THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 7, pp. 3346–3358, February 12, 2016

SOCS2 Balances Metabolic and Restorative Requirements during Liver Regeneration*

Received for publication, November 9, 2015, and in revised form, December 16, 2015 Published, JBC Papers in Press, December 23, 2015, DOI 10.1074/jbc.M115.703264

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After significant injury, the liver must maintain homeostasis during the regenerative process. We hypothesized the existence of mechanisms to limit hepatocyte proliferation after injury to maintain metabolic and synthetic function. A screen for candidates revealed suppressor of cytokine signaling 2 (SOCS2), an inhibitor of growth hormone (GH) signaling, was strongly induced after partial hepatectomy. Using genetic deletion and administration of various factors we investigated the role of SOCS2 during liver regeneration. SOCS2 preserves liver function by restraining the first round of hepatocyte proliferation after partial hepatectomy by preventing increases in growth hormone receptor (GHR) via ubiquitination, suppressing GH pathway activity. At later times, SOCS2 enhances hepatocyte proliferation by modulating a decrease in serum insulin-like growth factor 1 (IGF-1) that allows GH release from the pituitary. SOCS2, therefore, plays a dual role in modulating the rate of hepatocyte proliferation. In particular, this is the first demonstration of an endogenous mechanism to limit hepatocyte proliferation after injury.

Control of liver regeneration requires both stimulatory and inhibitory factors (1–11). Although it might be expected that stimulatory factors are induced and inhibitory factors repressed after partial hepatectomy, in fact, for certain genes, the reverse occurs. For example, suppressor of cytokine signaling 3 (SOCS3),[2] an inhibitor of liver regeneration, is induced after partial hepatectomy (2). This observation raises the possibility that mechanisms exist to limit the rate of liver regeneration after injury. What is the reason for this? One possible explanation considers that metabolic activity may be impaired in proliferating compared with non-proliferating hepatocytes. If true, after severe injury liver regeneration might need to be limited to a rate that balances the acute need to maintain the diverse functions of the liver and prevent the death of the organism with the long-term need for hepatocytes to proliferate to restore mass and maximum functional capacity.

Growth hormone (GH) signaling plays a central role in liver regeneration and may provide clues to how the rate of hepatocyte proliferation after injury is precisely regulated. Mice lacking GH activity display a diminished response to hepatectomy and do not restore liver mass even 7 days after partial hepatectomy (12). Loss of growth hormone receptor (GHR) impairs liver regeneration, and mice lacking Stat5, a transducer of GH signaling, demonstrate impaired hepatocyte proliferation after partial hepatectomy (13, 14).

GH is produced in the pituitary, stored in granules, and released into the circulation in response to growth hormone releasing hormone. This process is inhibited by a number of factors, including insulin-like growth factor 1 (IGF-1), which is produced primarily in the liver, and somatostatin (15).

In target tissues, GH binds to the GHR and modulates phosphorylation of important intracellular messengers including JAK2, signal transducer and activator of transcription 5b (STAT5b), STAT3, Erk1/2, and Akt (13).

Our preliminary studies demonstrated dramatic up-regulation of SOCS2 after hepatectomy (16), and we chose it for further study due to its important role inhibiting GH signaling and the apparent paradox that an inhibitor of proliferation would be dramatically induced after liver injury. First identified based on their ability to regulate cytokine signaling, SOCS genes function as feedback inhibitors for cytokine, growth factor, and hormone signaling (17, 18). Of the eight members of this family, SOCS2 is the most important regulator of GH action and critically influences organ and tissue size. SOCS2 inhibits GH signaling by targeting the GHR for ubiquitination and degradation. Loss of SOCS2 leads to gigantism (19–21).

In this study, we provide evidence that SOCS2 is induced early after liver injury to slow the rate of hepatocyte proliferation to allow the liver to meet metabolic demands. Further analysis demonstrates that liver regeneration can be divided into distinct phases: an early phase comprising the first round of hepatocyte proliferation in which SOCS2 inhibits hepatocyte proliferation by decreasing GHR levels, and a later phase in which SOCS2 enhances hepatocyte proliferation by modulating IGF-1 production in the liver, which in turn stimulates release of GH from the pituitary.

* This work was supported by National Institutes of Health Grant 7R01DK081387 (to S. J. K.) and Vanderbilt University Medical Center. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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[2] The abbreviations used are: SOCS, suppressor of cytokine signaling; GH, growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor 1; STAT, signal transducer and activator of transcription.

Experimental Procedures

Mice—C57BL/6 mice were purchased from Jackson Laboratories. *Socs2*-null mice were generously provided by Dr. Chris Greenhalgh and maintained on a C57BL/6 background backcrossed more than 20 times. Genotyping was performed as previously described (22).

Partial Hepatectomy—All surgeries were performed based on the National Institutes of Health (NIH) guidelines for the humane treatment of laboratory animals according to the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86–23) and with approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical School or Harvard Medical School. Prior to hepatectomy or for sham operations, mice were anesthetized with 60 mg/kg ketamine (Hospira) and 7 mg/kg xylazine (Phoenix Pharmaceutics) and positioned supine. A transverse incision was made inferior to the xiphoid process, which was excised. The median and left lateral lobes were eviscerated and ligated resulting in 60% liver removal. For the metabolic and synthetic experiments, the posterior left lobe was also removed. At tissue recovery, mice were anesthetized and weighed. Livers were excised, rinsed, blotted, and weighed. Sections were snap frozen in liquid nitrogen, fixed in 10% neutral buffered formalin, or preserved in RNALater (Qiagen). Mortality after hepatectomy was $<$5% and not associated with a particular genotype.

Immunohistochemistry—Staining for SOCS2 was performed using a rabbit polyclonal anti-SOCS2 antibody (Cell Signaling). Staining for GH in the pituitary was performed using a polyclonal anti-GH antibody (Abcam). Specimens were viewed with a brightfield microscope (Zeiss Axio Imager M2 with Spot Xplorer camera).

mRNA Isolation and Real-time RT-PCR —Total mRNA was purified from 30 mg of liver tissue preserved in RNALater using the RNeasy Mini kit (Qiagen). 1 μg of mRNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). StepOnePlus^{TM} (Applied Biosystems) was used for all real-time PCR. The cDNA template was diluted 1:5 and amplified using inventoried Taqman gene expression assays (Applied Biosystems) under standard conditions. Gene expression levels were normalized to GAPDH using the comparative CT method. Data were analyzed using StepOne^{TM} Software v2.1 (Applied Biosystems). Taqman probe and primers sets (Applied Biosystems) were: Jak2 (Mm01208489_m1), STAT5b (Mm00839889_m1), STAT3 (Mm01219775_m1), GHR (Mm00439093_m1), IGF-1 (Mm00439560_m1), SOCS2 (Mm00850544_g1), and GAPDH (Mm99999915_g1).

Quantitation of Hepatocyte Proliferation—To quantitate dividing hepatocytes, mice received intraperitoneal injection of 1 mg of 5-bromo-2-deoxyuridine (BrdU; BD Pharmingen). Staining for BrdU was performed on paraffin embedded liver tissue. Rat anti-mouse BrdU (Santa Cruz Biotechnology) was used for detection. The proliferation index was quantified as the percentage of labeled hepatocyte nuclei stained with BrdU over at least three high-power fields (200×).

Protein Sample Preparation, Western Blot Analysis—Whole cell liver lysates were prepared by homogenizing 50 mg of frozen tissue in lysis buffer (50 mM HEPES, 10% glycerol, 1 mM EDTA, 50 mM NaF, 1 mM DTT, and 0.1% Nonidet P-40) containing phosphatase and protease inhibitors (Sigma). Samples were sonicated for 10 s and clarified by centrifugation. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories). Protein was denatured and separated by SDS-PAGE in 4–12% or 12% NuPAGE Bis-Tris precast gels (Life Technologies) and transferred to PVDF membrane (Millipore). All antibody dilutions were done in 5% NFDM in TBS buffer. Primary antibodies used were: mouse anti-β actin (Abcam), rabbit anti-SOCS2 antibody (Santa Cruz Biotechnology), rabbit anti-GHR (Novus Biologicals), mouse anti-IGF-1 (Thermo Scientific), rabbit anti-JAK2 (Thermo Scientific), rabbit anti-pJAK2 (Thermo Scientific), rabbit anti-STAT5b (Abcam), rabbit anti-pSTAT5b (Abcam), mouse anti-STAT3 (Abcam), rabbit anti-pSTAT3 (Abcam), mouse anti-ERK1/2 (Santa Cruz Biotechnology), rabbit anti-pERK1/2 (Cell Signaling), rabbit anti-Akt (Cell Signaling), and rabbit anti-pAkt (Cell Signaling). HRP-conjugated species-specific secondary antibodies were obtained from Promega or Santa Cruz Biotechnology.

