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RESEARCH ARTICLE

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The CGRP receptor component RAMP1 links sensory innervation with YAP activity in the regenerating liver

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Abstract

The effectiveness of liver regeneration limits surgical therapies of hepatic disorders and determines patient outcome. Here, we investigated the role of the neuropeptide calcitonin gene-related peptide (CGRP) for liver regeneration after acute or chronic injury. Mice deficient for the CGRP receptor component receptor activity-modifying protein 1 (RAMP1) were subjected to a 70% partial hepatectomy or repeated intraperitoneal injections of carbon tetrachloride. RAMP1 deficiency severely impaired recovery of organ mass and hepatocyte proliferation after both acute and chronic liver injury. Mechanistically, protein expression of the transcriptional coactivators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) was decreased in regenerating livers of RAMP1-deficient mice. Lack of RAMP1 was associated with hyperphosphorylation of YAP on Ser127 and Ser397, which regulates YAP functional activity and protein levels. Consequently, expression of various YAP-controlled cell cycle regulators and hepatocyte proliferation were severely reduced in the absence of RAMP1. In vitro, CGRP treatment caused increased YAP protein expression and a concomitant decline of YAP phosphorylation in liver tissue slice cultures of mouse and human origin and in primary human hepatocytes. Thus, our results indicate that sensory nerves represent a crucial control element of liver regeneration after acute and chronic injury acting through the CGRP-RAMP1 pathway, which stimulates YAP/TAZ expression and activity.

KEYWORDS

CGRP, liver regeneration, RAMP1, sensory innervation, YAP/TAZ

Abbreviations: CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; ICER, inducible cAMP early repressor; LATS, large tumor suppressor kinase; RAMP1, receptor activity-modifying protein 1; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif.

Melanie Laschinger and Yang Wang contributed equally to this work.

1 | INTRODUCTION

The liver has a unique capacity to restore its original organ mass and function even after a massive loss of tissue.[1-3] The high-regenerative capacity of the liver allows for the treatment of hepatic disorders by surgical procedures, such as resection of primary and metastatic tumors or transplantation using split-liver donor organs. However, the remnant liver mass and the effectiveness of the regenerative process to rapidly restore organ mass and function do not only limit surgical therapy of many liver diseases, but also determine patient recovery and outcome.[4,5]

In the liver, neurons containing immunoreactive calcitonin gene-related peptide (CGRP) form a dense network in the fibromuscular layer of the biliary tree, surround the portal vein and are present in the connective tissue of the portal areas.[6] The neuropeptide CGRP exists in two isoforms, αCGRP and βCGRP, which show high amino acid sequence similarity and appear to be functionally redundant.[7] Consistent with anatomical studies, physiological levels of the neuropeptide CGRP were detected in normal livers of rat and human origin.[8,9] CGRP expression was found to increase in rat livers after partial hepatectomy suggesting that CGRP might exert paracrine functions during liver regeneration.[8,9]

The CGRP receptor has been identified as a heterodimer that is composed of a heptahelical G protein-coupled receptor subunit termed calcitonin receptor-like receptor (CLR) and the small single membrane-spanning subunit receptor activity-modifying protein 1(RAMP1).[7] Both, receptor expression at the cell surface and ligand binding require dimerization of CLR and RAMP1. Structural studies have shown that the RAMP1 subunit of the receptor heterodimer confers selectivity for binding to CGRP.[10] In addition, the RAMP1/CLR heterodimer associates with a protein termed receptor component protein, which is not involved in CGRP binding, but appears to enhance intracellular coupling of CLR to G proteins for signal transduction.[11]

Signaling of the CGRP receptor occurs through the CLR subunit and involves coupling to different G proteins.[7,12] Association of the CGRP receptor with Gs proteins activates adenylyl cyclase, leading to increased cellular levels of cAMP and activation of protein kinase A (PKA).[13] Catalytic PKA subunits translocate to the nucleus and activate the transcription factor cAMP response element-binding protein (CREB). In immune cells, activated CREB may promote expression of IL-10 and the early cAMP-inducible repressor (ICER). In addition, the CGRP receptor couples to Gq/11 proteins resulting in the activation of the β isoforms of phospholipase C.[13] Activated phospholipase C influences cell proliferation through mechanisms that may involve activation of protein kinase C and extracellular-signal regulated kinases (ERK). RAMP1 signaling through Gq/11 was reported in epithelial cells, osteoblasts, and astrocytes, but was not observed in immune cells, which, instead, show Gs-dependent signaling.[12] CGRP was found to stimulate proliferation of human lung carcinoma cells in an ERK-dependent manner.[14] In addition, CGRP was shown to have a potent vasodilatory activity that is suppressed in blood vessels of RAMP1-deficient mice.[15] Cellular responses to CGRP may be terminated by endocytosis of the RAMP1/CLR heterodimer through clathrin-coated vesicles.

In the present study, we investigated the role of the CGRP receptor subunit RAMP1 for liver regeneration after partial hepatectomy and CCl_4-mediated injury. Our results suggest that RAMP1 promotes liver regeneration through an increased expression and activity of the paralogous transcriptional coactivators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). The role of the CGRP-RAMP1 axis for regulating YAP was also demonstrated in precision-cut liver tissue of mouse and human origin and in primary human hepatocytes. Together, our findings suggest that the sensory nervous system acting through the CGRP-RAMP1 pathway defines an important control element for the regeneration of liver tissue after acute and chronic injury.

2 | MATERIALS AND METHODS

2.1 | Animals

RAMP1-deficient mice[15] were backcrossed to the C57BL/6N background for at least 10 generations and maintained in a specified pathogen-free facility (Charles River, Calco, Italy). C57BL/6N wild-type control mice were obtained from Charles River. All animal experiments were institutionally approved by the government of Upper Bavaria (licenses 55.2-2532. Vet_02-15-147 and 55.2-2532.Vet_02-15-125) and were performed in accordance with the guidelines and regulations.

2.2 | Partial hepatectomy

Male mice at the age of 10-12 weeks were subjected to 70% partial hepatectomy according to standard procedures.[16] According to this protocol, ligation and resection of the median lobe and the left lateral lobe was performed separately. Mice were anesthetized with isoflurane. Partial hepatectomies were performed between 8 and 10 AM. After the indicated time periods, mice were sacrificed and remnant livers were collected for further analyses.

2.3 | CCl_4-mediated chronic liver injury

Liver injury was induced using a CCl_4 model.[17] Male mice at the age of 10-12 weeks were subjected to a 4-week regimen of biweekly injections of CCl_4 (0.6 μL/g body weight, intraperitoneally; Merck, Darmstadt, Germany) diluted in

corn oil 1:7. Animals were euthanized 48 hours after the last injection.

2.4 | Western blotting

Liver samples were homogenized in a lysis buffer containing 1% of Triton X100, 150 mM of NaCl, 20 mM of Tris-HCl pH 7.5, and protease and phosphatase inhibitors using a TissueLyser II instrument (Qiagen). Lysates (40 μg protein) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with the following primary antibodies: cyclin B1, cyclin E1, phosphorylated ATF2 (T71), phosphorylated CREB (S133), phosphorylated c-Jun (S73), phosphorylated ERK1/2 (T202/T204), phosphorylated p38 (T180/T182), phosphorylated STAT3 (Y705), phosphorylated Rb (S807/S811), YAP/TAZ, YAP, phosphorylated YAP (S127), phosphorylated YAP (S397), GAPDH, and β-actin (all from Cell Signaling); CDK1, cyclin A2, and β-tubulin (all from Abcam); cyclin D1 (Santa Cruz). Second stage horseradish peroxidase-conjugated goat-anti-rabbit was obtained from Jackson ImmunoResearch. Antibody binding was visualized using the Pierce ECL western blotting detection system (Thermo Fischer Scientific) and a ChemStudio Plus instrument (Analytik Jena). Densitometric analyses were performed using the ImageJ software (http://rsb.info.nih.gov/ij).

