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Dynamic alterations in Hippo signaling pathway and YAP activation during liver regeneration

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Grijalva JL, Huizenga M, Mueller K, Rodriguez S, Brazzo J, Camargo F, Sadri-Vakili G, Vakili K. Dynamic alterations in Hippo signaling pathway and YAP activation during liver regeneration. *Am J Physiol Gastrointest Liver Physiol* 307: G196–G204, 2014. First published May 29, 2014; doi:10.1152/ajpgi.00077.2014.—The Hippo signaling pathway has been implicated in mammalian organ size regulation and tumor suppression. Specifically, the Hippo pathway plays a critical role regulating the activity of transcriptional coactivator Yes-associated protein (YAP), which modulates a proliferative transcriptional program. Recent investigations have demonstrated that while this pathway is activated in quiescent livers, its inhibition leads to liver overgrowth and tumorigenesis. However, the role of the Hippo pathway during the natural process of liver regeneration remains unknown. Here we investigated alterations in the Hippo signaling pathway and YAP activation during liver regeneration using a 70% partial hepatectomy (PH) rat model. Our results indicate an increase in YAP activation by 1 day following PH as demonstrated by increased YAP nuclear localization and increased YAP target gene expression. Investigation of the Hippo pathway revealed a decrease in the activation of core kinases Mst1/2 by 1 day as well as Lats1/2 and its adapter protein Mob1 by 3 days following PH. Evaluation of liver-to-body weight ratios indicated that the liver reaches its near normal size by 7 days following PH, which correlated with a return to baseline YAP nuclear levels and target gene expression. Additionally, when liver size was restored, Mst1/2 kinase activation returned to levels observed in quiescent livers indicating reactivation of the Hippo signaling pathway. These findings illustrate the dynamic changes in the Hippo signaling pathway and YAP activation during liver regeneration, which stabilize when the liver-to-body weight ratio reaches homeostatic levels.

liver; regeneration; Hippo signaling pathway; YAP; partial hepatectomy

THE LIVER HAS THE REMARKABLE ability to regenerate following hepatocyte loss (8). The endpoint of regeneration is replacement of lost hepatic mass until the near original liver-to-body weight ratio is restored. In clinical practice, this regulation can be observed following liver resection or partial liver transplantation after which the liver grows to reach the optimal liver-to-body weight ratio for the individual. In contrast, large size liver grafts tend to atrophy to reach the optimal liver-to-body weight ratio (4, 13). Even though numerous cytokines and growth factors have been shown to play a role in liver regeneration, the exact mechanisms that are responsible for the initiation and termination of liver regeneration are not fully understood.

Recent studies have demonstrated that the Hippo signaling pathway plays a role in liver size regulation through regulation of the transcriptional coactivator Yes-associated protein (YAP) (7, 20). While activation of the Hippo pathway leads to phosphorylation of YAP (p-YAP) (2, 9, 18) resulting in YAP cytoplasmic retention and degradation, attenuation of Hippo signaling leads to increased YAP nuclear localization and activation of a proliferative, antiapoptotic transcriptional program (2, 7). Deletion of Hippo pathway components in quiescent liver has been demonstrated to result in YAP-dependent hepatocyte proliferation and liver tumorigenesis (7, 20). In addition, overexpression of YAP in transgenic mice has been shown to result in increased hepatocyte proliferation, massive liver overgrowth, and hepatocellular carcinoma (HCC) development (1, 2). Together, these findings demonstrate that the Hippo pathway is a potent tumor suppressor pathway.

To date, the role of the Hippo signaling pathway in liver size regulation has been mainly investigated in knockout and transgenic models, which have demonstrated that YAP activation results in increased hepatocyte proliferation (1, 2, 20). Based on this evidence, we sought to examine alterations in the Hippo signaling pathway during a normal physiologic response such as liver regeneration. We utilized a rat partial hepatectomy (PH) model to investigate alterations in the Hippo pathway during the initiation, maintenance, and termination phases of liver regeneration. Our results indicate that YAP activation and nuclear localization are increased early during the regeneration process and decrease as the prehepatectomy liver-to-body weight ratio is reached. In addition, we found a specific decrease in activation of Hippo kinases Mst1/2 and Lats1/2 as well as adapter protein Mob1A/B (Mob1), a direct target of Mst1/2. Our findings demonstrate significant alterations in the Hippo signaling pathway during the entire process of liver regeneration and suggest that this pathway plays a role in modulating cell proliferation and liver size during liver regeneration.

MATERIALS AND METHODS

Animals. Male Lewis rats (*Rattus norvegicus*) weighing 300–350 g were obtained from Charles River Laboratories (Wilmington, MA). Animals were individually housed with food and water available ad libitum. A 12-h light/dark cycle was used with the lights on at 7:00 AM. The experimental protocols were all consistent with the guidelines issued by the U.S. National Institutes of Health and were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

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Partial hepatectomy. Animals were subjected to 70% PH as previously described (5) with minor modifications. Resected lobes represented quiescent tissue and served as matched controls for each animal. Following 70% hepatectomy, rats were killed at 1, 3, 7, 10, 14, 21, and 30 days. The weight of the regenerating liver remnant and body weight were measured at the time of death. The weight of the regenerating liver remnant was divided by body weight to calculate liver-to-body weight ratios. Sham animals underwent laparotomy and mobilization of the liver without liver resection.