Films were scanned and quantitated with Image J software. In all cases, at least three mice of *Socs2*-null or WT were used at each time after hepatectomy for quantitation. Where blots are shown, the images are representative of the individual experiments. Boxes denote separate blot photographs.

Primary Hepatocyte Cultures—Primary hepatocytes were cultured as previously described (11). Briefly, mice were anesthetized, and the portal vein was cannulated. The liver was perfused with preperfusion solution for 10 min and then with collagenase solution. The liver was then mechanically dissociated in MEM. Cells were filtered, collected, washed, resuspended, and plated.

Virus Infection—A previously described adeno-associated virus (AAV) vector plasmid bearing a hepatocyte-specific murine urinary protein (MUP) promoter (11) was used for *in vitro* transfection studies at 5.00×10^{11} DNase-resistant particles/ml for MUP-SOCS2-AAV8 and MUP-eGFP-AAV8.

Cell Proliferation Assay—Cells were grown for 2 days in serum-free medium with each AAV8. CyQUANT (Invitrogen) was used to measure proliferation according to the manufacturer's instructions.

Enzyme-linked Immunosorbent Assay (ELISA)—Commercial ELISA kits were used to detect serum RBP4 (ALPCO), GH (Millipore), IGF-1 (R&D), GHR (LSbio), and somatostatin levels (Phoenix).

Serum Direct Bilirubin Assay—Serum direct bilirubin concentration was assessed using Quantichrom^{TM} Bilirubin Assay kit (Bioassay Systems).

Ubiquitinated Protein Assay—Ubiquitinated proteins from mouse liver tissue were isolated using a ubiquitin enrichment kit (Pierce) according to the manufacturer's protocol. Briefly, mouse primary hepatocyte pellets after perfusion were homogenized in IP lysis buffer (Thermo) and sonicated for three 10-s pulses. 300 μg of total protein lysate were diluted 1:1 with TBS and incubated with 20 μl of polyubiquitin affinity resin in a column (Pierce). The polyubiquitin affinity proteins in the column were eluted with 4× SDS sample buffer and subjected to

SDS-PAGE. The membrane was probed with anti-GHR antibody.

Microarray Analysis—10 μg of high-quality total mRNA was pooled from three animals and run on an Affymetrix mouse 430 2.0 array as previously described (16). Two independent chips, each loaded with a different set of three pooled animals, were examined in duplicate. Genes with an LCB of >1.5 or less than <1.5 were considered up-regulated or downregulated, respectively, if the *p* value was less than 0.05. Ingenuity™ pathway analysis was performed as previously described (10). Microarray data are stored in minimum information about a microarray experiment (MIAME) format.

GH Administration—25 μg of recombinant human GH (Reprokine) dissolved in water was injected subcutaneously twice daily (23). The site of injection was rotated daily to minimize discomfort, and weight measurements were taken in the morning prior to injection.

IGF-1 Administration—5 mg/kg/day of recombinant human IGF-1 (Cell Sciences) dissolved in water was given continuously with osmotic pump (DURECT) or injected intraperitoneally three times per day.

Statistical Analysis—Comparisons were performed using a two-tailed unpaired Student's *t* test. All statistical tests used at least three different samples for each time point. A *p* value of less than 0.05 was considered statistically significant. All error bars are standard error of the mean.

Results

Socs2 Localization and Expression Levels after Partial Hepatectomy—Based on a preliminary screen suggesting increased SOCS2 transcription in the liver after partial hepatectomy, we determined the expression of SOCS2 in the liver by immunohistochemistry. Six hours after partial hepatectomy, SOCS2-positive cells were seen around the pericentral vein (Fig. 1*A*, *arrow*). In C57B/6 mice (wild type (WT)), real-time PCR demonstrated that transcription of *Socs2* rises rapidly (by 6 h) after 2/3 hepatectomy, but falls to baseline levels by 1 day and then remains close to baseline (Fig. 1*B*). In contrast, sham operated mice did not exhibit this early rise in *Socs2* mRNA. Protein levels of SOCS2 in the liver initially rise by 6 h and decrease through 48 h as determined by Western blot (Fig. 1*C*). SOCS2 is therefore induced by hepatectomy. Protein levels mirror mRNA levels, suggesting that SOCS2 is regulated at the transcriptional level.

Socs2 Controls the Rate of Liver Regeneration after Partial Hepatectomy—To determine the functional consequences of loss of SOCS2, comparison was made between the response of *Socs2*-null and WT mice to 60% hepatectomy. Loss of SOCS2 led to significant increases in hepatocyte proliferation 36, 48, and 72 h after partial hepatectomy as measured by BrdU incorporation (Fig. 2, *A* and *B*).

This early increase in hepatocyte proliferation produced increased liver to body weight ratio at 72 h after hepatectomy. Surprisingly, however, at later times this trend reversed. From 72 h through 1 week after hepatectomy there was minimal increase in liver mass in *Socs2*-null mice in contrast to the WT mice that demonstrated continued increase in liver mass (Fig. 2*C*). As a result, the liver to body weight ratio of *Socs2*-null mice fell below WT mice at 7 days. By 14 days after hepatectomy, the liver to body weight ratio in *Socs2*-null mice matched the controls.

These results demonstrate that the presence of SOCS2 slows down the rate of liver regeneration over the initial 3 days after hepatectomy, the time corresponding to the first round of hepatocyte proliferation. After this time period, loss of SOCS2 extends the time required for the liver to reach normal size to nearly 2 weeks.

Socs2 Slows Hepatocyte Proliferation in Primary Hepatocyte Cultures—To support these *in vivo* results, we examined the effect of expression of *Socs2* on proliferation in primary hepatocyte cultures using an AAV8 expression system (11). Primary hepatocyte cultures were infected with MUP-SOCS2-AAV8 or MUP-EGFP-AAV8 as a control. Successful expression with SOCS2 was demonstrated using real-time PCR (Fig. 2*D*). Infection with SOCS2 decreased hepatocyte proliferation by about 40% compared with control (Fig. 2*E*).

Socs2-null Mice Exhibit Impaired Metabolic Function—To explain why a growth inhibitor would be induced after hepatectomy, we hypothesized that SOCS2 might function to slow proliferation to preserve metabolic function. After 75% hepatectomy, a more stressful insult than 60% hepatectomy, we examined the metabolic and synthetic activity of Socs2-null mice compared with WT mice. We first looked at bilirubin. A major function of the liver is conjugation of bilirubin with glucoronic acid to enable excretion. Serum bilirubin levels therefore can be a measure of hepatocyte function. Whereas baseline bilirubin levels are not different between *Socs2*-null and WT mice, by 72 h after surgery, *Socs2*-null mice had significantly higher bilirubin, a nearly 6-fold increase, compared with WT mice (Fig. 3*A*). We then looked at retinol-binding protein (RBP) as a measure of liver synthetic function. RBP is produced in the liver and serves as a plasma carrier protein for retinol, the lipid alcohol of vitamin A. RBP has a short half-life of 12 h, suggesting it should be a sensitive marker of decreased liver synthetic function (24). RBP levels at baseline are equivalent, but 72 h after hepatectomy were lower in *Socs2*-null mice compared with WT mice (Fig. 3*B*). Taken together, these results suggest that loss of SOCS2 impairs liver metabolic and synthetic function.

SOCS2 Controls Liver GHR Levels and Inhibits Hepatocyte Proliferation Early after Hepatectomy via Ubiquitination—To determine the mechanism by which SOCS2 influences liver regeneration during the initial round of hepatocyte proliferation, we examined the effect of loss of SOCS2 on GHR levels in the liver. Consistent with this known function, we found that the level of GHR in the liver 6 h after hepatectomy is higher in *Socs2*-null mice compared with WT, with a trend toward decreased levels at later time points as measured by Western blot (Fig. 3*C*).