2.5 | Quantitative reverse transcriptase PCR

Liver samples were snap frozen in liquid nitrogen immediately after explantation and stored in −80°C until use. RNA was prepared using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized from 2 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR analyses were performed using the Universal Probe Library (Roche Diagnostics). The primers were: *Calca* sense, 5′-TGC AGG ACT ATA TGC AGA TGA AA-3′; *Calca* antisense, 5′-GGA TCT CTT CTG AGC AGT GAC A-3′; *Calcb* sense, 5′-CCT GCA GGC CTG AGT CAC-3′; *Calcb* antisense, 5′-GGC ATG GTG AGT TCA ACT TTA TG-3′; *Calcrl* sense, 5′-GCC AAT AAC CAG GCC TTA GTG-3′; *Calcrl* antisense, 5′-GCC CAT CAG GTA GAG ATG GA-3′; *Ccna2* sense, 5′-CTT GGC TGC ACC AAC AGT AA-3′; *Ccna2* antisense, 5′-CAA ACT CAG TTC TCC CAA AAA CA-3′; *Ccnb1* sense, 5′-GCT TAG CGC TGA AAA TTC TTG-3′; *Ccnb1* antisense, 5′-TCT TAG CCA GGT GCT GCA TA-3′; *Ccnd1* sense, 5′-TCT TTC CAG AGT CAT CAA GTG TG-3′; *Ccnd1* antisense, 5′-GAC TCC AGA AGG GCT TCA ATC-3′; *Foxm1* sense, 5′-AGC TAA GGG TGT GCC TGT TC-3′; *Foxm1* antisense, 5′-CTG TTG TCC AGC GTG CAG-3′; *Gna11* sense, 5′-CAC TGG CAT CAT CGA GTA CC-3′; *Gna11* antisense, 5′-GAT CCA CTT CCT GCG CTC T-3′; *Gnaq* sense, 5′-GAC TAC TTC CCA GAA TAT GAT GGA C-3′; *Gnaq* antisense, 5′-TCA GGA TGA ATT CTC GAG CTG-3′; *Gnas* sense, 5′-GCA GAA GGA CAA GCA GGT CT-3′; *Gnas* antisense, 5′-GCT TTT GCC AGA CTC TCC AG-3′; *Ramp1* sense, 5′-TGA CTA TGG GAC TCT CAT CCA G-3′; *Ramp1* antisense, 5′-CGT GCT TGG TGC AGT AAG TG-3′; *Socs3* sense, 5′-ATT TCG CTT CGG GAC TAG C-3′; *Socs3* antisense, 5′-AAC TTG CTG TGG GTG ACC AT-3′. RNA levels were normalized to those of β-actin and are depicted as relative quantification ($2^{-\Delta ct}$) or as fold difference relative to liver samples of untreated mice. Accumulation of PCR amplicons was quantified on a LightCycler 480 Real-Time PCR system (Roche Diagnostics).

2.6 | IL-6 serum levels

IL-6 protein concentrations in serum were determined using a Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.7 | Liver histology and immunohistochemistry

For detection of hepatocyte proliferation, mice were given a single, ip, injection of 100 μg/g BrdU (Roche Applied Science) 2 hours before sacrifice. Liver samples were fixed in 4% of paraformaldehyde followed by dehydration and embedded in paraffin. Tissue sections were incubated with BrdU-specific antibodies (Abcam) or anti-Ki-67 (BD Pharmingen). For visualization, horseradish peroxidase-labeled rabbit anti-mouse (Abcam) was used and detection was performed using the Bond Polymer Refine Detection Kit (DS9800) on a Bond Max staining robot (both from Leica). Individual specimens were stained with hematoxylin and eosin according to standard protocols. Stained sections were analyzed using an Axiolab attached to an AxioCamMRc5 5MPx camera, an CP-Achromat 5×/0.12 NA, an EC-Plan-Neofluar 10×/0.3 NA, and an Achroplan 20×/0.45 NA objective (all Zeiss Microscopy). For quantification, five random high-power fields for each animal were counted and the fraction of stained hepatocyte nuclei was calculated using the AxioVision Software (Zeiss Microscopy).

2.8 | Precision-cut liver tissue slices and primary human hepatocytes

All experiments with human liver samples were approved by the ethics committee at the Technical University of Munich. In addition, the study protocol conformed to the ethical guidelines

of the World Medical Association (WMA) Declaration of Helsinki. Research using human liver tissue was performed in accordance with the regulations of the ethics committee and the ethical guidelines of the WMA Declaration of Helsinki. Informed written consent was obtained from each patient.

Precision-cut liver tissue slices were prepared with minor modifications as described.[18] Briefly, murine liver tissues were embedded in 4% of low gelling agarose (Sigma-Aldrich) in PBS, and precision-cut tissue slices (200-300 μm) were prepared in ice-cold Custodiol using a vibratome (VT1000S, Leica). Liver tissue slices were incubated in HEPES-buffered William's Medium E (PAN-Biotech) in 24 well plates (Falcon) and were untreated or stimulated with 100 nM of αCGRP (Bachem) for the indicated time periods. During the incubation period, liver tissue slices were maintained on a rocking platform in a humidified incubator at 5% of CO_2 and 37°C. For further analysis, protein lysates were prepared as described above.

Primary human hepatocytes were isolated by perfusion of liver tissue via a central vessel. Digestion was accomplished using 0.05% of collagenase (Serva). Non-perfused parts were discarded using a 100 μm filter. Viable hepatocytes were enriched using a percoll gradient and viability was determined by trypan blue dye exclusion. Cells were seeded on plates coated with rat tail collagen (Collagen R, Serva) in Williams E medium containing insulin-transferrin-selenium, 1.5% of bovine serum albumin, GlutaMAX™, nonessential amino acids, 100 U/mL of penicillin/streptomycin (all supplements from Gibco) in the presence of 10% of FCS for 16 hours. Hepatocytes were maintained in medium lacking FCS for 2 to 5 days and stimulated with 100 nM of CGRP (Bachem) for the indicated time periods in a humidified incubator at 5% of CO_2 and 37°C.

2.9 | Statistical analysis

All data are presented as mean ± standard error. Statistical differences were analyzed using the two-tailed unpaired Student's *t* test or the Mann-Whitney test. A difference between experimental groups was considered significant for a *P* value less than .05.

3 | RESULTS

3.1 | RAMP1 deficiency impairs recovery of liver tissue and hepatocyte proliferation following partial hepatectomy

To elucidate the role of the sensory neuropeptide CGRP for liver regeneration, mice deficient for the specific CGRP receptor component RAMP1 were examined. Histomorphological analyses of livers, including hepatocytes, biliary ducts, blood vessels, and immune cell infiltration, did not reveal any differences between wild-type and RAMP1-deficient mice in the untreated state (Figure S1). Moreover, liver-to-body-weight ratios of untreated mice were not influenced by RAMP1 (Figure 1A). Thus, liver development appears normal in the absence of RAMP1.

The role of RAMP1 for liver regeneration was examined in a murine model of 70% hepatectomy. As shown in Figure S2, CGRP and its receptor components as well as G proteins associating with the CGRP receptor were regulated after an acute loss of liver tissue. We observed a significant and sustained upregulation of βCGRP, but not αCGRP. Hepatic levels of RAMP1 mRNA were elevated between 40 and 72 hours after partial hepatectomy, whereas expression of the shared CGRP receptor component CLR showed an early, but transient, increase at 2 hours. The G proteins Gαq and Gαs showed a biphasic regulation of mRNA expression (Figure S2). However, whereas Gαq was markedly upregulated, expression of Gαs showed an inverse response with a significant downregulation. In addition, Gα11 was transiently downregulated between 4 and 24 hours after partial hepatectomy.

Importantly, the recovery of liver mass after partial hepatectomy was severely impaired in the absence of RAMP1 (Figure 1A). Liver-to-body weight ratios were significantly reduced in RAMP1-deficient mice as compared with wild-type mice throughout an extended time period (24-168 hours) and only reached wild-type levels at the 336 hours time point. In contrast, wild-type mice showed a complete recovery of liver mass as early as at 168 hours after partial hepatectomy (Figure 1A).