Western blotting. Western blots were carried out as previously described (10, 11). Briefly, 40 μg of protein were resuspended in sample buffer, boiled at 95°C for 5 min, and fractionated on a 4–20% glycine gel or 10–20% tricine gel (Invitrogen, Carlsbad, CA) for 90 min at 120 V. Proteins were transferred to PVDF membranes and then blocked with 5% milk or 5% BSA in Tris-buffered saline with Tween 20 (TBST) before immunodetection with the following antibodies: anti-YAP (Cell Signaling, Beverly, MA), phosphorylated YAP (Cell Signaling), TAZ (Cell Signaling), phosphorylated TAZ (Santa Cruz Biotechnology, Santa Cruz, CA) Mst1 (Cell Signaling), phosphorylated Mst1/2 (Cell Signaling), phosphorylated Lats1/2 (Bioss, Boston, MA), phosphorylated Mob1(Cell Signaling), and GAPDH (Millipore, Billerica, MA). Primary antibody incubation overnight was followed by four washes (15–30 min at room temperature) in TBST before incubation with secondary antibody for 1 h (horseradish peroxidase-conjugated goat anti-rabbit IgG; Cell Signaling). After four washes in TBST, proteins were visualized using the ECL detection system (NEN, Boston, MA). Coomassie gels were used to ensure equal protein loading. Band densities were analyzed using AlphaEase FC software version 4.1.0 (Alpha Innotech) to determine relative protein expression and were then normalized to GAPDH band densities. The Student's *t-test* was used for comparison of band intensities. One-way ANOVA was used for comparison of the same variable across multiple time points. Each animal served as its own control.

Immunostaining. Sections of fresh frozen liver tissue were cut at 8 μm and fixed in 4% paraformaldehyde for 1–2 min. Following fixation, sections were washed in PBS and blocked in 1% normal goat serum (Vector Laboratories, Burlingame, CA) with 0.1% Triton-100 in PBS for 30 min. Sections were washed in PBS and then incubated overnight in primary antibody anti-YAP (Cell Signaling) and Ki-67 (DAKO, Carpinteria, CA). Sections were then washed in PBS and incubated in secondary antibody Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and Alexa Flour 488 donkey anti-mouse (Life Technologies, Grand Island, NY) for 1 h. Sections were washed in PBS and incubated in DAPI for 30 min and then mounted in Vectashield (Vector Laboratories) and imaged using fluorescence microscopy. Open source software from the National Institutes of Health (ImageJ) (12) was used for data analysis.

RNA extraction and reverse transcription. RNA was extracted from liver tissue using a RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions and as previously described (10, 11). Reverse transcription reactions were performed using Superscript First Strand Synthesis System (Invitrogen) in an iCycler (Bio-Rad, Waltham, MA; 25°C for 10 min, 42°C for 50 min, 70°C for 15 min). To quantify the amount of gene expression, cDNA samples were used in quantitative real time-PCR (qPCR) using 50 PCR cycles (95°C for 30 s, 57°C for 60 s, 72°C for 90 s) in an iCycler (Bio-Rad) with the use of SYBR-green PCR Master Mix (Bio-Rad) with the following primers: connective tissue growth factor (CTGF): forward 5′- ATGATGCGAGCCAACTGC-CTG-3′ and reverse 5′-CGGATGCACTTTTTGCCCTTCTTAATG-3′; cysteine-rich angiogenic protein 61 (Cyr61): forward 5′-GTGCCGCCTG-GTGAAAGAGA-3′ and reverse 5′-GCTGCATTTCTTGCCCTTTTT-TAG-3′; and angiomotin-like protein 2 (AmotL2): forward 5′-GACTCTTTCTGGAGATCGGA-3′ and reverse 5′-ACTGATCCTCT-GTCCTTCCT-3′. The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. For each replicate, mRNA levels were normalized to their respective GAPDH mRNA levels.

Chromatin immunoprecipitation assay. A modified chromatin immunoprecipitation (ChIP) technique was adapted from previously published studies from our laboratory to analyze DNA/protein complexes (10, 11). Five micrograms of anti-YAP antibody (Cell Signaling) were added to isolated protein-DNA complexes from liver tissues. CTGF, Cyr61, and AmotL2 promoter DNA was detected in the resulting ChIP-DNA by qPCR using specific primers for the CTGF, Cyr61, and AmotL2 promoters. The following primer sequences were used for qPCR: CTGF: forward 5′-CAAATGTGTCTTCCAGTCGG-3′ and reverse 5′-AGGGGAAATTGTCCTATCCG-3′; Cyr61: forward 5′-AGACT-GAAAAGTTCCGGGG-3′ and reverse 5′-CACCAGGAGTGTCTC-TATCC-3′; and AmotL2 forward 5′-CGACTCTTTCTGGAGATC-GG-3′ and reverse 5′- CCCAGGATCAATCCTCGAAA-3′. Threshold amplification cycle numbers (T_c) using iCycler software were used to calculate immunoprecipitation DNA quantities as percentage of corresponding inputs.