These increases in GHR levels provide a possible explanation for the early increase in hepatocyte proliferation after hepatectomy in *Socs2*-null mice due to increased GH signaling. If true, this would predict that high GHR levels in *Socs2-null* hepatocytes would render them hyper-responsive to GH. To test this possibility, we injected GH subcutaneously twice a day after hepatectomy to *Socs2*-null and WT mice. *Socs2*-null mice with GH administration exhibited higher hepatocyte proliferation

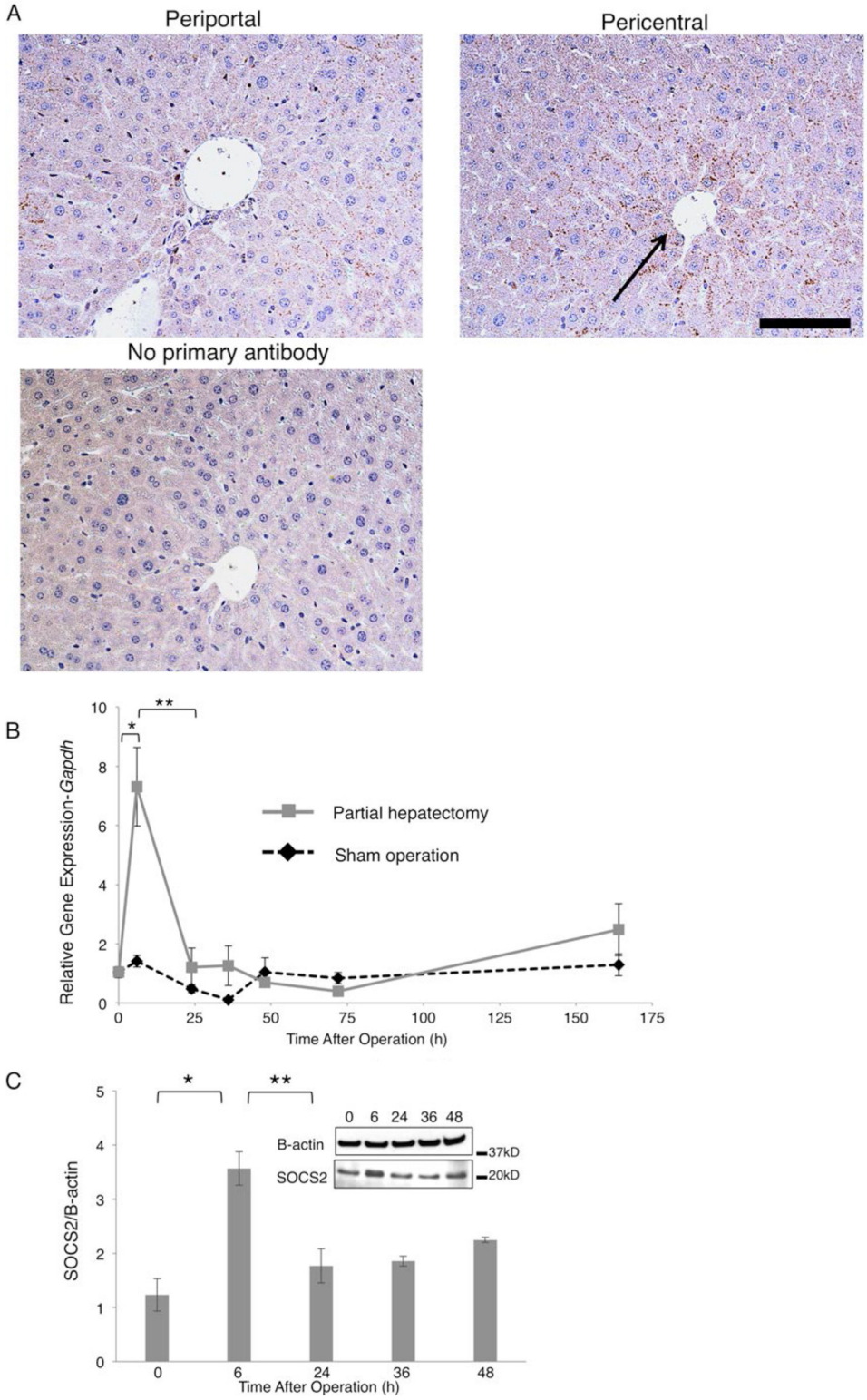

FIGURE 1. **SOCS2 localization and expression over time after hepatectomy.** *A*, immunohistochemistry demonstrates SOCS2-positive cells scattered throughout the liver and concentrated around the pericentral vein (*arrow*). Scale bar, 50 μM. *B*, real-time PCR of *Socs2* mRNA in liver samples at various intervals after hepatectomy (*, $p = 0.04$; **, $p = 0.03$). *Socs2* is increased 6 h after 2/3 hepatectomy and returns to baseline by 24 h. Sham operated mice do not show an increase at 6 h. Each time point consists of at least three animals. *C*, Western blot analysis of total protein from liver at various time after hepatectomy. Protein levels of SOCS2 in the liver initially rise by 6 h but then fall by 24 h after hepatectomy by Image J analysis (*, $p = 0.006$; **, $p = 0.01$). β-Actin demonstrates equal protein loading. Data are mean $\pm$ S.E. from 3 mice in each group.

compared with WT mice with GH administration 48 h after hepatectomy (Fig. 3, *D* and *E*). GH administration had no effect on hepatocyte proliferation in WT mice but increased proliferation in the *Socs2*-null mice (compare Figs. 2*A* and 4*B*, $p = 0.035$). These results suggest loss of SOCS2 induces a state of hyper-responsiveness to GH.