The recovery of liver tissue requires extensive hepatocyte proliferation. In wild-type mice, the fraction of hepatocytes showing BrdU incorporation rapidly increased from 24 to 40 hours after partial hepatectomy and decreased thereafter (Figure 1B,C). In RAMP1-deficient mice, the fraction of hepatocytes showing active DNA synthesis remained at low levels until the 40 hours time point and only increased after 48 hours (Figure 1B,C). However, wild-type peak levels of BrdU incorporation were not reached in RAMP1-deficient livers at any time point tested (Figure 1B,C). Together, these results indicate that the lack of the CGRP receptor component RAMP1 severely impairs hepatocyte proliferation and markedly delays the recovery of liver tissue following partial hepatectomy.

3.2 | Reduced expression and activity of YAP/TAZ proteins in regenerating livers of RAMP1-deficient mice

The RAMP1/CLR receptor is known to signal through G proteins of the $G_{q/11}$ or G_S families,[19-21] which may act as regulators of the paralogous transcriptional coactivators YAP and TAZ.[22-24] Interestingly, the loss of YAP and TAZ leads to profound defects in liver regeneration.[25] Therefore, we addressed

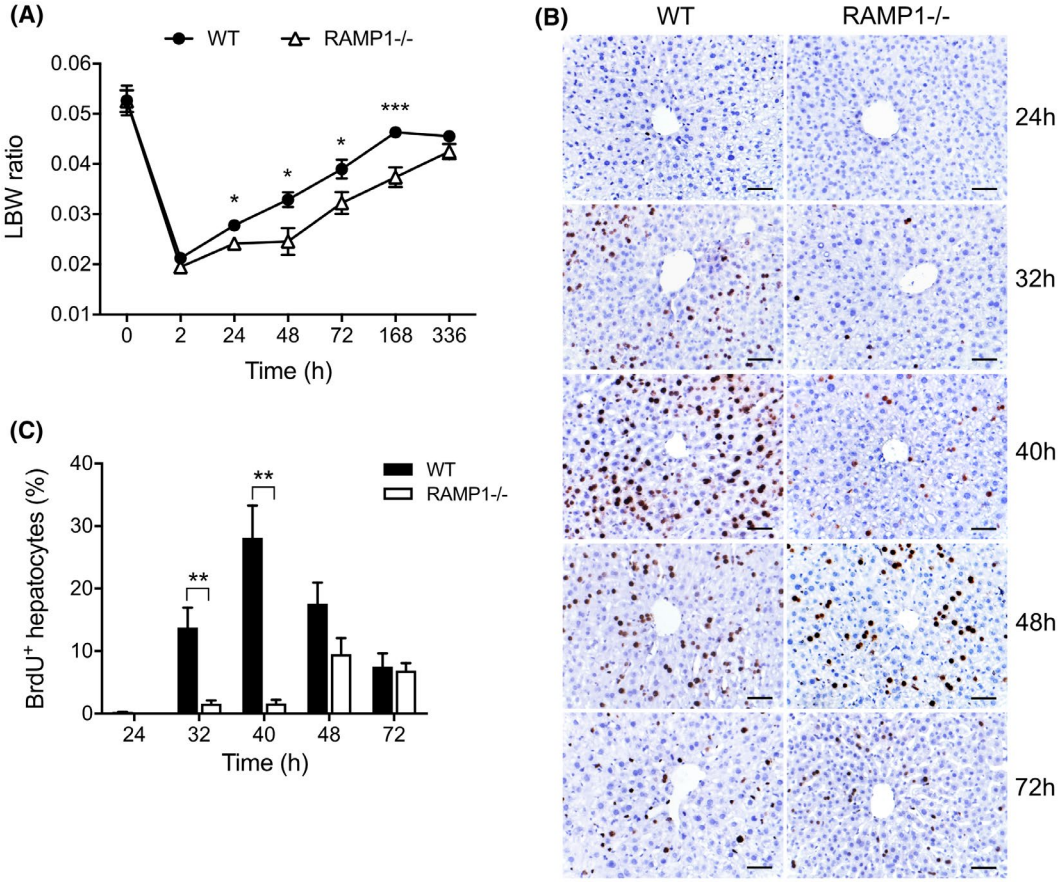

FIGURE 1 RAMP1 deficiency impairs recovery of liver tissue and hepatocyte proliferation following partial hepatectomy. A, Liver-to-body weight (LBW) ratios were determined at the indicated time points following partial hepatectomy (n = 6-9 mice per time point in each group). Representative images of immunohistochemical staining for BrdU (B) and the results of the quantification of BrdU-positive hepatocytes (C) are depicted (n = 5-8 mice per time point in each group). Scale bars indicate 50 μm. *$P < .05$, **$P < .01$, ***$P < .001$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

the hypothesis that RAMP1 may promote liver regeneration through influencing YAP/TAZ expression and/or activity. As shown in Figures 2 and 3, RAMP1 deficiency was associated with significantly reduced hepatic levels of both YAP and TAZ proteins at 32 and 40 hours following partial hepatectomy. YAP and TAZ proteins are known to be negatively regulated by phosphorylation events. While YAP phosphorylation on Ser127 causes its cytoplasmic retention and inactivation,[26] phosphorylation on Ser397 initiates the proteasome-mediated degradation of YAP.[27] It was therefore interesting to find out that reduced YAP protein levels correlated with a significant increase of YAP-Ser397 phosphorylation in RAMP1-deficient livers at 32 and 40 hours after partial hepatectomy (Figure 2). In addition, the fraction of Ser127-phosphorylated YAP was significantly elevated in RAMP1-deficient as compared with wild-type mice from 32 to 72 hours after hepatectomy suggesting diminished YAP activity for an extended time period (Figure 2). Thus, during liver regeneration after partial hepatectomy, the lack of RAMP1 results in a reduced expression of YAP and TAZ proteins and an enhanced phosphorylation of YAP on both Ser397 and Ser127.

Active YAP and TAZ function as potent stimulators of cell proliferation[28-30] by inducing the expression of numerous genes involved in cell cycle control, such as cyclins A and B, CDK1, and FoxM1.[31-33] Thus, we examined the expression of cell cycle regulators to address the question whether RAMP1 may also influence the functional activity of YAP and TAZ. The results in Figure 4A show that, in wild-type livers, cyclin E1 protein levels increased from 24 to 32 hours after partial hepatectomy and declined thereafter. In contrast, RAMP1-deficient mice failed to upregulate cyclin E1 in regenerating livers. Similarly, phosphorylation of Rb protein was strongly elevated in wild-type livers both 32 and 40 hours after partial hepatectomy, but was markedly attenuated in RAMP1-deficient mice at these time points (Figure 4A). Rb phosphorylation was comparable between both mouse strains at later time points, but levels in RAMP1-deficient livers did not reach wild-type peak values. CDK1 protein expression was also significantly decreased in livers of RAMP1-deficient as compared with wild-type mice at 40 and 48 hours after partial hepatectomy (Figure 4A). Moreover, upregulation of cyclin A2 and B1 as well as FoxM1 mRNA levels was substantially