Statistical analysis. Data are presented as means ± SE. Student's *t*-test was used for comparisons between groups. One-way ANOVA and Tukey's honestly significance difference test were used for comparison of the same variable across multiple time points. Statistical comparisons were made between regenerating and quiescent (control) livers. Each animal served as its own control. $P < 0.05$ was considered statistically significant. The data were evaluated with JMP Pro 10 statistical software (SAS Institute, Cary, NC).

RESULTS

Liver-to-body weight ratio during liver regeneration. To assess liver growth following PH, the liver-to-body weight ratio of the regenerating liver was calculated at 1, 3, 7, 10, 14, and 21 days post-PH (Fig. 1). One-way ANOVA indicated a significant effect of days post-PH on liver-to-body weight ratio in regenerating liver [$F(6,22) = 84.36$, $P < 0.0001$]. Post hoc comparisons using Tukey's honestly significance difference test indicated the liver-to-body weight ratio in regenerating liver reached pre-PH size [mean = 2.79, 95% confidence interval(2.68, 2.90)] by 7 days post-PH [mean = 2.86, 95% confidence interval(2.55, 3.15), $P = 0.99$]. In addition, 28.4 ± 2.3 and 69.4 ± 4.2% (SE) of the pre-PH liver weight was regained by 1 and 3 days, respectively.

Alterations in YAP and p-YAP protein levels during liver regeneration. Previous studies have demonstrated that YAP overexpression results in increased hepatocyte proliferation

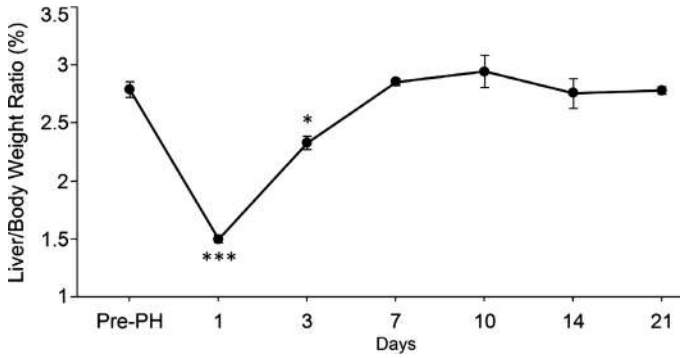

Fig. 1. Liver-to-body weight ratio following hepatectomy. The liver-to-body weight ratio in regenerating rat liver was calculated at 1 ($n = 8$), 3 ($n = 8$), 7 ($n = 4$), 10 ($n = 4$), 14 ($n = 4$), and 21 ($n = 4$) days following 70% partial hepatectomy (PH). Regenerating liver reached pre-PH size 7 days post-PH (Tukey's honestly significant difference, $P = 0.99$). Mean estimated liver-to-body weight ratio before PH for all animals = 2.79 ± 0.07 (SE). *$P < 0.05$, ***$P < 0.001$.

(1, 2); however, its role during liver regeneration has not been investigated. We therefore sought to determine whether YAP activation is increased during liver regeneration. With the use of Western blot analysis, levels of total YAP and its inactive form, phosphorylated YAP, were assessed in regenerating livers at 1, 3, 7, 10, 14, 21, and 30 days post-PH. One-way multivariate ANOVA indicated a significant effect of days post-PH on total YAP [$F(6,20) = 5.08$, $P = 0.026$] and p-YAP levels [$F(6,23) = 3.62$, $P = 0.011$] in regenerating liver. Total YAP levels were significantly increased at 1 day post-PH [$t(2) = 9.40$, $P = 0.011$] and remained elevated through *day 3* [$t(3) = 4.17$, $P = 0.025$] in the regenerating group compared with control group (Fig. 2*A*). In addition, there was a progressive and significant decrease in p-YAP levels starting at *day 3* post-PH [$t(3) = -3.50$, $P = 0.02$] through *day 14* [$t(3) = -3.42$, $P = 0.02$] in the regenerating group compared with control group (Fig. 2*B*). Investigation of total YAP and p-YAP levels following sham operation revealed no significant change in total YAP levels [*day 1*: $t(4) = 0.61$, $P = 0.57$; *day 7*: $t(3) = 0.59$, $P = 0.60$] or p-YAP levels [*day 1*: $t(3) = 1.40$, $P = 0.26$; *day 7*: $t(3) = 2.11$, $P = 0.12$] compared with the control group. These findings indicate that YAP activation is specifically increased following PH.

