscle RAS oncogene homolog (MRAS), a RRAS-related protein, is upregulated during HSC activation. Among the different members of RAS family, only MRAS can interact with SHOC2 in a ternary complex with protein phosphatase 1 (PP1), which dephosphorylates autoinhibited CRAF and thereby activates the CRAF-MEK-ERK cascade (77). These findings and data obtained in this study suggests that MRAS may be responsible for the high levels of p-MEK and p-ERK in activated HSCs due to RAF kinase activation. The cytoplasmic p-ERK alternatively phosphorylates Mnk1 and p90RSK and thereby promotes protein synthesis and cell growth (84,85) (ii) PDGF and IGF1 are the most potent mitogens for activated HSCs and induce activation of MAPK pathways (7,86). (iii) The expression of SATB1, a cellular inhibitor of the RAS-RAF-MEK-ERK pathway, significantly declines during HSC activation (61).

Putative role of the ERAS-RASSF5-MST1/2-LATS1/2-YAP axis in HSCs—We observed a moderate interaction between ERAS and RAS-association domain (RA) of RASSF5A (Fig. 6A). Previously, we showed that the switch I region of ERAS is important for ERAS-RASSF5 interaction and mutation in this region impairs ERAS binding to RASSF5 (23). RAS-association domain family (RASSF) proteins are recognized as specific RAS effectors with tumor suppressor function (87,88). MST1/2 (mammalian orthologues of HIPPO), which are expressed in HSCs, interact and form heterodimers with RASSF1/5A and WW45 through their SARAH (SAV/RASSF/HPO) domain (89). This complex phosphorylates and activates LATS1/2, which in turn promotes phosphorylation, sequestration, and proteasomal degradation of YAP in the cytoplasm (Supplementary Fig. S3A) (90,91). YAP is a transcriptional co-activator that promotes transcription of CTGF and NOTCH2, which are involved in cell development and differentiation (92-95). It has been shown that

Proliferation, growth, and differentiation of activated HSCs—In comparison with quiescent HSCs, activated HSCs are proliferative cells and can pass through cellular checkpoints (82). One of the candidate pathways is the RAF-MEK-ERK cascade that can be stimulated via different growth factors. Consist with previous studies, we detected high levels of p-MEK and p-ERK in culture-activated HSCs (7,83). Three scenarios may explain the elevated RAF-MEK-ERK activity in activated HSCs: (i) As discussed above, MRAS with SHOC2 and PP1 is able to activate the CRAF-MEK-ERK pathway (80). Phospho-ERK translocates to the nucleus and phosphorylates different transcriptional factors, including Ets1 and c-Myc thereby eliciting cell cycle progression and proliferation. The cytoplasmic p-ERK alternatively phosphorylates Mnk1 and p90RSK and thereby promotes protein synthesis and cell growth (84,85) (ii) PDGF and IGF1 are the most potent mitogens for activated HSCs and induce activation of MAPK pathways (7,86). (iii) The expression of SATB1, a cellular inhibitor of the RAS-RAF-MEK-ERK pathway, significantly declines during HSC activation (61).
the HIPPO-YAP pathway plays a distinct role in differentiated parenchymal and undifferentiated liver progenitor cells, respectively. Most recently, van Grunsven and colleagues reported that transcriptional co-activator of YAP controls in vitro and in vivo activation of HSCs (13). Consistent with this study we observed hardly any YAP protein in quiescent HSCs in comparison to activated HSCs (Fig. 6C). Thus, our data suggest that YAP degradation through RASSF5-MST1/2-LATS1/2 may be triggered by binding and recruitment of RASSF5 to the plasma membrane via ERAS-GTP (Figs. 6B and 8).

Cell survival and anti-apoptotic pathways—One of the most important features of activated HSCs is their survival and anti-apoptotic response during liver injury and regeneration (96). Here, we demonstrated elevated p-AKT levels not only in quiescent, but also in activated HSCs, the latter leading to pro-survival responses such as phosphorylation of FOXO1 (Fig. 4G). Additionally, we detected moderate levels of p-STAT3 implicating that the JAK1-STAT3-SOCS3 axis may control the anti-apoptotic pathway in activated HSCs.