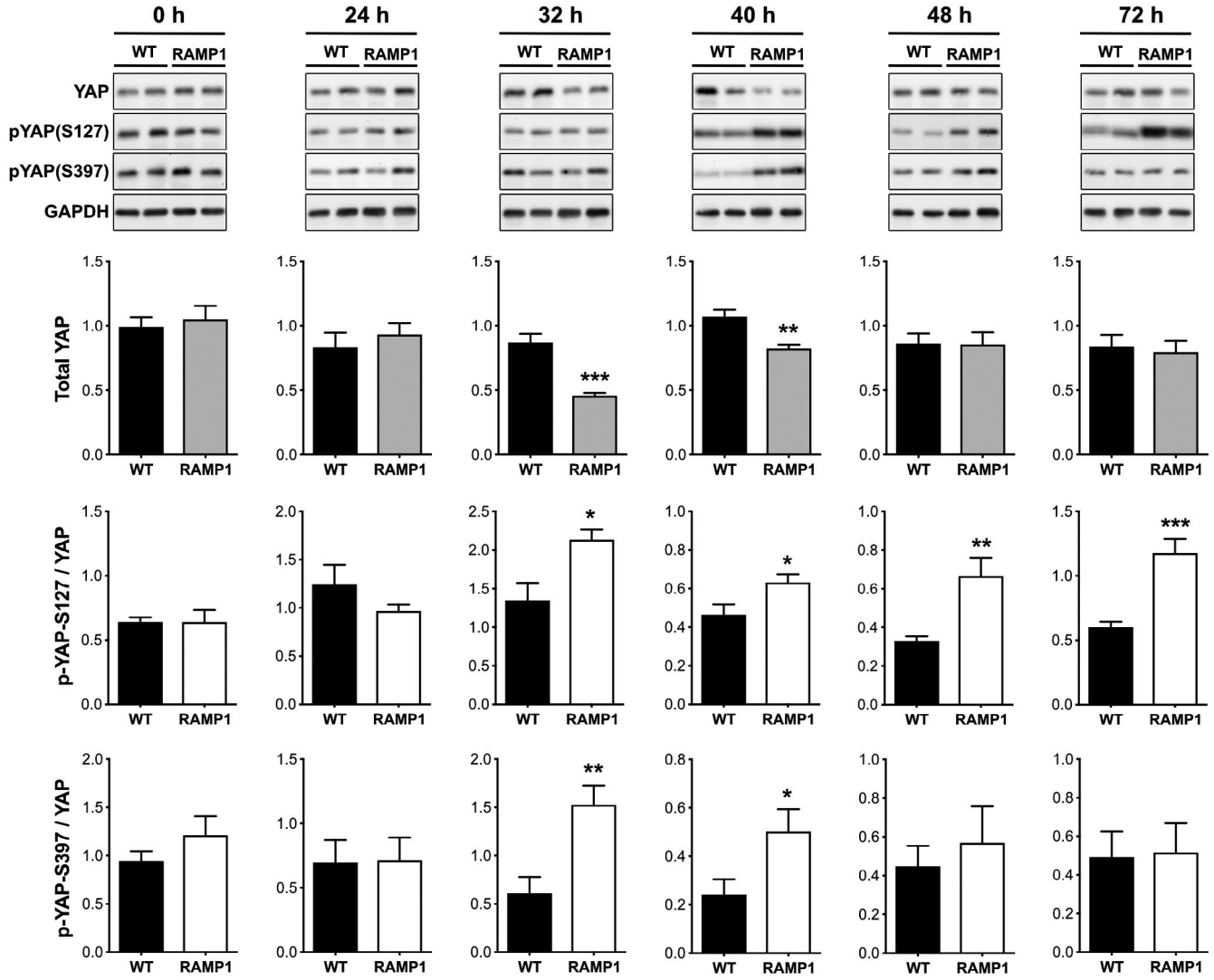

FIGURE 2 Livers of RAMP1-deficient mice show reduced YAP protein levels and increased YAP phosphorylation after partial hepatectomy. Liver samples were analyzed by Western blotting for YAP total protein expression. In addition, YAP phosphorylation on Ser127 or Ser397 was determined and normalized against total YAP protein levels. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from 5-6 independent mice were analyzed. $*P < .05$, $**P < .01$, $***P < .001$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

delayed and reduced in the absence of RAMP1 (Figure 4B). Together, these results are consistent with the conclusion that, in the absence of RAMP1, reduced expression of YAP/TAZ is also associated with an impaired functional activity of these transcriptional coactivators during liver regeneration.

3.3 | Influence of RAMP1 on the initial phase of liver regeneration after partial hepatectomy

Our results indicate that the sustained defect of RAMP1-deficient mice in recovering liver tissue after partial hepatectomy is linked with an impaired expression and activity of YAP/TAZ during the peak proliferation phase of hepatocytes. However, we also observed a reduced liver-to-body weight ratio in RAMP1-deficient mice even prior to this period (24 hours time point; Figure 1A) suggesting additional functions of RAMP1 during the initiation phase of liver regeneration.[9] We found that, in wild-type mice, expression of cyclin D1 protein, which is upregulated during the early G_1 phase of the cell cycle, was significantly reduced at the 12 and 24 hours time points in RAMP1-deficient as compared with wild-type livers (Figure 5A). Cyclin D1 mRNA expression showed a biphasic regulation in wild-type livers with a first increase at 8 hours followed by a decline to baseline levels, and a second increase between 32 and 72 hours after partial hepatectomy (Figure 5B). Notably, the early, but not the late, increase in cyclin D1 mRNA levels was absent in RAMP1-deficient livers (Figure 5A). The cyclin D1

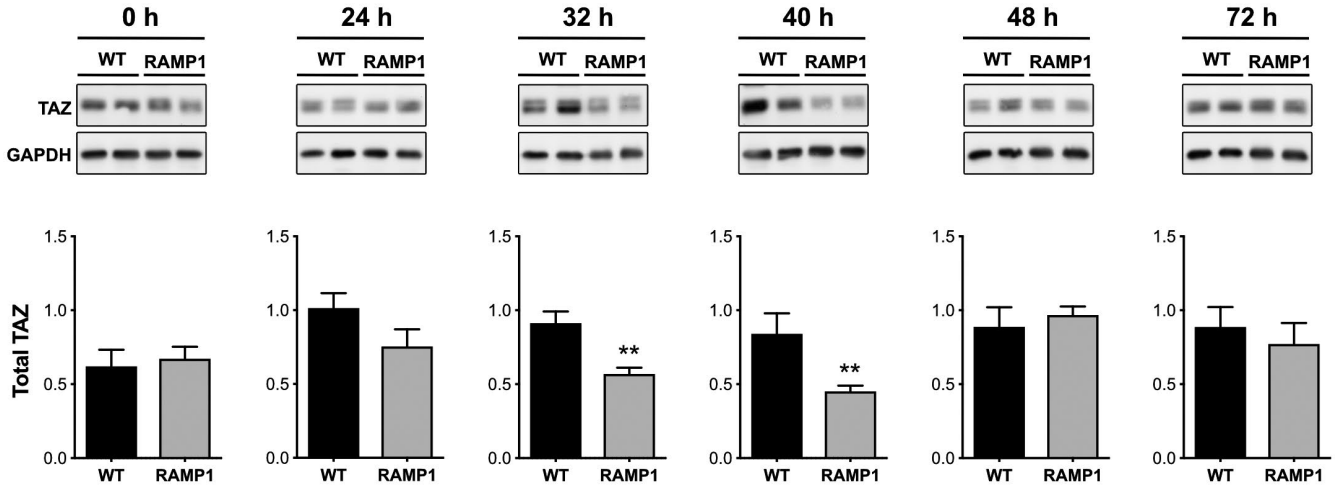

FIGURE 3 Reduced TAZ protein levels in livers of RAMP1-deficient mice. Liver lysates were analyzed by Western blotting for TAZ protein expression. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from 5-6 independent mice were analyzed. $^{**}P < .01$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