The YAP paralog, transcriptional coactivator with PBZ binding motif (TAZ), has been shown to share similar functions with YAP in mediating cell proliferation. In addition, TAZ activity is regulated by the Hippo signaling pathway (6). Given our finding of increased YAP activation, we sought to investigate whether TAZ activation was also altered during liver regeneration. Using Western blots, we measured alterations in total TAZ and inactive phosphorylated TAZ (p-TAZ) levels in regenerating liver at 1, 3, and 7 days post-PH. Our results indicate that there was no significant change in total TAZ [*day 1*: $t(3) = 0.25$, $P = 0.82$; *day 7*: $t(2) = -2.96$, $P = 0.10$] or inactive p-TAZ [*day 1*: $t(3) = 0.05$, $P = 0.97$; *day 7*: $t(2) = 0.98$, $P = 0.43$] in the regenerating group compared with the control group (Fig. 3).

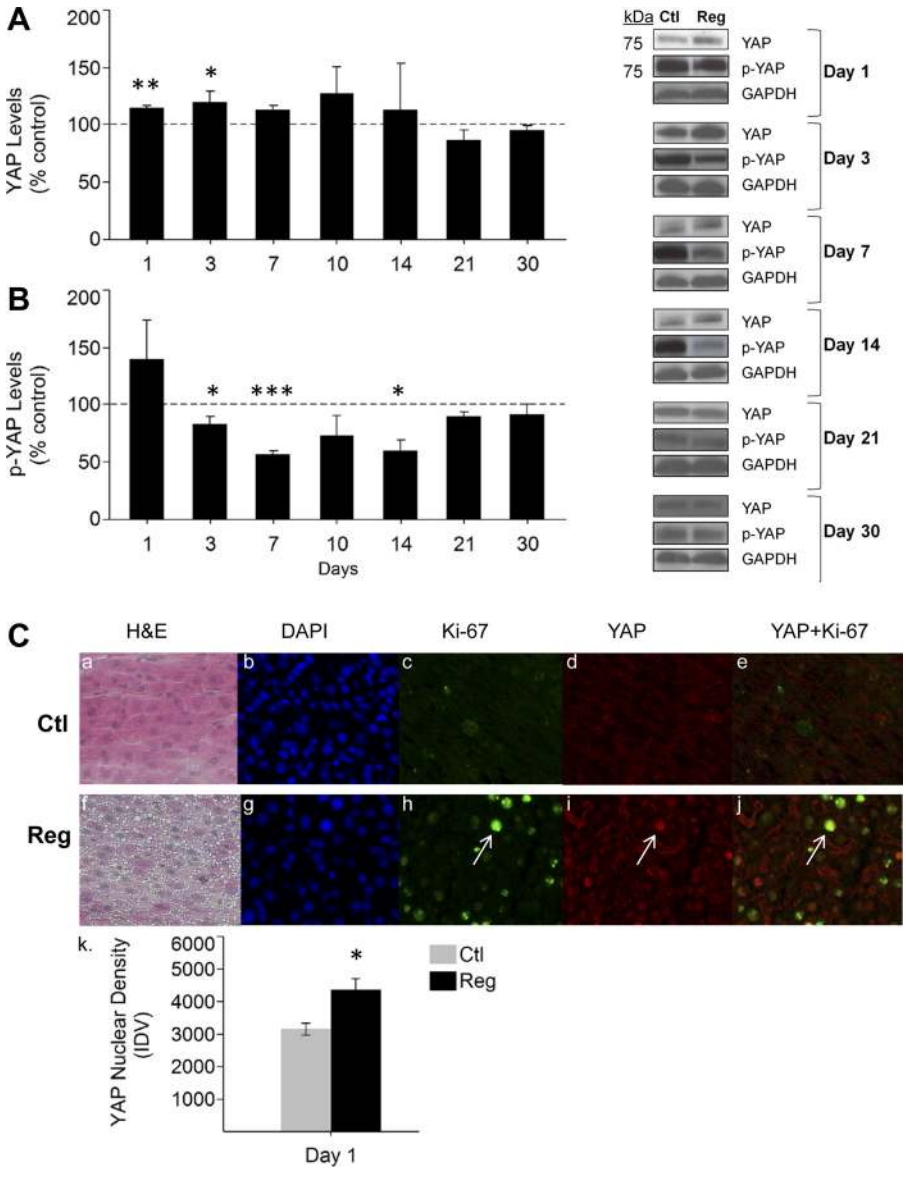

Fig. 2. Activation of Yes-associated protein (YAP) is increased during liver regeneration. *A*: Western blot analysis demonstrating an increase in total YAP levels from *days 1–3* following PH in regenerating liver (Reg) compared with controls (Ctl). *B*: phosphorylated (p)-YAP (inactive form) levels were decreased from *days 3-14* following PH in Reg liver compared with Ctl as measured by Western blot analysis. Dashed line represents YAP and p-YAP levels in Ctl group ($n = 4$/group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *C*: representative histologic and immunohistochemical comparison of quiescent (Ctl) and regenerating (Reg) liver tissue at 1 day following PH. *a* and *f*: Representative hematoxylin and eosin (H&E) sections. *b* and *g*: Nuclear staining using DAPI. Ki-67 staining demonstrates increased proliferation in the Reg liver (*h*) compared with control (*c*). Increased YAP nuclear localization in Reg (*i*) compared with Ctl (*d*; $n = 3$; $P < 0.05$). Merged immunofluorescence of Ki-67 and YAP in Reg (*j*) and Ctl (*e*) livers. A representative cell positive for Ki-67 and nuclear YAP is marked by arrow. *k*: Bar graph represents quantitative analysis of YAP nuclear density demonstrating a significant increase in Reg livers compared with controls at day 1 following PH ($n = 3$/group; $^*P < 0.05$).