Lastly, the high levels of YAP transcriptional activity in activated HSCs, which might result from the inhibitory activities of AKT and mTOR on MST1/2 (97), may contribute to increased cell survival, proliferation, and development of activated HSCs (13) by causing antagonistic effects to the pro-apoptotic RAS-RASSF5-MST1/2-LATS1/2 pathway (Fig. 8).

Functional similarity between human and rat ERAS—We observed sequence deviations between human and rat ERAS, especially at their extended N-termini (Fig. 2A). Therefore, we compared the signaling activity of different human and rat ERAS variants. However, so far, we did not observe remarkable functional differences (Figs. 4 and S4). ERAS function in human diseases is poorly understood. Its expression profile ranges from embryonic stem cells to tumors (20,21). Yamanaka and colleagues have introduced ERAS as critical factor for the maintenance of growth of embryonic stem cells (21). Kaizaki and colleagues reported ERAS expression in 45% of gastric cancer tissues and observed a correlation between ERAS-negative patients and poorer prognosis (20). In addition, ERAS may promote transforming activity and chemoresistance in neuroblastoma patients (19).

In summary, expression analysis revealed a different pattern of RAS and RAS-signaling components in quiescent vs. activated HSCs. Among different RAS family members, we identified ERAS, p110α and p110γ to be mainly expressed in quiescent HSCs and MRAS, RRAS, RAP2A, RALA, p110β, p110δ, YAP, CTGF and NOTCH2 expressed in activated HSCs. Our data suggest that an increased activity via PI3K-AKT-mTORC1 and HIPPO signaling in quiescent HSCs. Therefore, this study adds ERAS signaling to the remarkable features of quiescent HSCs and the cellular outcome of these signaling pathways would maintain the quiescent state of HSCs via inhibition of proliferation (HIPPO pathways, G0 arrest) and apoptosis (PI3K-PDK1 and mTORC2) (see Fig. 8). On the other hand, activated HSCs exhibit YAP-CTGF/NOTCH2 and RAS-RAF-MEK-ERK activity, which both are involved in HSC proliferation and development (Fig. 8). Finally we would like to point out that our study is based on the ex vivo activation of HSCs, which is a known model for the in vivo activation process (72,98). However, there may be some aspects, which could be different in the ex vivo model and the in vivo situation. Therefore, future studies should also address the ERAS networking in an in vivo models of liver injury.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS:
MRA conceived and coordinated the study. MRA, SNR designed the study and wrote the paper. SNR, HN, SG, IS, CK, MJH, MF designed, performed and analyzed the experiments. All authors reviewed the results and approved the final version of the manuscript.
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Abbreviations:
α-SMA, α-smooth muscle actin; aa, amino acid; CTGF, connective tissue growth factor; ECM, extracellular matrix; ERAS, embryonic stem cell-expressed RAS; ERK, extracellular regulated kinase; EYFP, enhanced YFP; FOXO1, forkhead transcription factor; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFAP, glial fibrillary acidic protein; GST, glutathion-S-transferase; GTPase, guanosine triphosphatase; HRAS, Harvey rat sarcoma; HSCs, hepatic stellate cells; HVR, hypervariable region; IB, immunoblot; IGF, insulin-like growth factor; IP, immunoprecipitation; KCs, Kupffer cells; KRAS, Kirsten rat sarcoma; MDCK II, Madin-Darby canine kidney cells; MEK, MAP/ERK kinase; MMP, matrix metalloproteinases; MRAS, Muscle RAS; mTORC, mammalian target of rapamycin; NRAS, neuroblastoma RAS; PCs, parenchymal cells; PD, pull-down; PDGF, platelet-derived growth factor; PDK1, 3-phosphoinositide-dependent protein kinase; PI3K, phosphoinositol 3-kinase; PIP3, phosphoinositol 3,4,5-trisphosphate; PLC, phospholipase C; PPARγ, peroxisome proliferative-activator receptor-γ; PTM, post-translational modification; RBD, RAS binding domain; RA, RAS association; RAL, RAS like; RALGDS, guanine nucleotide dissociation stimulator; RAP2, RAS related protein 2; RAS, rat sarcoma; RASSF5, RAS-association domain family; RHEB, RAS homolog enriched in brain; RHO, RAS homolog; RRAS, related RAS viral; SDS-PAGE, dodecyl sulfate polyacrylamide gel electrophoresis; SECs, sinusoidal endothelial cell; SREBP, sterol regulatory element-binding protein; STAT3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor beta; TSC, tuberous sclerosis; YAP, Yes-associated protein.
The FLAG-proteins by immunoblotting (C) and in MDCKII cells as FLAG-tagged protein by confocal imaging (D).