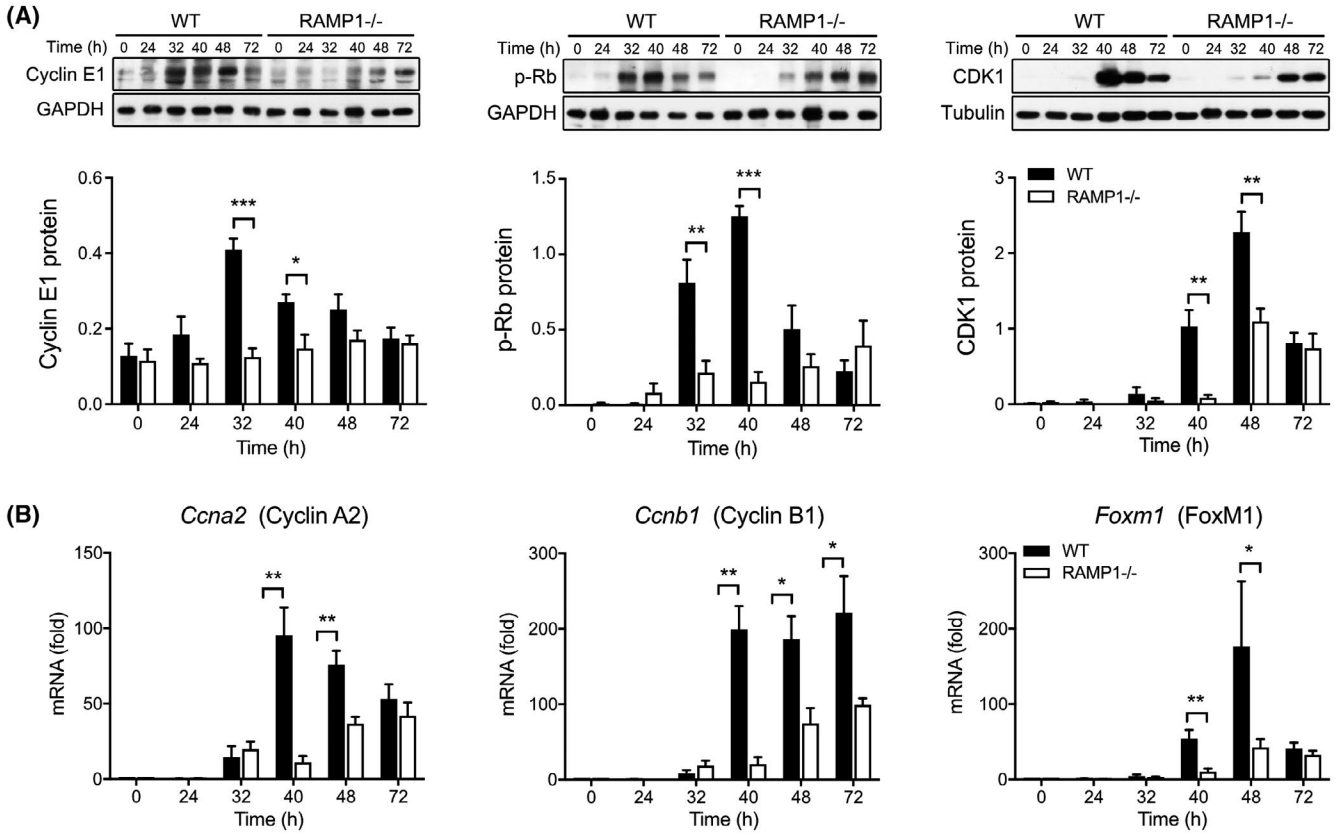

FIGURE 4 Hepatic cell cycle progression is impaired in RAMP1-deficient mice after partial hepatectomy. A, Total protein extracts of liver samples were analyzed by Western blotting using antibodies against cyclin E1, phosphorylated Rb protein, and CDK1. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from 5-6 independent mice were analyzed. B, Hepatic mRNA levels of cyclin A2 (*Ccna2*), cyclin B1 (*Ccnb1*), and FoxM1 (Foxm1) after partial hepatectomy are depicted as fold difference relative to untreated controls. For each time point and in each group samples from 5-6 independent mice were analyzed. $^{*}P < .05$, $^{**}P < .01$ $^{***}P < .001$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

promoter contains an evolutionary conserved CRE site that was found to bind ATF2/CREB or ATF2/c-Jun transcription factor complexes.[34] As shown in Figure 5C, phosphorylation of ATF2, but not CREB or c-Jun, was significantly reduced in RAMP1-deficient livers 8 hours after partial hepatectomy. ATF2 may be activated by sequential phosphorylation events

involving the MAP kinases ERK1/2 and p38.[35] Notably, 4 hours after partial hepatectomy and preceding ATF-2 activation, phosphorylation of both ERK1/2 and p38 was significantly reduced in RAMP1-deficient as compared with wild-type mice (Figure 5C). Thus, RAMP1 appears to promote the transcriptional upregulation of cyclin D1 during the initiation phase of liver regeneration through a mechanism involving activation of MAP kinases and ATF2.

RAMP1 is also known as a potent modulator of innate immune responses,[20] and thereby, could influence the onset of hepatocyte proliferation through this activity.[1-3] As shown in Figure 5D, serum levels of IL-6 were transiently elevated

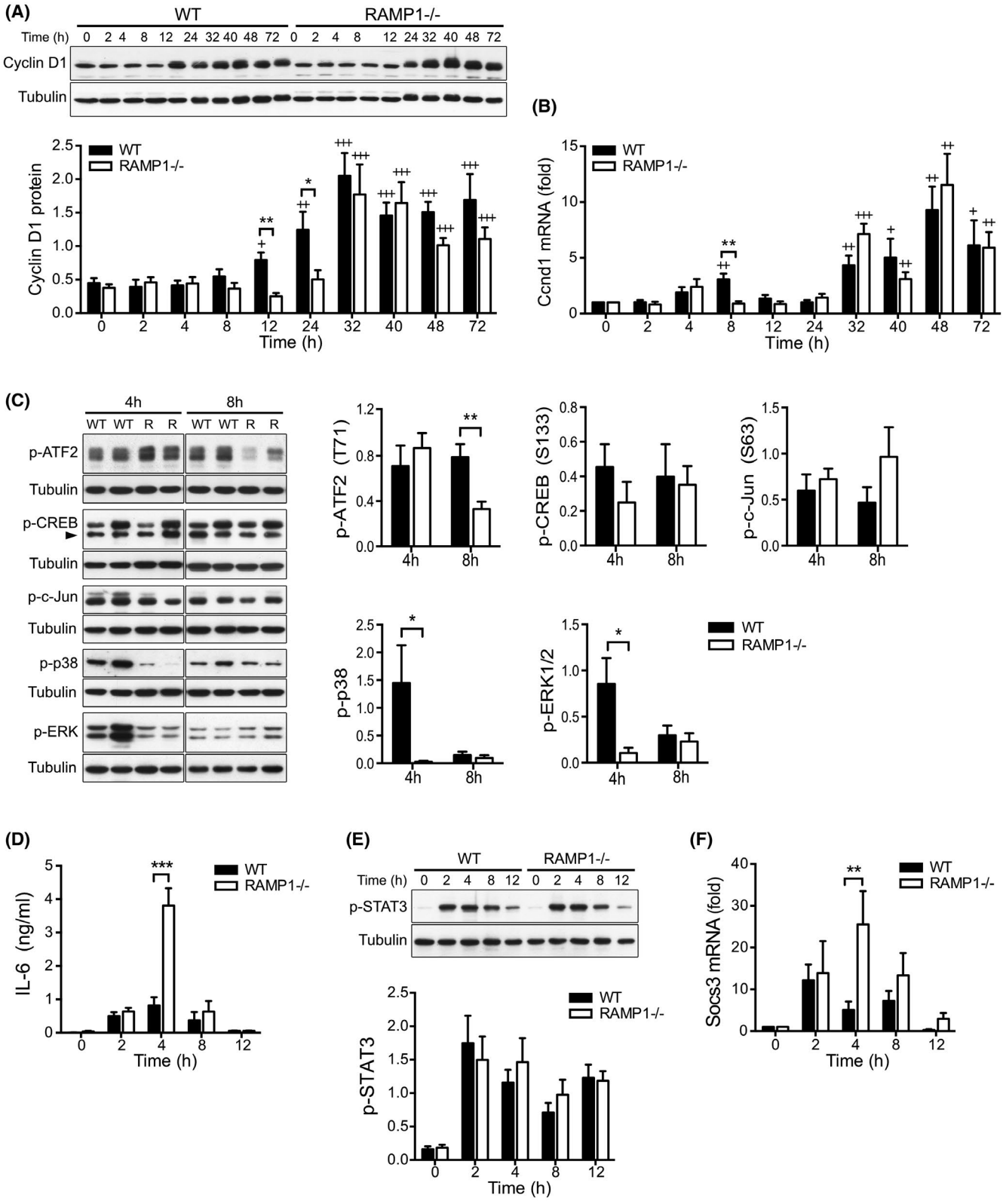

FIGURE 5 Lack of RAMP1 results in delayed induction of cyclin D1 that is linked with an impaired activation of MAP kinases and ATF2. A, Total protein extracts of liver samples were analyzed for cyclin D1 expression. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from six independent mice were analyzed. B, Hepatic mRNA expression of cyclin D1 (*Ccnd1*) following partial hepatectomy is depicted as fold difference relative to untreated controls. For each time point and in each group samples from 4-8 independent mice were analyzed. C, Total protein extracts of liver samples were analyzed by Western blotting using the indicated antibodies. The arrow head marks p-ATF-1, which is also recognized by the p-CREB antibody. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from 5-8 independent mice were analyzed. D, Systemic levels of IL-6 were measured using serum samples from 4-6 independent mice for each time point and group. E, Hepatic levels of phosphorylated STAT3 were determined by Western blotting. Representative gels and densitometric analyses are depicted. For each time point and in each group samples from 4-6 independent mice were analyzed. F, Expression of SOCS3 mRNA in livers following partial hepatectomy is depicted as fold difference relative to untreated livers. For each time point and in each group samples from 5-8 independent mice were analyzed. $*P < .05$, $**P < .01$, $***P < .001$ (WT vs RAMP1$^{-/-}$); $^{+}P < .05$, $^{++}P < .01$, $^{+++}P < .001$ (hepatectomy vs 0 hours)