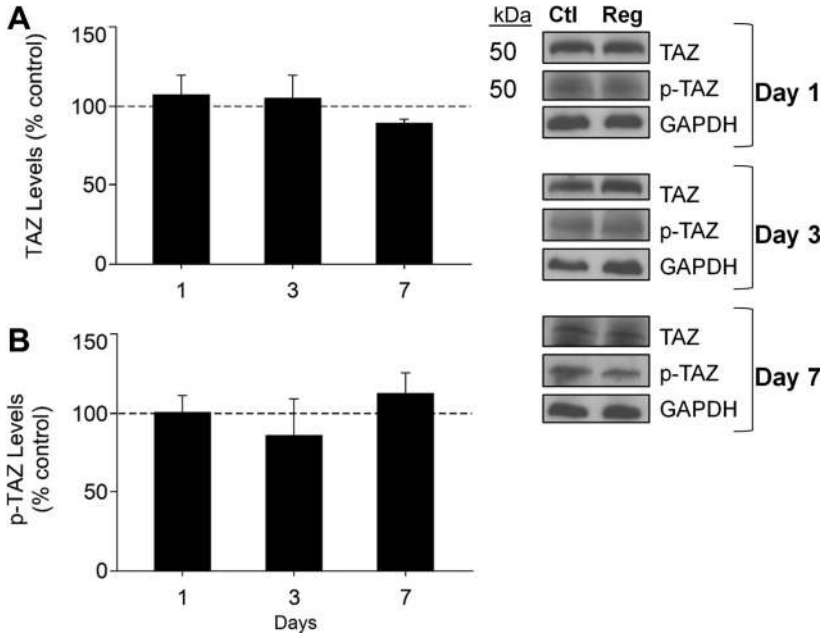

Fig. 3. TAZ activity is not altered during liver regeneration. Levels of total TAZ (*A*) and inactive p-TAZ (*B*) were not significantly altered at 1 through 7 days following PH as measured by Western blot analysis. Dashed lines represent TAZ and p-TAZ levels in Ctl group (n = 4/group).

YAP nuclear localization is increased during liver regeneration. The subcellular localization of YAP has been demonstrated to be regulated by its phosphorylation state (18). Phosphorylation of YAP increases its cytoplasmic retention and, hence, inhibits its function as a transcriptional coactivator. Our Western blot findings of decreased YAP phosphorylation during liver regeneration suggest increased YAP activation. Therefore, to determine whether YAP activation subsequently results in its nuclear localization, YAP immunostaining was performed on regenerating liver sections at 1, 7, and 21 days post-PH and compared with quiescent liver tissue. We analyzed four sections per animal from three animals at each time point. Our results indicate that there was a significant increase in YAP nuclear localization at 1 day [$t(2) = 4.15$, $P = 0.05$] and 7 days post-PH [$t(2) = 6.50$, $P = 0.02$] (Fig. 2*C*). In addition, YAP nuclear levels were comparable to levels observed in quiescent tissue by 21 days post-PH. To determine whether YAP nuclear localization was present in actively proliferating cells, coimmunostaining with the cell cycle marker Ki-67 (3) was performed. As expected, there was a significant increase in Ki-67 expression in regenerating liver compared with control (24.8% regenerating vs. 7.0% control) at 1 day post-PH [$t(3) = 3.62$, $P = 0.04$]. In addition, there was a significant increase in colocalization of nuclear YAP and Ki-67 in regenerating liver at 1 day post-PH compared with control [$t(3) = 4.60$, $P = 0.02$]. Furthermore, investigation of YAP nuclear localization in regenerating liver revealed that 44.5% of Ki-67-positive cells did not exhibit increased nuclear YAP.