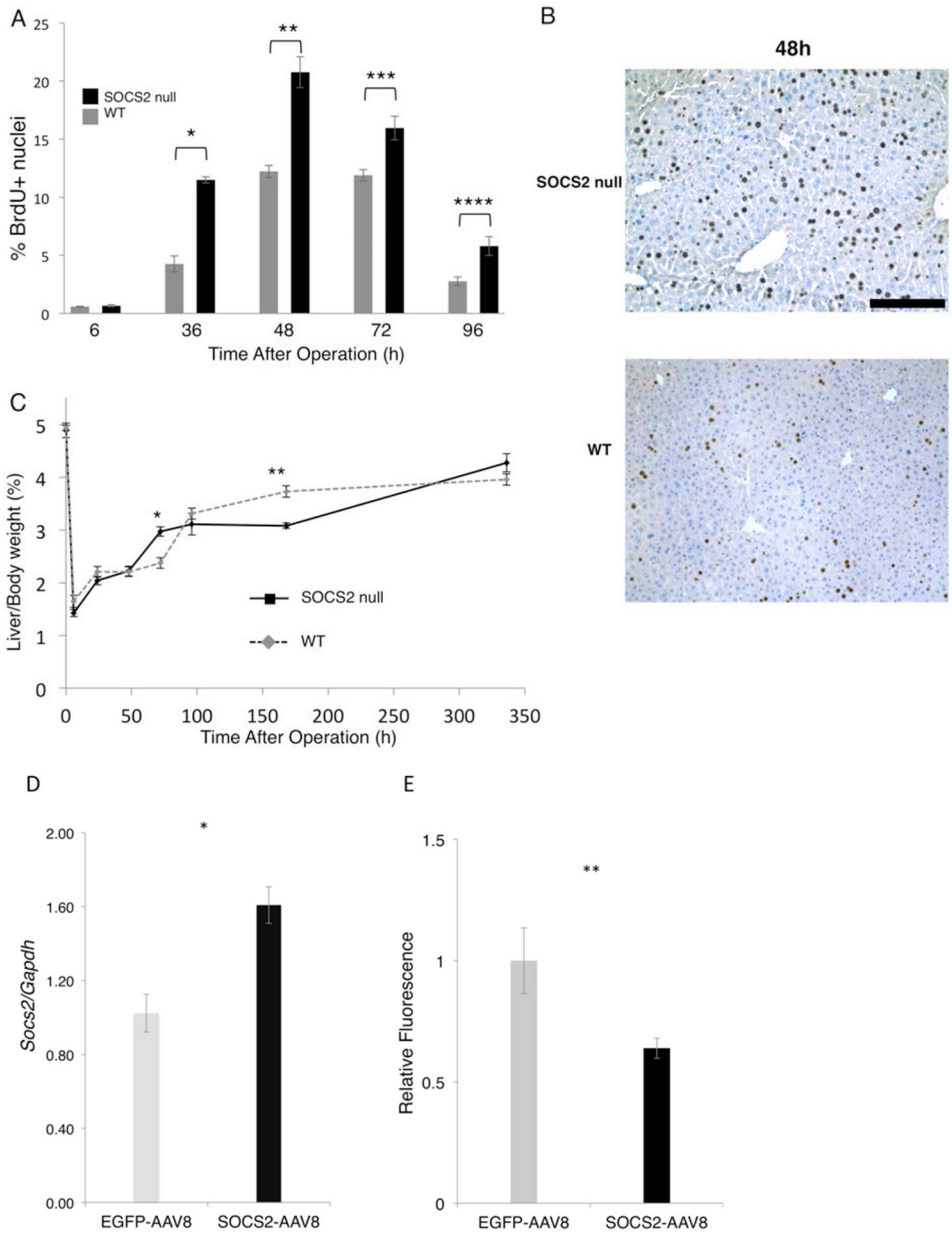

FIGURE 2. **Loss of SOCS2 modulates liver regeneration and hepatocyte proliferation after hepatectomy.** *A*, BrdU incorporation in hepatocytes is quantitated at indicated time points after 2/3 hepatectomy. *Socs2*-null mice demonstrate increased hepatocyte proliferation (*, $p = 0.004$; **, $p = 0.01$; ***, $p = 0.03$; ****, $p = 0.046$). *B*, representative micrograph of BrdU incorporation demonstrates hepatocyte proliferation. Scale bar = 200 μM. *C*, restoration of liver mass was measured by liver/body weight ratio at the indicated time points. Loss of SOCS2 initially increases then inhibits restoration of liver mass after hepatectomy (*, $p = 0.005$; **, $p = 0.005$). Data are mean ± S.E. from at least 3 mice ($n = 3$–7) in each group. *D*, successful infection of primary hepatocyte cultures with SOCS2-AAV8 as measured by SOCS2 gene expression (*, $p = 0.002$). *E*, SOCS2 expression in primary hepatocyte culture decreases hepatocyte proliferation (**, $p = 0.038$).

SOCS2 is known to ubiquitinate the GHR, targeting it for destruction. To determine whether this mechanism of action holds in hepatocytes, we examined the effect of SOCS2 on GHR. In primary hepatocyte culture, infection with MUP-SOCS2-AAV8 decreased GHR protein levels by ~30% (Fig. 3*F*). To determine whether ubiquitination might be the mechanism of this decrease, we compared ubiquitination of GHR *in vivo* between *Socs2-null* and WT mice. Immunoprecipitation using an antibody to ubiquitin and then probing for GHR revealed markedly decreased ubiquitination in *Socs2*-null mice (Fig. 3*G*). These results suggest SOCS2 ubiquitinates GHR, leading to degradation and lower protein levels.

SOCS2 Enhances Hepatocyte Proliferation Late after Hepatectomy—Hyper-responsiveness to GH does not explain the almost complete failure of liver growth between three and 7 days after hepatectomy. To examine this finding, we hypothesized that this might be due to changes in the level of circulating GH. To test this, we examined serum levels of GH after hepatectomy. WT mice increase serum levels of GH after hepatectomy (Fig. 4*A*). In contrast, serum GH levels in *Socs2*-null mice remain very low after hepatectomy. As a result, serum GH levels are 20–30 times higher in WT compared with *Socs2*-null mice from 24 through 72 h after hepatectomy.

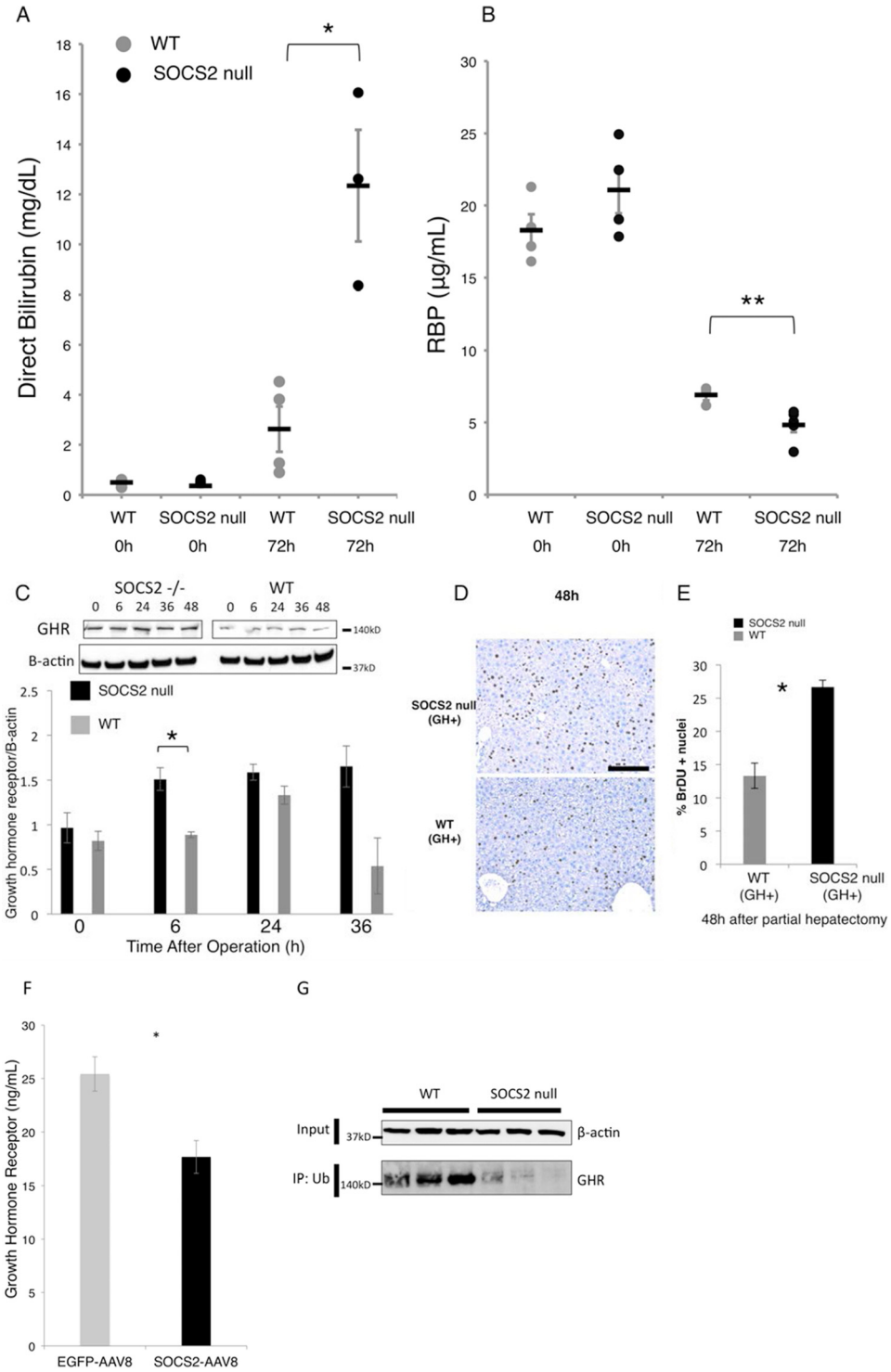

FIGURE 3. **Metabolic and synthetic activity and GH signaling after partial hepatectomy.** *A*, serum direct bilirubin was equal at baseline but higher 72 h after hepatectomy in *Socs2*-null *versus* WT mice (*, $p = 0.03$). *B*, RBP levels at baseline were equivalent but at 72 h after hepatectomy were lower in *Socs2*-null mice compared with WT mice (**, $p = 0.015$). Each individual data point is represented by a *circle*. *Solid bar* designates mean and error bars are standard error. Data are mean ± S.E. from at least 3 mice ($n = 3$–5) in each group. *C*, Western blot and Image J analysis demonstrate loss of SOCS2 leads to increased levels of GHR 6 h after hepatectomy ($p = 0.03$). *D* and *E*, administration of GH increases hepatocyte proliferation 48 h after hepatectomy in *Socs2*-null compared with WT mice ($p = 0.008$). Scale bar = 200 µM. Data are mean ± S.E. from 3 mice in each group. *F*, expression of SOCS2 in primary hepatocyte cultures decreased GHR protein levels by ~25% (*, $p = 0.003$). *G*, immunoprecipitation using an antibody to ubiquitin and then probing for GHR revealed markedly decreased ubiquitination of GHR in Socs2-null mice.