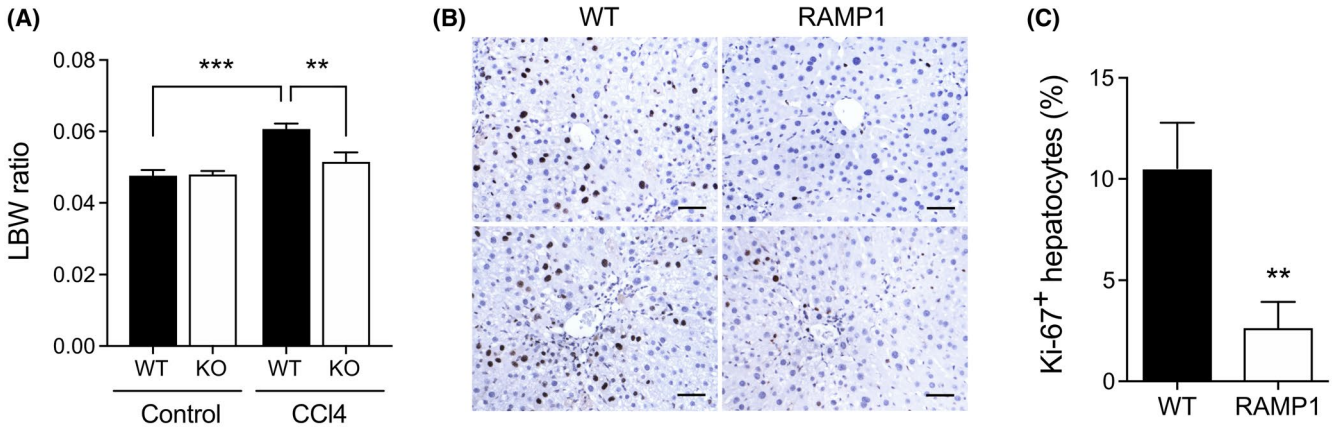

FIGURE 6 Lack of RAMP1 leads to reduced liver mass and hepatocyte proliferation upon CCl_4 treatment. A, Liver-to-body weight (LBW) ratios were determined in the untreated state and following CCl_4 treatment (n = 5-9 mice in each group). Representative images of immunohistochemical staining for Ki-67 (B) and the results of the quantification of Ki-67-positive hepatocytes (C) are depicted (n = 8-9 mice per time point in each group). Scale bars indicate 50 μm. $**P < .01$, $***P < .001$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

in RAMP1-deficient as compared with wild-type mice at the 4 hours time point. However, phosphorylation of STAT3, which mediates signal transduction through the IL-6 receptor, was not influenced by the absence of RAMP1 (Figure 5E) suggesting that RAMP1 does not affect STAT3-dependent cytokine responses. This finding may be explained by an elevated expression of SOCS3 in regenerating livers of RAMP1-deficient mice (Figure 5F), because SOCS3 is known to inhibit hepatectomy-induced phosphorylation of STAT3.[36]

3.4 | Lack of RAMP1 is linked with impaired hepatocyte proliferation and enhanced YAP phosphorylation following chronic liver injury

To elucidate whether RAMP1 may have a more general role for regulating the expression and/or activity of YAP/TAZ during liver regeneration, chronic liver injury was induced by biweekly injections of the hepatocellular toxin CCl_4 for 4 weeks. The CCl_4 treatment caused a significant upregulation of αCGRP and RAMP1 mRNA levels, but did not affect expression of βCGRP and CLR (Figure S3), confirming earlier studies where increased αCGRP or RAMP1 mRNA expression levels were observed upon bile duct ligation or CCl_4-induced liver cirrhosis.[37-39] In addition, expression of Gα11, but not of Gαq and Gαs was significantly increased after CCl4 treatment of mice (Figure S3). Importantly, liver-to-body weight ratios were significantly increased after 4 weeks of CCl_4 administration in wild type, but not RAMP1-deficient mice, resulting in a markedly reduced relative liver mass of CCl_4-treated RAMP1-deficient mice as compared with wild-type controls (Figure 6A). To further examine the proliferative response of hepatocytes, expression of Ki-67 was quantified. As shown in Figure 6B,C, substantial hepatocyte proliferation was observed in wild-type mice, which may compensate for the repetitive loss of cells caused by CCl_4 administration. However, in RAMP1-deficient mice, hepatocyte proliferation in response to CCl_4 treatment was markedly diminished (Figure 6B,C). Consistent with these findings, hepatic expression of the cell cycle regulators cyclin A2, cyclin B1, and CDK1 was significantly reduced in the absence of RAMP1 (Figure 7A,B).

Cyclins A and B as well as CDK1 may be regulated by YAP/TAZ[31-33] and our findings in the partial hepatectomy model