YAP target gene expression is increased during liver regeneration. YAP is a transcriptional coactivator that promotes cellular proliferation and inhibits apoptosis through its interaction with transcription factors (e.g., TEAD family of transcription factors) (2). Given our finding of increased YAP activation and nuclear localization, we examined whether the expression of known YAP target genes, Cyr61, AmotL2, and CTGF (2, 19), is also altered during liver regeneration. With the use of reverse transcription followed by RT-qPCR, transcript levels of Cyr61, AmotL2, and CTGF were measured in regenerating liver at 1, 3, and 7 days post-PH. One-way multivariate ANOVA indicated a significant effect of days post-PH on Cyr61 transcript levels [$F(2,8) = 4.57$, $P = 0.0475$] and AmotL2 transcript levels [$F(1,12) = 6.97$, $P = 0.022$]. There was a significant increase in Cyr61 transcript levels at 1 day [$t(2) = 6.80$, $P = 0.01$] and 3 days [$t(3) = 2.45$, $P = 0.045$] post-PH compared with quiescent tissue (Fig. 4). In addition, there was a significant increase in AmotL2 transcript levels at 1 day [$t(5) = 2.51$, $P = 0.027$] and 3 days [$t(5) = 2.47$, $P = 0.028$] post-PH compared with quiescent tissue. Investigation of CTGF expression during liver regeneration revealed a nonsignificant increase in CTGF transcript levels at 1 [$t(4) = 1.01$, $P = 0.19$] and 3 days [$t(4) = 1.69$, $P = 0.08$] post-PH.

Chromatin immunoprecipitation using YAP antibody followed by qPCR was used to determine whether increases in gene expression were due to an increase in YAP binding to the Cyr61 and AmotL2 promoters. There was a significant increase in YAP association with the Cyr61 [$t(4) = 2.02$, $P = 0.05$] and AmotL2 [$t(4) = 2.24$, $P = 0.04$] gene promoters 1 day following PH compared with quiescent tissue (Fig. 5), indicating a direct YAP-mediated transcriptional upregulation of Cyr61 and AmotL2.

Hippo kinase Mst1/2 and Lats1/2 activity is attenuated during liver regeneration. Given that there is a decrease in p-YAP levels during liver regeneration (Fig. 2), we aimed to identify the Hippo kinases that played a role in this process. The Hippo pathway includes the core kinases Sterile 20-like kinases Mst1 and Mst2 (Mst1/2) and the large tumor suppressor homolog kinases Lats1 and Lats2 (Lats1/2) that function to regulate YAP activity through modulation of its phosphorylation state (2).

Mst1/2 kinases are constitutively active in quiescent liver, and their deletion results in increased YAP levels, decreased p-YAP levels, increased YAP nuclear localization, and liver overgrowth (7, 20). These findings indicate that Mst1/2 plays a

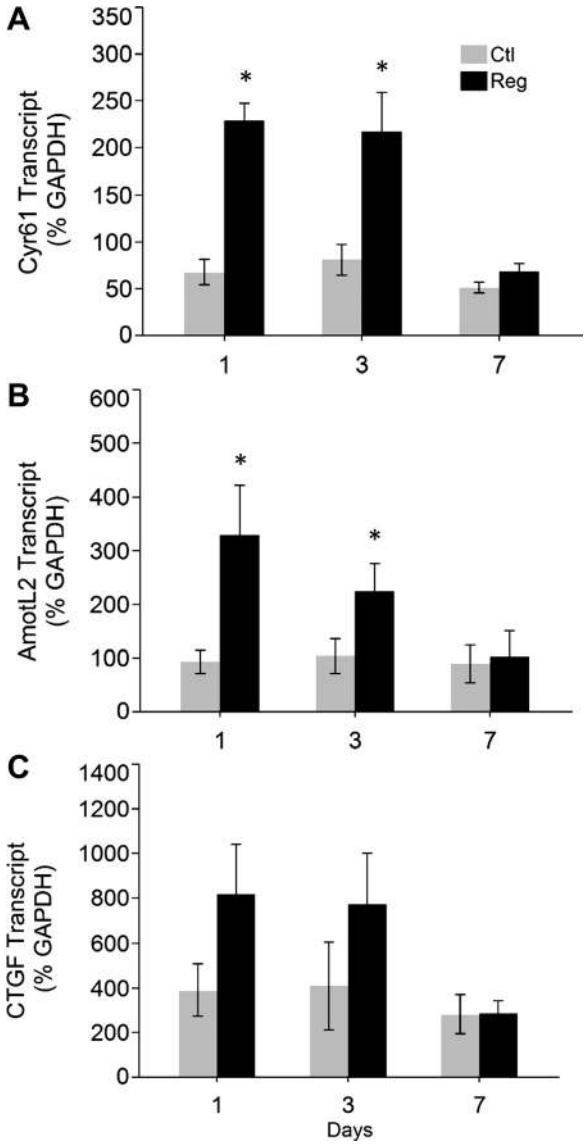

Fig. 4. Expression of YAP target genes is increased during liver regeneration. Expression of YAP target genes cysteine-rich angiogenic protein 61 (Cyr61; *A*), angiomotin-like protein 2 (AmotL2; *B*), and connective tissue growth factor (CTGF; *C*) was measured by quantitative RT-PCR in Reg liver at 1, 3, and 7 days following PH. Transcript levels of Cyr61 and AmotL2 were significantly increased at 1 and 3 days following PH ($n = 4–6$/group; $^*P < 0.05$).