recognized NRAS, HRAS and KRAS, respectively, but not ERAS. HRAS and KRAS do not contain the no cross-reactivity against other RAS species. Two other antibodies were used as controls, which only recognized NRAS, HRAS and KRAS, respectively, but not ERAS. HRAS and KRAS do not contain the HVR and are therefore smaller as compared for example to NRAS. (C-D) Anti-ERAS antibody (clone HVR and are therefore smaller as compared for example to NRAS. (C-D) Anti-ERAS antibody (clone 6.5.2) recognized recombinant rnERAS, but not hsERAS, overproduced in COS-7 cells as YFP-fusion proteins by immunoblotting (C) and in MDCKII cells as FLAG-tagged protein by confocal imaging (D). The FLAG-rnERAS construct, lacking the N-terminal 38 amino acids of rnERAS, was used as negative control. Scale bar = 10 μm.

Figure 3. ERAS protein in HSCs. (A) Immunoblot analysis of isolated liver cell lysates detected ERAS in hepatic stellate cells (HSCs), but not in other liver cells. PCs, parenchymal cell; KCs, Kupffer cell; SECs, sinusoidal endothelial cell. (B) Immunoblot analysis of ERAS from freshly isolated (d0) and activated HSCs maintained in monolayer up to eight days (d8). GFAP and desmin were used as markers for quiescent HSCs (d0), and α-SMA as marker for activated HSCs (d8). α-actin and γ-tubulin served as loading controls. (C) Confocal imaging of ERAS and GFAP in HSC monocultures from d0 to d8. The level of ERAS and also GFAP is significantly reduced in the course of cell culture with a trace amount of ERAS in the nucleus. Scale bar = 10 μm. (D) ERAS showed a diverse subcellular distribution pattern in HSCs day 0 as revealed by subcellular fractionation analysis. HSCs were fractionated into four distinct fractions, including heavy membrane (plasma membrane and rough endoplasmic reticulum), light membrane (polysomes, Golgi apparatus, smooth endoplasmic reticulum), cytoplasm (cytoplasm and lysosomes) and enriched nucleus.

Figure 4. Highly active PI3K-AKT and mTORC2-AKT pathways in quiescent HSCs may controlled by ERAS. (A) Transiently expressed FLAG-tagged rat ERASWT and human HRASWT were pulled down from COS-7 cell lysates with well-known RAS effectors, including CRAF-RBD, RALGDS-RA, PLCε-RA, PI3Kα-RBD, and RASSF5-RA as GST-fused proteins. Immunoblots (IB) of total cell lysates were used as a control to detect FLAG-RAS. (B) FLAG-tagged RAS constructs used in this study including ERASWT, ERASN (N-terminal truncated, aa 39-227), ERASV (palmitoylation-deficient), ERASAN/S (N-terminal truncated and palmitoylation-deficient), as well as HRASWT and HRASV12. G stands for G domain. (C) PI3Kα-RBD-derived pull-down (PD) of GTP-bound ERAS variants and their signaling activities were analyzed by immunoblotting of FLAG-tag and AKT phosphorylation (p-AKT) at positions T308 and S473. (D) Quantitative mRNA expression analysis of PI3K isoforms α, β, γ and δ in quiescent and activated HSCs (d0-d8). (E) Immunoblot of the PI3K isoforms in quiescent and activated HSCs (d0-d8). (F) Co-immunoprecipitation analysis of the PI3K isoforms with ERASWT, HRASWT and HRASV12 overexpressed as EYFP-fusion proteins in COS-7 cells. TCL stands for total cell lysate. (G) Immunoblot of the phosphorylated signaling proteins downstream of the PI3K-AKT and mTORC2 axis in quiescent and activated HSCs (d0-d8). Total AKT, FOXO1 and STAT3 served as controls.