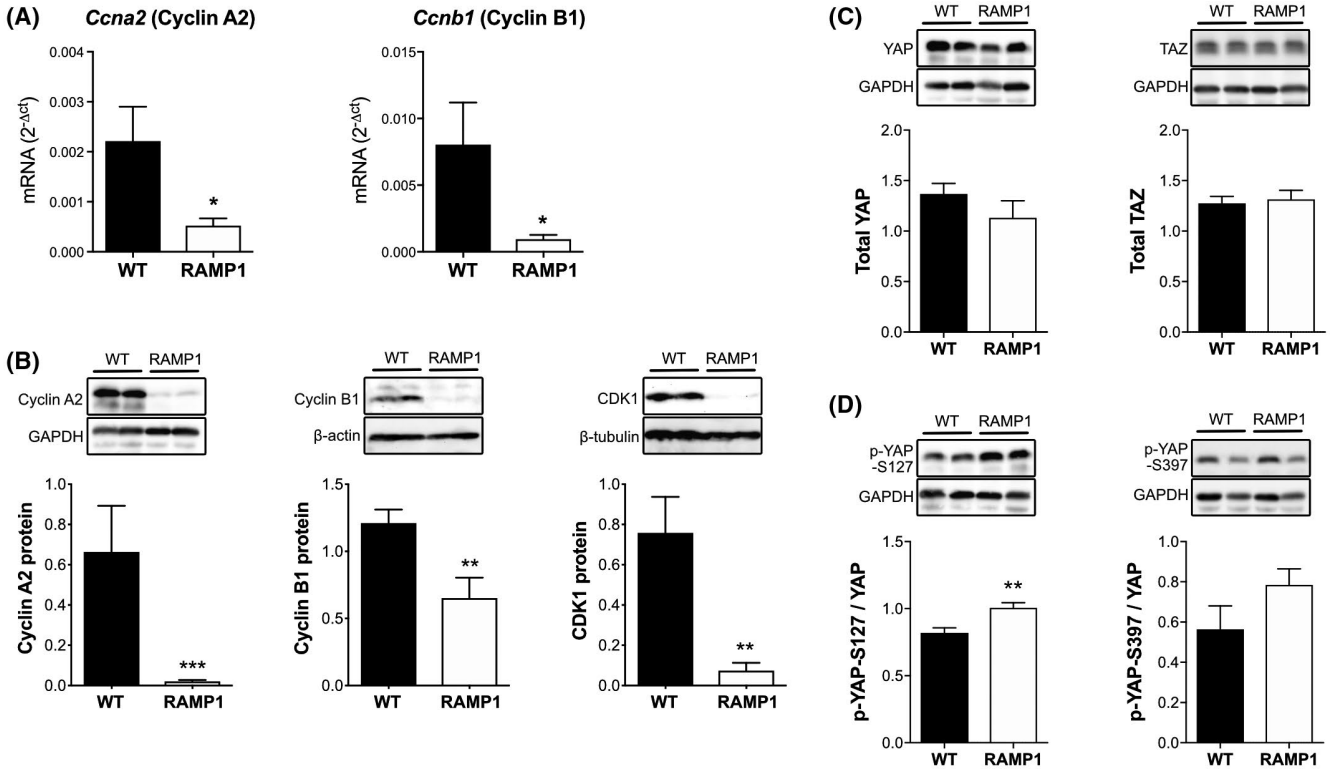

FIGURE 7 Impaired hepatic cell cycle progression and enhanced YAP phosphorylation in RAMP1-deficient mice after CCl_4 treatment. A, Hepatic mRNA levels of cyclin A2 (*Ccna2*) and cyclin B1 (*Ccnb1*) were determined after CCl_4 treatment and are depicted as relative quantification. Total protein extracts of liver samples were analyzed by Western blotting using antibodies against cyclin A2, cyclin B1, and CDK1 (B) or YAP and TAZ (C). In addition, YAP phosphorylation on Ser127 or Ser397 was determined and normalized against total YAP protein levels (D). Representative gels and densitometric analyses are depicted (n = 8-9 mice per group). $^*P < .05$, $^{**}P < .01$ $^{***}P < .001$ (two-tailed unpaired Student's *t* test or Mann-Whitney U test)

indicate that RAMP1 modulates YAP/TAZ expression and activity. Therefore, we analyzed the influence of RAMP1 on the expression and phosphorylation of YAP/TAZ after chronic liver injury caused by CCl_4. As shown in Figure 7C,D, the lack of RAMP1 did not influence total protein levels of YAP and TAZ or YAP-Ser397 phosphorylation in livers of CCl_4-treated mice. It should be noted, however, that phosphorylation of YAP on Ser127 was significantly upregulated in RAMP1-deficient as compared with wild-type livers (Figure 7D). Importantly, Ser127 phosphorylation of YAP was previously linked with cytoplasmic retention and functional inactivation of YAP.[26] Therefore, these data suggest that, in chronic CCl_4-mediated liver injury, RAMP1 is required for normal YAP activity rather than for maintaining YAP protein levels.

3.5 | CGRP treatment upregulates YAP protein expression and reduces YAP phosphorylation in liver tissue and primary hepatocytes

To further examine whether the sensory neuropeptide CGRP directly controls YAP expression and activity through RAMP1 signaling, precision-cut liver slices were used as an in vitro organ model.[18] The results depicted in Figure 8A demonstrate that stimulation of wild-type murine liver slices with CGRP resulted in a time-dependent and significant increase of YAP protein levels. In parallel, phosphorylation of YAP on both Ser127 and Ser397 was significantly decreased by the CGRP treatment (Figure 8A). Analysis of liver tissue slices derived from RAMP1-deficient mice confirmed that the stimulatory effect of CGRP on YAP protein expression was dependent on the presence of RAMP1 (Figure 8B). Importantly, upregulation of YAP protein expression and downregulation of YAP phosphorylation in response to CGRP was also demonstrated using precision-cut organ slices derived from liver samples of human patients (Figure 8C). Thus, the CGRP-RAMP1 pathway enhances the expression of active YAP protein both in murine and human liver tissue.

In additional experiments, primary hepatocytes were isolated from liver tissue of human patients and stimulated with CGRP. Consistent with the results obtained from precision-cut liver slices, we found that treatment of primary hepatocytes with CGRP also caused an increase in total YAP protein expression that was linked with a reduced phosphorylation of

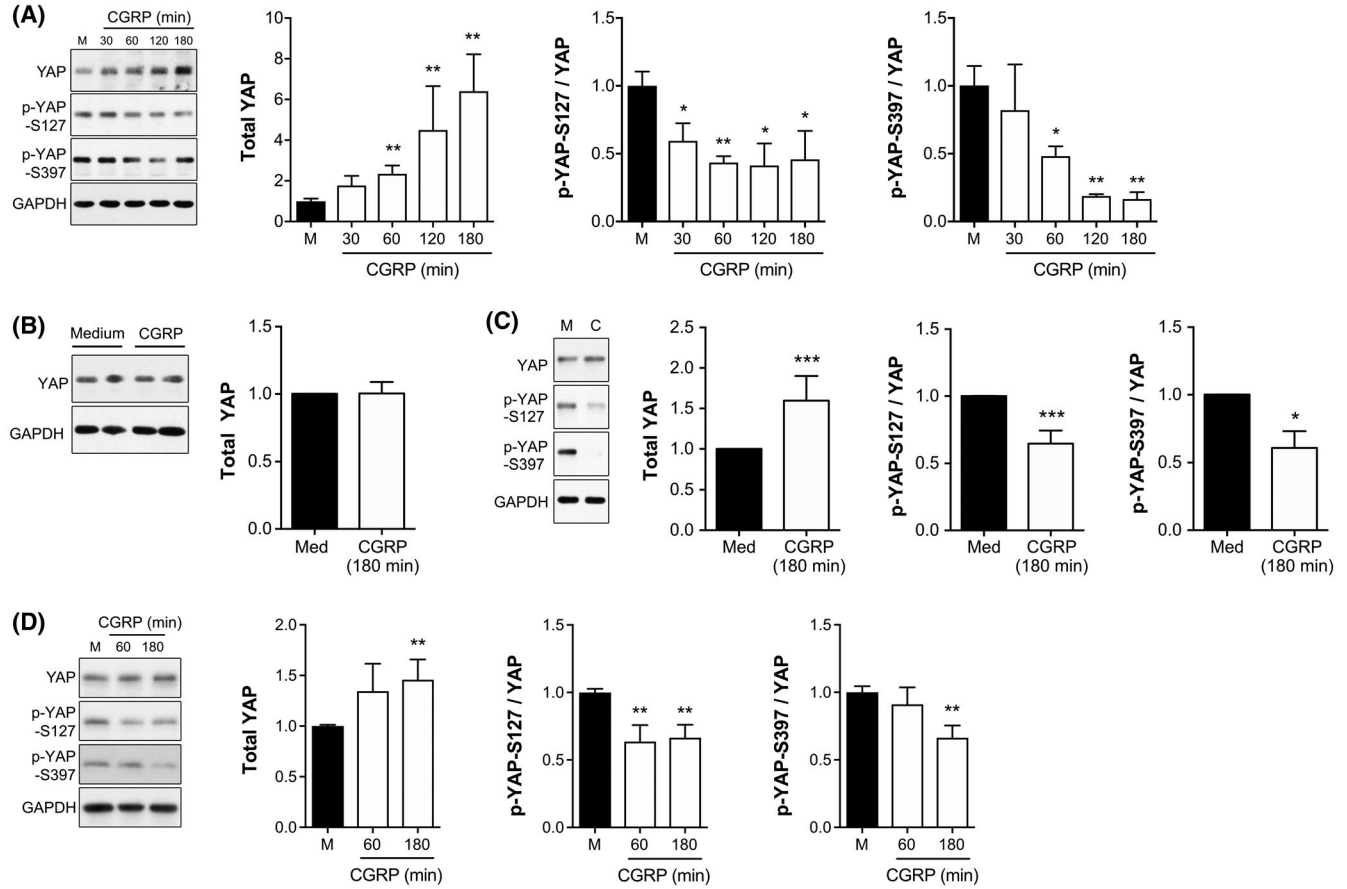

FIGURE 8 CGRP treatment enhances YAP protein expression and attenuates YAP phosphorylation in liver tissue and primary hepatocytes. Precision-cut liver slices derived from wild-type mice (A), RAMP1-deficient mice (B), or human patient samples (C), as well as primary human hepatocytes (D) were stimulated with 100 nM of CGRP for the indicated time periods. Total protein extracts were analyzed by Western blotting for the expression of YAP and phosphorylation of YAP on Ser127 or Ser397. Levels of YAP phosphorylation were normalized against total YAP protein levels. Representative gels and densitometric analyses are depicted. The results are derived from four independent experiments, respectively. $*P < .05$, $**P < .01$ (CGRP stimulation vs medium control; two-tailed unpaired Student's *t* test or Mann-Whitney U test)

YAP on Ser127 and Ser397 (Figure 8D). Together, these results therefore demonstrate that stimulation of RAMP1 signaling by the agonistic neuropeptide CGRP directly leads to attenuated phosphorylation and increased protein expression of YAP both in an in vitro organ culture model and in isolated primary hepatocytes.