role in regulating the phosphorylation state of YAP. Using Western blot analysis, we measured active phosphorylated Mst1/2 (p-Mst1/2) and inactive nonphosphorylated Mst1 (Mst1) levels in regenerating liver tissue at 1, 3, 7, 10, and 14 days post-PH. One-way multivariate ANOVA indicated a significant effect of days post-PH on p-Mst1/2 levels [$F(4,22) = 3.65$, $P = 0.02$] in the regenerating group compared with the control group. There was a significant decrease in p-Mst1/2 levels at 1 day [$t(7) = -3.19$, $P = 0.008$] and 3 days post-PH [$t(6) = -2.32$, $P = 0.03$; Fig. 6*A*]. In addition, levels of inactive, nonphosphorylated Mst1 were significantly increased by 7 days post-PH [$t(3) = 3.24$, $P = 0.047$]. To compare the levels of active vs. inactive Mst kinase, the ratio of p-Mst1/2 (active) to Mst1 (inactive) was quantified. One-way multivariate ANOVA indicated a significant effect of days post-PH on active p-Mst1/2-to-inactive Mst1 ratios [$F(2,12) = 4.15$, $P = 0.043$]. The relative amount of p-Mst1/2 was significantly reduced at 3 [$t(6) = -3.28$, $P = 0.019$] and 7 [$t(3) = -10.92$, $P = 0.002$] days post-PH in regenerating liver, indicating a decrease in the active form of Mst1/2 kinase (Fig. 6*B*).

Previous studies have demonstrated that Mst1/2 and Lats1/2 kinases function in a signaling cascade to phosphorylate and thereby inhibit YAP activity (2, 9). In addition, activated Mst1/2 kinases catalyze the phosphorylation of Mob1, promoting Mob1 binding to Lats1/2 kinases and augmenting Mst1/2-mediated activation of Lats1/2 kinases (9). We therefore sought to determine whether decreased levels of active Mst1/2 kinases in regenerating liver were coupled with alterations in Mob1 and Lats1/2 kinase activation. Using Western blot analysis, we measured active phosphorylated Lats1/2 (p-Lats1/2) and phosphorylated Mob1 (p-Mob1) levels in regenerating liver at 1, 3, and 7 days post-PH. One-way multivariate ANOVA indicated a significant effect of days post-PH on p-Mob1 levels [$F(2,8) = 8.96$, $P = 0.009$] and p-Lats1/2 levels [$F(2,8) = 6.74$, $P = 0.019$] in the regenerating group compared with the control group. There was a significant decrease in p-Mob1 levels at 3 days post-PH [$t(3) = -5.77$, $P = 0.01$; Fig. 7*A*]. Furthermore, there was a significant decrease in active p-Lats1/2 levels at 3 days [$t(3) = -3.55$, $P = 0.04$] and 7 days post-PH [$t(2) = -6.68$, $P = 0.02$; Fig. 7*B*]. These findings suggest that alterations in Mst1/2 phosphorylation in regenerating liver are coupled with decreased phosphorylation and activation of Mob1 and Lats1/2 kinases.

DISCUSSION

The Hippo signaling pathway has been shown to play a role in liver size regulation and inhibition of its signaling results in massive liver overgrowth (20). In addition, prolonged inactivation of the Hippo pathway and YAP overexpression result in tumorigenesis (2, 7). Importantly, restoration of Hippo signaling is sufficient to reverse liver overgrowth and return the liver to its near normal liver-to-body weight ratio. Despite numerous studies demonstrating the role of the Hippo pathway in modulating hepatocyte proliferation and apoptosis, the physiologic role of the Hippo pathway during liver regeneration has not been elucidated. In this study, we aimed to investigate whether the Hippo pathway is altered during liver regeneration. In brief, we found that the Hippo pathway is inhibited early during liver regeneration, resulting in increased YAP activation, as dem-

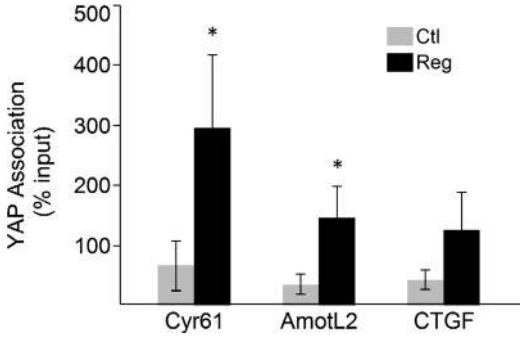

Fig. 5. YAP associates with Cyr61 and AmotL2 promoter during liver regeneration. Chromatin immunoprecipitation (ChIP) analysis demonstrated a significant increase in the association of YAP with Cyr61 and AmotL2 gene promoters 1 day following PH ($n = 5$/group; $^*P < 0.05$).