There are two likely explanations for this result. Either *Socs2*-null mice do not produce GH, or they do not release it in response to hepatectomy. To distinguish between these possibilities, we dissected the pituitary glands of three WT and *Socs2*-null mice and performed immunohistochemistry for GH. The pituitaries of 8-week-old male *Socs2*-null mice appeared histologically normal. Staining for GH was roughly equivalent between the WT and Socs2-null mice (Fig. 4*B*).

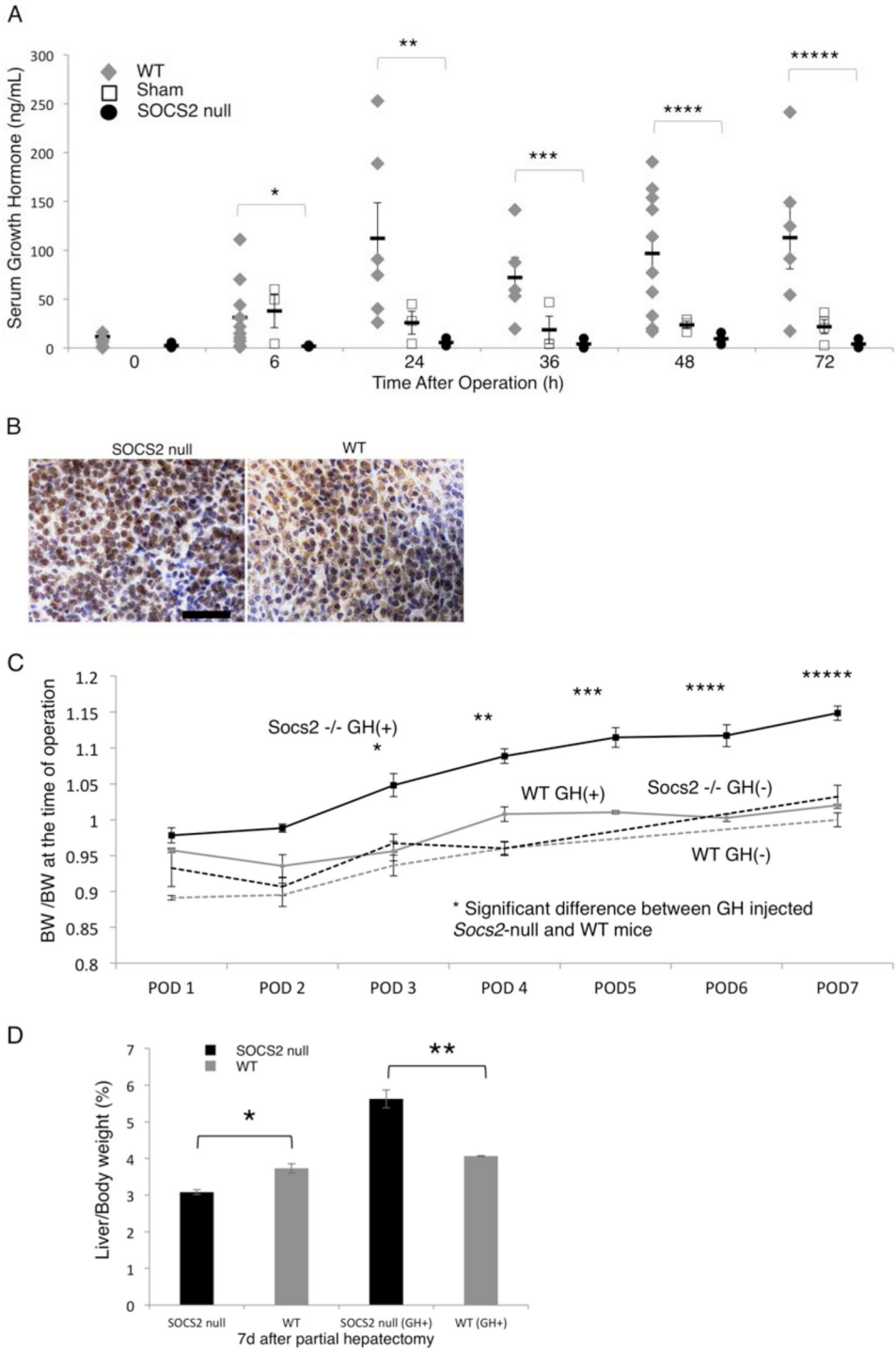

FIGURE 4. **Loss of SOCS2 induces a state of hyper-responsiveness to GH early after hepatectomy.** *A*, serum GH levels are 20–30× higher in WT compared with *Socs2*-null mice from 24 through 72 h after hepatectomy (*, $p = 0.03$; **, $p = 0.03$; ***, $p = 0.03$; ****, $p = 0.002$; *****, $p = 0.02$). Each individual data point is shown. *Solid bar* designates mean and error bars are standard error. *B*, immunohistochemistry for GH in pituitary glands in *Socs2*-null and WT mice show similar staining. *Brown color* represents antibody staining. Scale bar, 50 μM. *C*, administration of GH causes a dramatic increase in body weight in *Socs2*-null mice compared with WT mice given GH and WT or *Socs2*-null mice in the absence of GH (*, $p = 0.01$; **, $p = 0.005$; ***, $p = 0.02$; ****, $p = 0.01$; *****, $p = 0.002$). *D*, administration of GH increases liver to body weight ratio after hepatectomy in *Socs2*-null compared with WT mice (*, $p = 0.005$; **, $p = 0.02$). Data are mean ± S.E. from at least 3 mice ($n = 3$–10) in each group.

Taken together, these results suggest that SOCS2 is necessary for the release of GH from the pituitary, but not for its production. This further provides an explanation for the slow return of liver mass after hepatectomy in *Socs2*-null mice as a consequence of suppressed circulating GH levels.

Delay in long-term growth could be due to low circulating GH or changes in responsiveness to GH. To distinguish between these possibilities, we administered GH to *Socs2*-null or WT mice after 2/3 hepatectomy for an extended period of time. Body weight was measured every day at the same time.