Figure 5. RAF-MEK-ERK signaling in activated HSCs. (A) CRAF-RBD-derived pull-down (PD) of GTP-bound HRAS and ERAS variants and their signaling activities towards MAPK pathway were analyzed by immunoblotting (IB) of the FLAG-tag and phosphorylation of MEK1/2 (p-MEK1/2) and ERK1/2 (p-ERK1/2) using total cell lysates derived from transfected COS-7 cells. γ-tubulin was used as the loading control. (B) Co-immunoprecipitation analysis of the RAF isoforms with ERASWT, HRASWT and HRASV12 overexpressed as EYFP-fusion proteins in COS-7 cells. TCL stands for total cell lysate. (C) Quantitative mRNA expression analysis of the RAF, MEK and ERK isoforms in quiescent and activated HSCs (d0-d8). (D) Immunoblot analysis of the component of the MAPK
pathway, including RAF isoforms, p-MEK1/2 and p-ERK1/2 in quiescent and activated HSCs (d0-d8). Total RAS was detected using a panRAS antibody. Total amounts of MEK1/2 and ERK1/2 as well as γ-tubulin served as loading controls.

**Figure 6.** ERAS-RASSF5 interaction may repress YAP that is highly active in activated HSCs. (A) RASSF5-RA pull-down (PD) of HRAS WT, hsERAS, rnERAS overexpressed in COS-7 cells. Total amounts of FLAG-tagged RAS proteins as well as α-tubulin were detected as loading controls. (B) Immunoblot of YAP and p-YAP at S127 of COS-7 cell lysate overexpressing wild-type ERAS and HRAS as well as the constitutive active variant of HRAS (HRASV12). α-tubulin was used as loading controls. (C) Immunoblot of YAP and p-YAP at S127 of quiescent (d0) and activated HSCs (d8). γ-tubulin served as a control. (D) qPCR analysis of MST1 and 2 as well as YAP and its target genes CTGF and NOTCH2 in quiescent (d0) vs. activated HSCs (d8).

**Figure 7.** DNA methylation analyses of ERAS in quiescent and activated primary HSCs. (A) Results of genome-wide DNA methylation analysis of quiescent HSCs (d0) and early activated HSCs (d3) at the ERAS promoter region. DNA methylation of individual CpG dinucleotides is depicted in percent (methylated CpG/total numbers of CpG) and displayed with a *color code from red (0%) to light green (100% DNA methylation). (B) ERAS promoter methylation analyzed by direct bisulfite sequencing exhibited a significant increase of DNA methylation during HSCs activation (n=3-5, t-test. *p<0.05). (C) HSC (day 3) were treated with 10 µM 5-AZA-2′-deoxycytidine (5-AZA) and/or 5 µM SAHA for 4 days and the expression of ERAS was analyzed with qPCR at day 8.

**Figure 8.** Schematic view of the proposed model on a reciprocal ERAS/RAS dependent signaling pathways in quiescent vs. activated HSCs (for details see discussion). ECM, extracellular matrix; ERAS, embryonic stem cell-expressed RAS; ERK, extracellular regulated kinase; FOXO1, forkhead transcription factor; HGF, hepatocyte growth factor; HSCs, hepatic stellate cell; IGF, insulin-like growth factor; JAK1, Janus kinase 1; LIF1, Leukemia inhibitory factor; MEK, MAP/ERK kinase; MMP, matrix metalloproteinases; mSin1, mammalian stress-activated MAP kinase-interacting protein 1; mTORC, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PDK1, 3-phosphoinositide-dependent protein kinase; PI3K, phosphoinositide 3-kinase; RAS, rat sarcoma; RASSF5, Ras-association domain family; SATB1, special AT-rich binding protein 1; SPRY1, sprouty homolog 1; STAT3, signal transducer and activator of transcription 3; TSC, tuberous sclerosis; YAP, Yes-associated protein.
Nakhaei-Rad et al., Figure 4
Nakhaei-Rad et al., Figure 5

A

B

C

D
Nakhai-Rad et al., Figure 6
Nakhaei-Rad et al., Figure 8
The role of embryonic stem cell-expressed RAS (ERAS) in the maintenance of quiescent hepatic stellate cells
Saeideh Nakhaei-Rad, Hossein Nakhaeizadeh, Silke Götzte, Claus Kordes, Iris Sawitza, Michèle J. Hoffmann, Manuel Franke, Wolfgang A. Schulz, Jürgen Scheller, Roland P. Piekarz, Dieter Häussinger and Mohammad R. Ahmadian

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