4 | DISCUSSION

The liver contains a dense network of dorsal root sensory neurons expressing the neuropeptide CGRP.[6] In the present report, we provide evidence that CGRP signaling depending on the receptor component RAMP1 is crucial for liver regeneration after both acute and chronic organ damage. Deficiency of RAMP1 caused a prolonged defect in the recovery of liver tissue and hepatocyte proliferation following an acute loss of tissue due to partial hepatectomy. When chronic liver injury was induced by repetitive CCl_4 administration, hepatocyte proliferation was also markedly impaired in the absence of RAMP1. Mechanistically, we found RAMP1 to be required for normal protein expression and functional activity of the paralogous transcriptional coactivators YAP and TAZ during the peak proliferation phase of hepatocytes. Evidence is provided that the CGRP-RAMP1 axis stimulates YAP expression not only in murine livers, but also in human liver tissue and primary hepatocytes. Thus, the results of the present report link the sensory innervation of the liver and the neuropeptide CGRP with the expression and activity of YAP and TAZ during liver regeneration.

YAP and TAZ regulate the expression of genes important for the control of cell proliferation and survival.[28,30] Active YAP appears to be important for the sustained expression of cell cycle genes in hepatocytes,[40] and genetic ablation of YAP and/or TAZ impairs hepatocyte proliferation during liver regeneration.[25,41] The findings of the present study suggest that sensory nerves are able to control YAP/TAZ-mediated hepatocyte proliferation via the CGRP-RAMP1 signaling pathway. We demonstrate that following partial hepatectomy, deficiency of the CGRP receptor component

RAMP1 results in a markedly attenuated expression of YAP and TAZ during the peak of the proliferation phase. Reduced YAP protein levels may be explained by an increase of YAP phosphorylation at Ser397, which was reported to enhance ubiquitination and proteasome-mediated degradation of YAP.[27] In hepatectomized mice lacking RAMP1, YAP was also hyperphosphorylated on Ser127 both during the proliferation phase and at later time points. In CCl_4-treated mice, deficiency of RAMP1 did not alter expression of YAP/TAZ or Ser397 phosphorylation of YAP, but resulted in a significant increase of YAP-Ser127 phosphorylation. Because, Ser127 phosphorylation of YAP is linked with functional inactivation through cytoplasmic retention,[26] these findings strongly suggest that the absence of RAMP1 causes a prolonged reduction of YAP function after both acute and chronic liver injury. This assumption is further strengthened by our findings showing that expression levels of cell cycle regulators, such as cyclins A and B, CDK1, and FoxM1, which are known transcriptional targets of YAP/TAZ,[31-33] are reduced in RAMP1-deficient mice.

Previous studies have identified various pathways that may regulate the transcriptional coactivators YAP and TAZ during liver regeneration. Whereas integrin-linked kinase and glypican-3 may function as negative regulators of hepatic YAP activity,[42,43] hedgehog signaling in hepatic stellate cells was reported to enhance YAP activity in hepatocytes.[44] In the present study, we identify the sensory nervous system acting through the CGRP-RAMP1 pathway as a previously unknown regulator of YAP activity in the regenerating liver. Regenerating livers of RAMP1-deficient mice exhibit a reduced YAP/TAZ protein expression, an increased phosphorylation of YAP and an impaired expression of YAP-controlled cell cycle regulators. Importantly, experiments with murine and human precision-cut liver slices and primary human hepatocytes directly demonstrated that in vitro stimulation of tissues and cells with the RAMP1 agonistic neuropeptide CGRP caused an upregulation of YAP that was associated with an attenuated YAP phosphorylation. Hence, the present report identifies the CGRP-RAMP1 pathway as a novel inducer of YAP expression and activity in the regenerating liver.

YAP is known to be regulated by signaling through G protein-coupled receptors. Thus, the Gα subunits $G_{12/13}$ and $G_{q/11}$ were found to stimulate the activity of YAP,[22,24] while signaling through the G_S subunit was reported to repress YAP function.[22,23] The receptor for CGRP belongs to the class-B family of G protein-coupled receptors.[19,20] It has been shown that the RAMP1/CLR receptor complex may associate with Gs and Gq/11, but not with G12/13 proteins. RAMP1/CLR signaling in epithelial cells was reported to occur through Gq/11 proteins leading to activation of phospholipase C-β.[13] In immune cells, RAMP1/CLR signaling was found to involve Gs proteins, which cause increased cellular cAMP levels and an activation of PKA. In the present study, we show that RAMP1 directly stimulates the expression and activity of YAP during liver regeneration after acute and chronic injury. In addition, we found that hepatectomy causes a biphasic upregulation of Gαq proteins and a parallel downregulation of Gαs, while hepatic Gα11 expression is increased after CCl4 treatment of mice. Thus, our findings suggest that RAMP1 activates YAP through a mechanism that involves Gq/11 protein-mediated signaling. Studies showing that signaling through Gq/11 proteins enhances cell cycle progression during liver regeneration support this concept.[45]

We also found RAMP1 to be required for the upregulation cyclin D1 mRNA and protein during the initial phase of liver regeneration after partial hepatectomy, when hepatocytes first enter the G_1 phase of the cell cycle. Mechanistically, the lack of early cyclin D1 induction in RAMP1-deficient mice was associated with a reduced phosphorylation of ERK1/2 and p38 that was followed by an impaired activation of ATF-2. Attenuated cyclin D1 induction and impaired ERK1/2 phosphorylation following partial hepatectomy were previously observed in rats treated with the RAMP1 antagonist iCGRP.[9] However, whereas Mizutani and coworkers reported that iCGRP treatment of rats attenuated expression of c-Fos, c-Jun, and c-Myc, we found, instead, an impaired activation of ATF2. It is conceivable that these differences may be related to differences in the species investigated and the approaches used to block RAMP1 activity. It seems important to note, however, that the lack of RAMP1 affected cyclin D1 expression only at the very first entry of hepatocytes into the proliferation phase, but not at later time points suggesting that regulation of early cyclin D1 expression may not represent a major mechanism to explain the long-term influence of RAMP1 on liver regeneration. Considered together, these findings therefore support the concept that CGRP-RAMP1 signaling may promotes liver regeneration after partial hepatectomy in a biphasic manner. During the initial phase, the CGRP-RAMP1 pathway may contribute to hepatocyte cell cycle entry through induction of ERK1/2 and cyclin D, whereas, during the subsequent phase of major hepatocyte proliferation, it may be crucial for sustained hepatocyte cell cycle progression and active proliferation through induction of YAP/TAZ expression and activity.

In summary, our results identify the CGRP-RAMP1 pathway as a novel inducer of the expression and activity of the transcriptional coactivators YAP and TAZ that operates during liver regeneration after acute and chronic organ injury. Genetic inactivation of the CGRP receptor component RAMP1 severely impairs the recovery of liver tissue, the proliferation of hepatocytes and the induction of important cell cycle regulators. Using primary cultures of liver tissue and isolated hepatocytes of both mouse and human origin, CGRP was directly demonstrated to increase YAP protein

expression and to attenuate inhibitory YAP phosphorylation. Thus, the present study supports the concept that the sensory nervous system acting through the CGRP-RAMP1 pathway represents a crucial control element of liver regeneration that may also be relevant in patients with liver disease.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

B. Holzmann, M. Laschinger, N. Hüser, D. Hartmann designed research; M. Laschinger, Y. Wang, G. Holzmann, B. Wang, C. Stöß, M. Laschinger, M. Brugger, A. Schneider, S. Schulze, F. Altmayr performed research; B. Holzmann, M. Laschinger, Y. Wang, P. Knolle, K. Tsujikawa, D. Wohlleber, K. Steiger, H. Friess analyzed data; B. Holzmann, M. Laschinger, N. Hüser, D. Hartmann, Y. Wang wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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