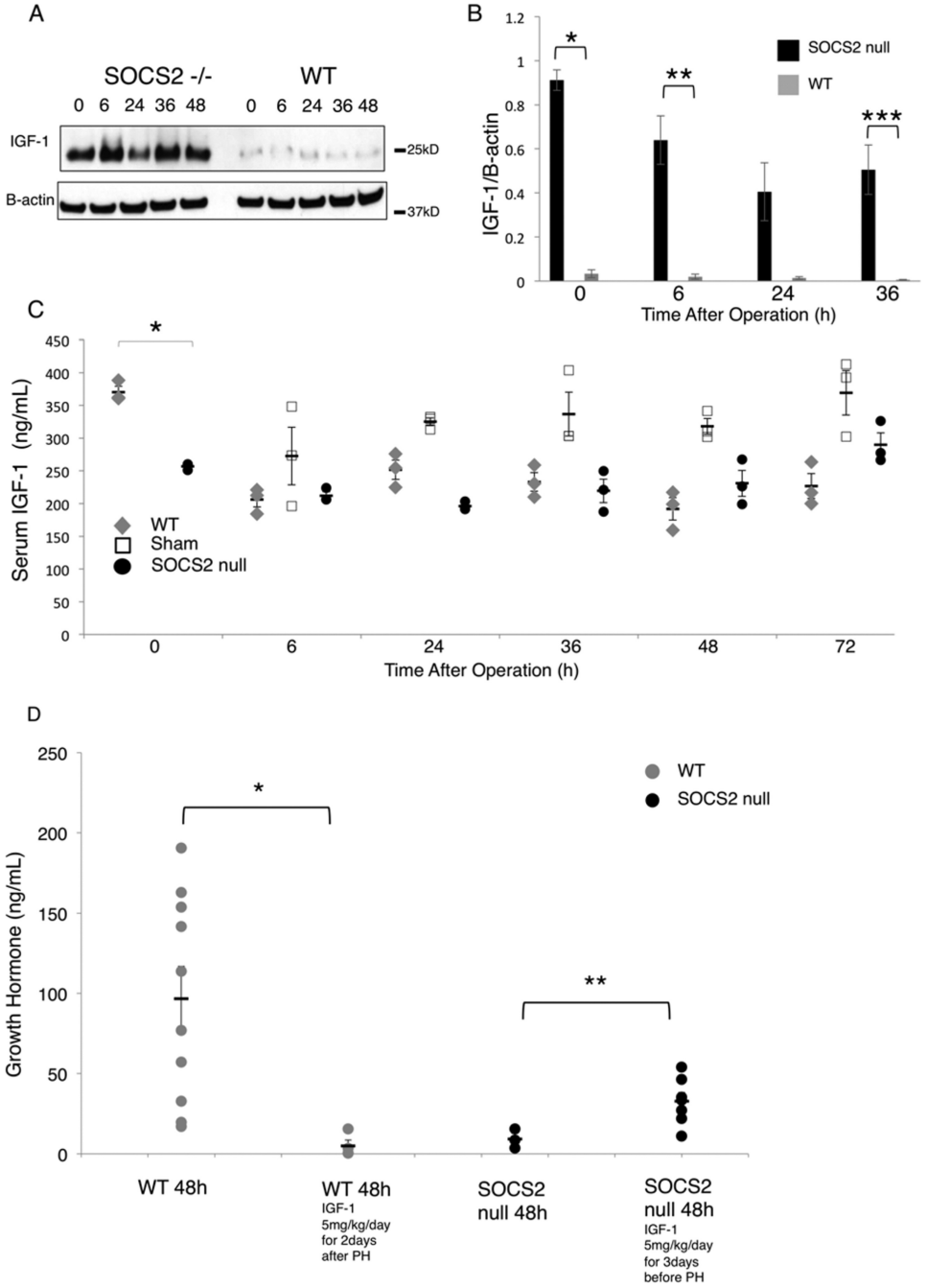

FIGURE 5. **IGF-1 signaling after partial hepatectomy and the effect of administration of IGF-1 on serum GH levels.** *A* and *B*, Western blot and Image J analysis show increased IGF-1 protein in the liver of *Socs2*-null compared with WT mice (*, $p < 0.001$; **, $p = 0.03$; ***, $p = 0.047$). *C*, serum IGF-1 levels are decreased at baseline in *Socs2*-null compared with WT mice (*, $p = 0.003$). Levels decrease over time in WT mice but remain constant in *Socs2-null* mice after 2/3 hepatectomy. Data are mean ± S.E. from 3 mice in each group. *D*, WT mice in which IGF-1 was administered after hepatectomy did not increase GH levels after hepatectomy (*, $p = 0.001$). In contrast, with abrupt discontinuation of IGF-1, *Socs2*-null mice are able to increase serum GH level (**, $p = 0.007$). Data are mean ± S.E. from at least 3 animals ($n = 3$–10) in each group.

GH administration markedly increased body weight in *Socs2*-null mice compared with WT beginning as early as 3 days after hepatectomy and continuing through 7 days (Fig. 4*C*). Similarly, 7 days after hepatectomy, liver to body weight ratio was highest in *Socs2*-null mice that received GH compared with WT mice that received GH and either genotype that did not receive GH (Fig. 4*D*).

These data indicate that loss of SOCS2 creates a state of hyper-responsiveness to GH that continues throughout the period of liver regeneration, and is consistent with loss of circulating GH as a cause of the stunted long-term return of liver mass in *Socs2*-null mice.

SOCS2 Is Required for Regulation of Serum IGF-1 but Not Somatostatin Levels—IGF-1 and somatostatin both inhibit GH secretion. We next investigated if and how these proteins might explain the mechanism by which SOCS2 modulates GH release. Since the majority of IGF-1 is produced in the liver, we first examined protein levels of IGF-1 in the liver in WT *versus*

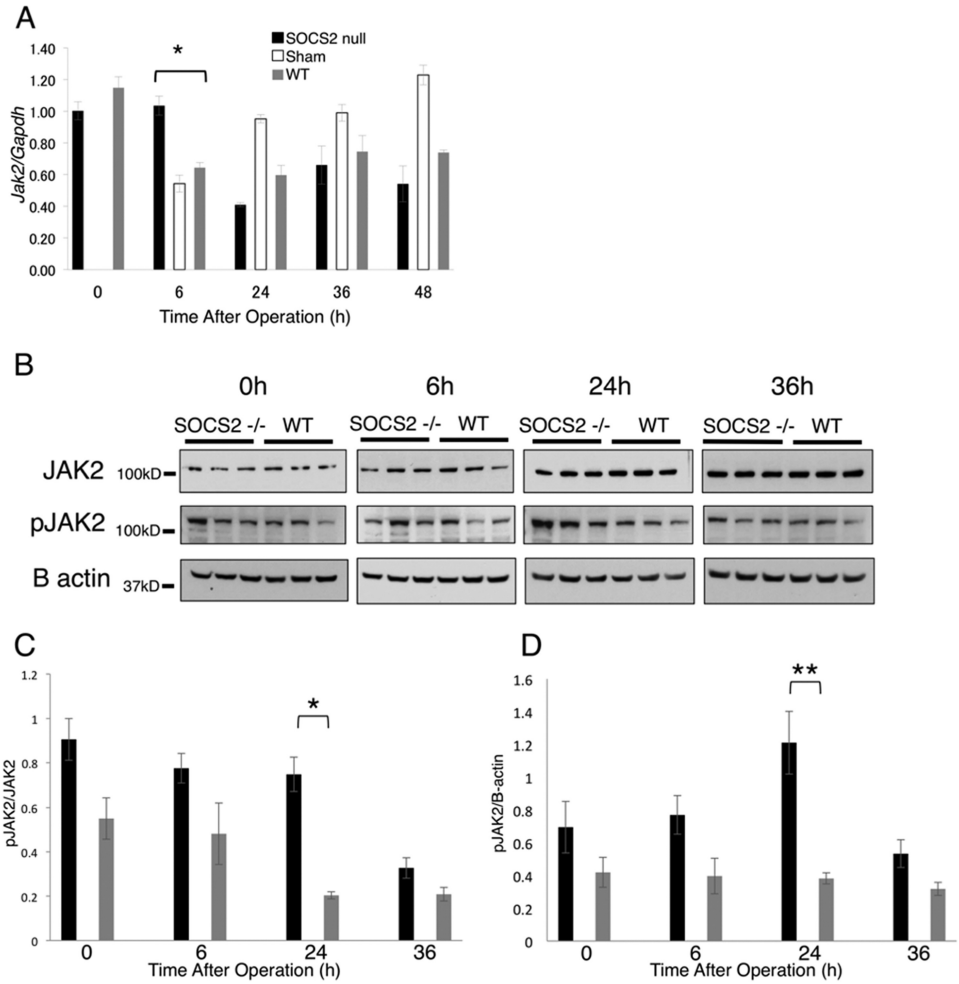

FIGURE 6. **Jak2 signaling after partial hepatectomy.** *A*, real-time PCR demonstrates delayed decrease in *Jak2* mRNA in *Socs2*-null mice compared with WT after partial hepatectomy (*, $p = 0.01$). *B—D*, Western blot and Image J analysis demonstrate similar Jak2 levels but increased Jak2 phosphorylation in *Socs2*-null *versus* WT mice 24 h after hepatectomy (*, $p = 0.016$; **, $p = 0.046$). Data are means ± S.E. from 3 mice in each group.

Socs2-null mice. *Socs2*-null mice exhibited dramatically increased IGF-1 protein levels in the liver compared with WT mice (Fig. 5, *A* and *B*). Surprisingly, however, baseline serum IGF-1 levels were lower in *Socs2*-null compared with WT mice (Fig. 5*C*). Importantly, in WT mice, IGF-1 levels decrease by 6 h after hepatectomy, whereas in *Socs2*-null mice, levels remain relatively constant out to 72 h. Serum levels of somatostatin were not different between the WT and *Socs2*-null groups (Data not shown). These results suggest that SOCS2 suppresses IGF-1 production by the liver, and serum levels of IGF-1 are maintained by extra-hepatic production in the *Socs2*–null mice. With the dramatic differences in IGF-1 production in the liver, a major source of serum IGF-1, these results argue that loss of SOCS2 disrupts the normal production of IGF-1 and therefore its regulation of GH.

To explore the mechanism by which SOCS2 regulates IGF-1, we examined IGF-1 mRNA expression in both intact liver and in primary hepatocyte cultures. We found no significant difference in either when comparing *Socs2*-null with wild type (data not shown), suggesting SOCS2 regulates IGF-1 at the post-transcriptional level.

To further explore these results, we hypothesized decreases in serum IGF-1 drive GH release from the pituitary during liver regeneration. We modeled decreases in serum IGF-1 levels by injecting exogenous IGF-1 into *Socs2*-null mice for 3 days prior to hepatectomy followed by discontinuation to produce an abrupt decrease in serum IGF-1 levels. Under this condition, *Socs2*-null mice are able to increase serum GH levels (Fig. 5*D*). We next administered IGF-1 after hepatectomy as a control and confirmed IGF-1 suppressed GH levels after hepatectomy. These results are consistent with the explanation that decreases in IGF-1 drive GH release from the pituitary, which in turn drives hepatocyte proliferation. We conclude, therefore, that SOCS2 modulates GH release via effects on IGF-1.

GH Signaling Is Increased Early in Socs2-null Mice after Partial Hepatectomy—In the liver, activated JAK2 phosphorylates GHR target genes, activating four major pathways: the STAT5/IGF-1 axis, STAT3, Ras/MAPK/ERK, and phosphatidylinositol 3-kinase (PI3K)/Akt (13). To determine how loss of SOCS2 influences GH signaling, we performed real-time PCR to determine mRNA levels for Jak2, STAT3, and 5, Erk 1/2, and Akt, and Western blot to measure protein levels and phosphorylation states.

Six hours after hepatectomy, *JAK2* mRNA was significantly higher in *Socs2*-null mice compared with WT (Fig. 6*A*). Although JAK2 protein levels were similar, JAK2 phosphoryla-

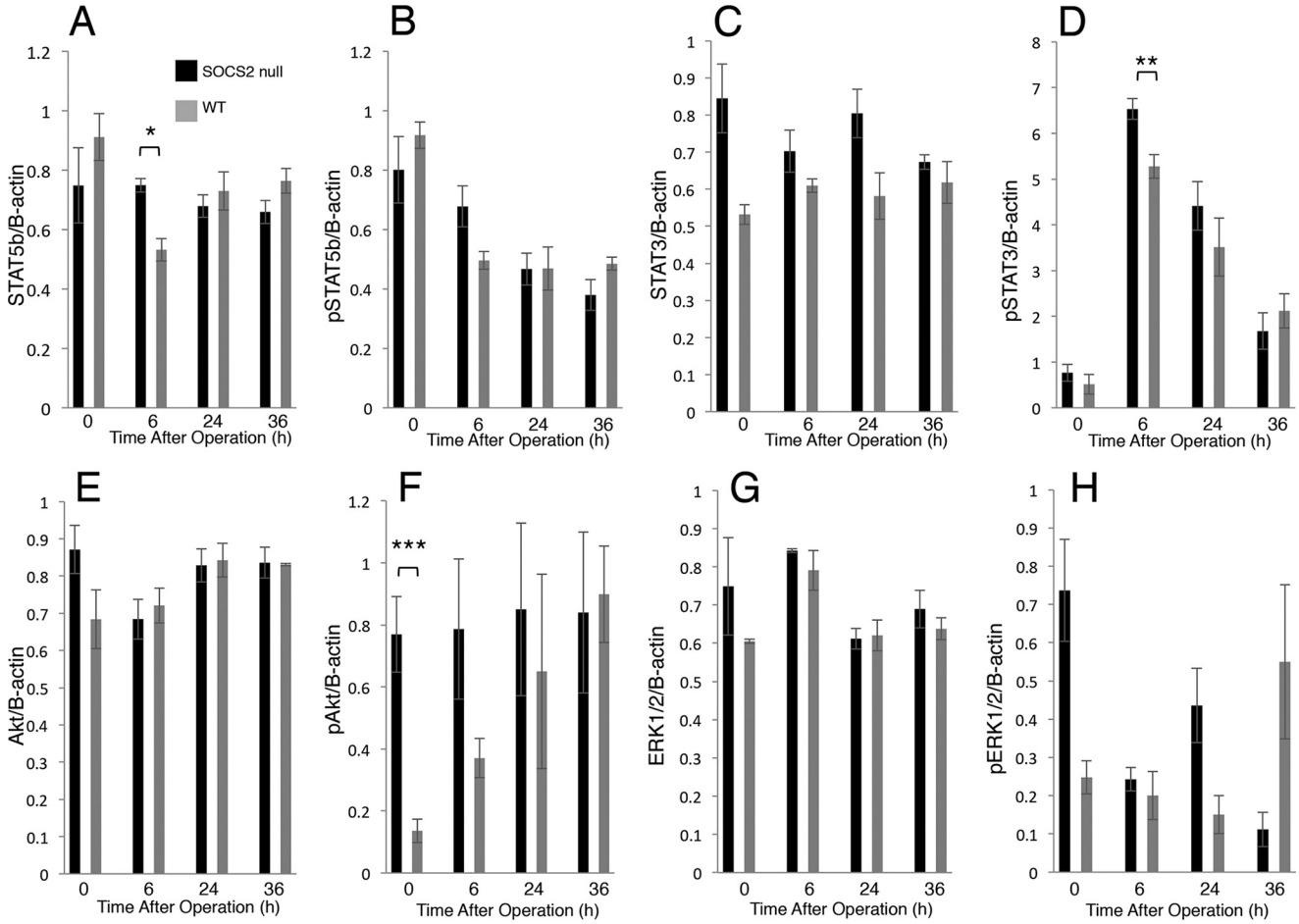

FIGURE 7. **STAT5b, STAT3, AKT, and ERK1/2 signaling after partial hepatectomy.** *A–F*, image J analysis of Western blots reveal increased STAT5b 6 h after hepatectomy (*A*, *B*), increased phosphorylated STAT3 6 h after hepatectomy (*C*, *D*), and increased phosphorylated Akt at baseline (*E*, *F*) (*, $p = 0.01$; **, $p = 0.02$; ***, $p = 0.03$) in *Socs2*-null compared with WT mice. ERK1/2 shows a trend toward increased phosphorylation in the *Socs2*-null mice compared with WT. Data are means ± S.E. from 3 mice in each group.

tion was higher in *Socs2*-null mice compared with WT mice 24 h after hepatectomy (Fig. 6, *B--D*; differences in the 6-h time point did not reach statistical significance). Six hours after hepatectomy, STAT5b, and phosphorylated STAT3 were increased in *Socs2*-null mice compared with WT (Fig. 7, *A–D*). Erk1/2 did not demonstrate differences either in mRNA or in protein or phosphorylation between *Socs2*-null and WT mice after hepatectomy (Fig. 7, *E*, *F*, and data not shown). Akt protein levels were similar between *Socs2*-null and WT mice, however AKT phosphorylation was higher at baseline in *Socs2*-null mice.

These results suggest GH pathway signaling is activated by loss of SOCS2 as GH targets STAT5b and phosphorylation of JAK2, STAT3, and Akt, are increased in *Socs2*-null mice early after hepatectomy.

GH Pathway Alterations due to SOCS2—To further understand the mechanism by which SOCS2 increases early liver regeneration, we performed microarray analysis of *Socs2*-null mice at 24 and 36 h after hepatectomy (Fig. 8, *A* and *B*). Ingenuity™ pathway analysis software was used to graphically represent genes induced and activated in the GH pathway. Fig. 8*A* denotes genes up-regulated (red) or downregulated (green), and proteins predicted to be activated (orange) or inhibited (blue) in the WT mice 36 h after hepatectomy. Fig. 8*B* denotes the same information in *Socs2*-null mice. Red and green colors represent actual microarray data whereas orange and blue colors are predictions based on the overall expression data.

Comparing baseline WT to *Socs2*-null gene expression pattern changes 36 h after hepatectomy demonstrates that based on transcriptional patterns, the GH signaling pathways is predicted to be inhibited in WT mice compared with *Socs2*-null mice after hepatectomy. In contrast, loss of SOCS2 is predicted to activate GH signaling, specifically, STAT1 and 3 leading to α2 microglobulin and c-fos through SRF and Elk1. Array data for GHR was consistent with real-time PCR data (data not shown). Similar data were obtained 24 h after hepatectomy (data not shown).

Discussion

Based on preliminary results suggesting SOCS2 is highly induced after hepatectomy, we examined the effect of loss of SOCS2 on liver regeneration. We find that *Socs2* mRNA and protein are increased after hepatectomy, and that the increased SOCS2 limits the rise of GHR via ubiquitination, slows hepatocyte proliferation, and preserves metabolic function during the initial round of hepatocyte proliferation after hepatectomy. SOCS2 has multiple effects on GH signaling, including sup-

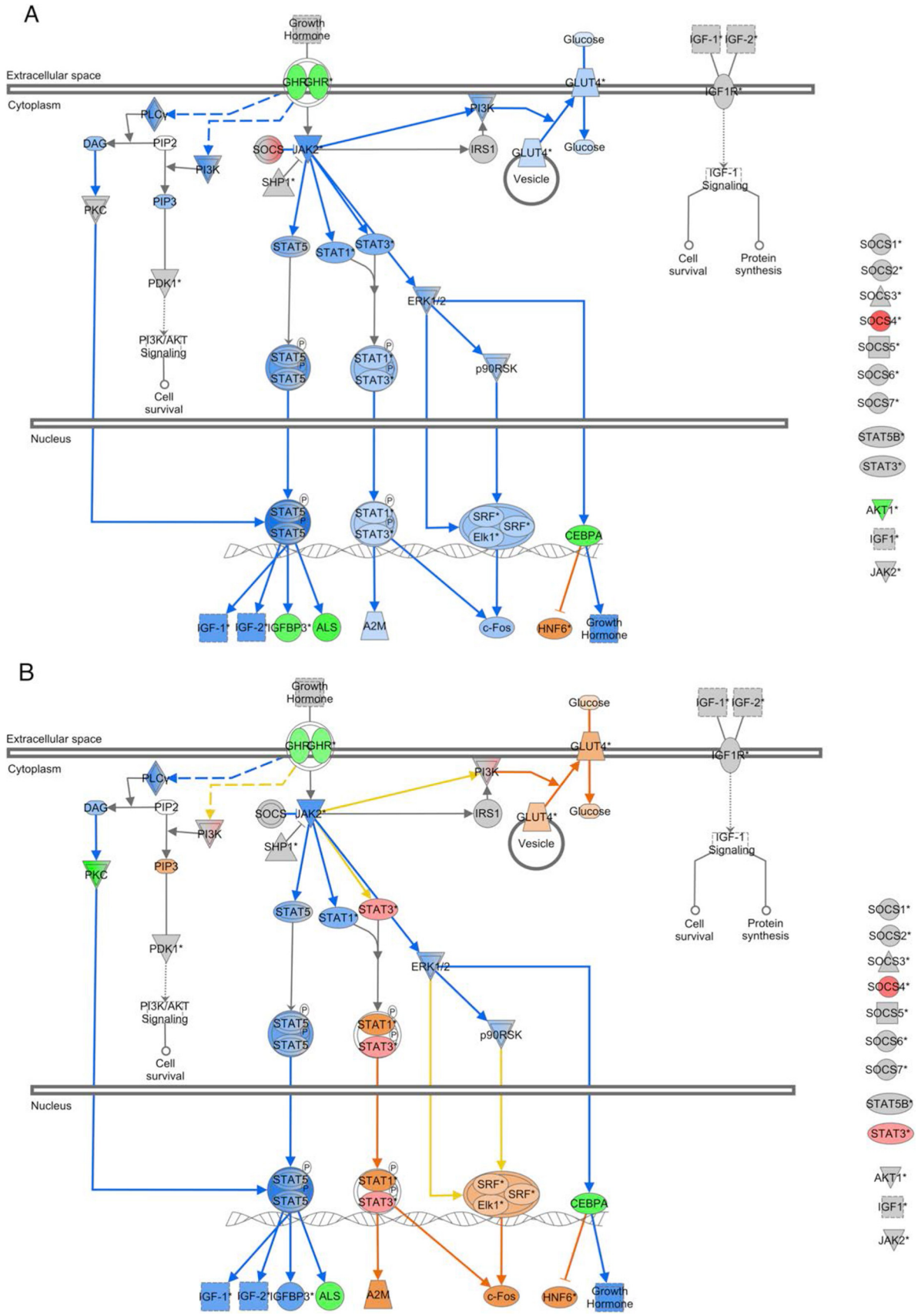

FIGURE 8. **Ingenuity pathway analysis of GH signaling after hepatectomy.** *A* and *B*, 36 h after hepatectomy, compared with WT mice (*A*), *Socs2*-null mice (*B*) demonstrate increased *STAT3* mRNA levels (*red*) and predicted activation of STAT family proteins and downstream targets. Overall GH signaling is decreased after hepatectomy in WT mice 36 h after hepatectomy compared with pre-hepatectomy values.

pression of IGF-1 production in the liver, and is required to modulate changes in serum IGF-1 levels that drive GH release from the pituitary and are necessary for normal restoration of liver mass by 1 week after hepatectomy.

These results are significant in that they support the existence of a mechanism to limit the rate of hepatocyte proliferation after hepatectomy to preserve metabolic function. Although the hepatectomy model as presented has low mortality, it is tempting to speculate that in a more severe injury model, loss of SOCS2 might carry higher mortality. To our knowledge this is the first demonstration of a mechanism that specifically limits the rate of hepatocyte proliferation after injury to preserve metabolic function.

Recent advances demonstrate that liver regeneration is best thought of as balance of stimulatory and inhibitory factors, and our present findings are supportive of this view. We previously demonstrated that down-regulation of inhibitory factors is required for normal liver regeneration (10, 11) and others have shown that inhibitory factors, specifically SOCS3, can be up-regulated after liver injury (2). The question is why would an anti-proliferative factor be up-regulated in a setting that requires liver regeneration? In the case of SOCS2, this seemingly paradoxical regulation as part of the early response to 2/3 hepatectomy results in improved metabolic and synthetic function as measured by bilirubin levels and RBP after resection.

Our findings are consistent with other published reports. In particular, SOCS2 is known to be an important part of an ubiquitination complex that binds to the GHR and targets it for degradation (19). We demonstrate this directly. SOCS2 was previously shown to be preferentially expressed in the pericentral regions of the liver, also consistent with our data (25). Normal expression of GH in the pituitary of *Socs2*-null mice is consistent with previous findings. Our serum levels show high variability due to the pulsatile nature of GH secretion (26).

This study has a number of limitations. Since we have not selectively knocked out SOCS2 in the liver, we cannot conclude that SOCS2 production in the liver alone is responsible for changes in serum IGF-1. The liver is not the sole source of IGF-1 (15), and though IGF-1 production in the liver is increased in *Socs2*-null mice, plasma levels are decreased, suggesting that IGF-1 production is dysregulated in *Socs2*-null mice. In addition, it is possible that metabolic deficiency after hepatectomy in *Socs2*-null mice is due to effects unrelated to the rate of hepatocyte proliferation.

Active induction of SOCS2 to decrease GH levels after hepatectomy implies that the rate of liver regeneration is tightly controlled and not simply modulated by a growth signal. This study, therefore, defines for the first time to our knowledge a physiologic mechanism to limit the rate of liver regeneration after injury. Clinical therapies to enhance liver regeneration may be useful in a variety of settings. These include treatments for acetaminophen overdose or to expand the ability to perform extensive liver resections for cancer which leave a very small remnant liver mass. Our findings suggest enhancing hepatocyte proliferation in the setting of injury may leave patients vulnerable to metabolic compromise and this may need to be addressed when developing new therapies.

Author Contributions—R. M. provided conceptual input and designed and performed the majority of the experiments, R. S. Z. provided conceptual input and performed many of the preliminary experiments. M. T. V. designed and performed the genomic experiments. D. T. designed and performed experiments. Y. O. designed and performed experiments. K. C. R. designed and performed experiments. S. J. K. designed the study, provided project oversight, final assurance of scientific integrity, and wrote the manuscript. All authors reviewed, commented on, and approved the manuscript.

Acknowledgment—We thank Dr. Michael Sirover for critical review of the manuscript.

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