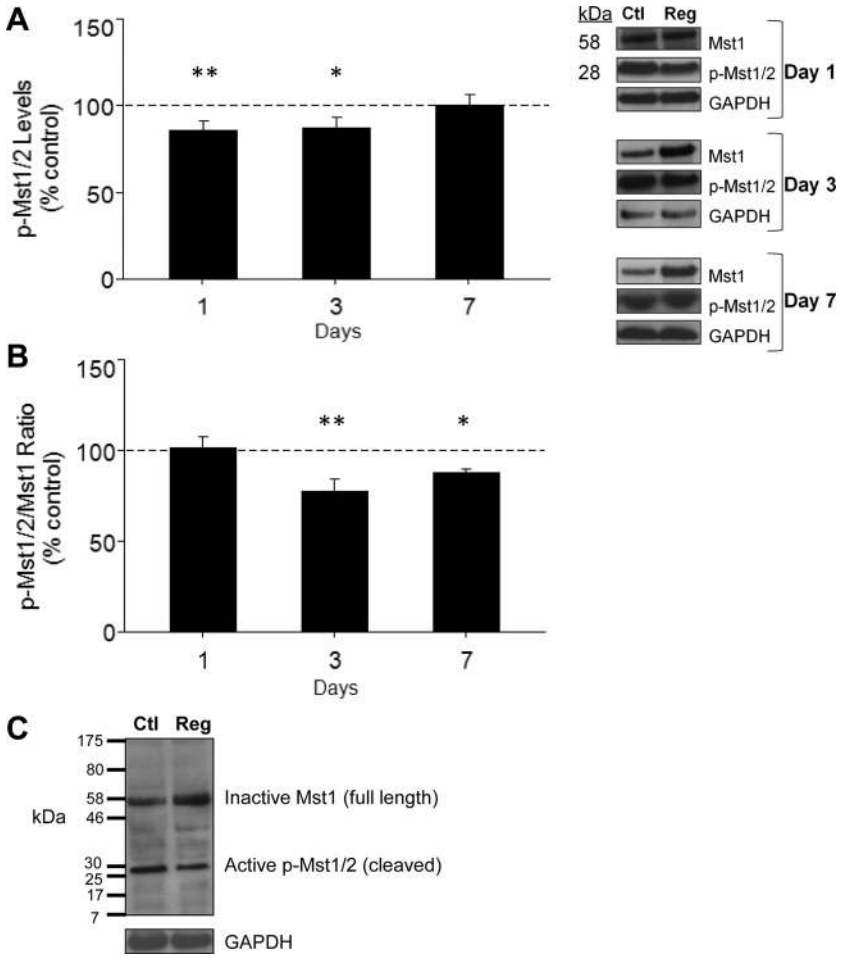

Fig. 6. Mst1/2 kinase activation is decreased during liver regeneration. *A*: Western blot analysis revealed decreased active p-Mst1/2 levels in regenerating (Reg) liver at 1 and 3 days post-PH and increased inactive Mst1 levels at 7 days post-PH. *B*: ratio of active p-Mst1/2 to inactive Mst1 was quantified and revealed a significant decrease in the relative amount of activated Mst1/2 kinase in regenerating liver at 3 and 7 days post-PH. Dashed line represents p-Mst1/2 levels and p-Mst1/2-to-Mst1 ratio in Ctl group ($n = 4–8$/group; $*P < 0.05$, $**P < 0.01$). *C*: representative immunoblot of inactive Mst1 (full length) and active phosphorylated Mst1/2 (cleaved) fragment in control and regenerating liver at 3 days following PH.

onstrated by decreased inactive p-YAP levels and increased YAP nuclear localization. This effect was specific to YAP as there was no change in TAZ and p-TAZ levels in regenerating liver. We also found that increased YAP nuclear localization was associated with increased YAP target gene expression. Finally, following the termination of liver regeneration, the Hippo pathway is no longer inhibited and levels of active core Hippo kinases Mst1/2 and inactive phosphorylated YAP return to levels measured in quiescent liver tissue.

We utilized the well-established 70% PH rat model to study alterations in Hippo signaling during liver regeneration. This model allowed us to monitor the time course and rate of liver growth following hepatectomy. We found that the liver-to-body weight ratio of the regenerating liver reached prehepatectomy levels by 7 days following 70% hepatectomy. These results are consistent with previous reports (5). Additionally, ~30% of the prehepatectomy liver weight was regained by 1 day and 70% by 3 days following 70% hepatectomy, indicating that the highest rate of liver growth occurs within the first 3 days.

Given the tremendous rate of regeneration seen in the first 7 days following PH, we focused our attention on evaluating alterations in the Hippo signaling pathway and YAP activity during this time period. Our results indicate that YAP protein levels were significantly increased during the first 3 days post-PH (Fig. 2*A*) compared with quiescent liver tissue. Wu et al. (16) observed similar findings when measuring YAP levels following PH. In addition, evaluation of inactive phosphorylated YAP levels revealed a significant decrease from 3 days post-PH through 14 days (Fig. 2*B*). Previous investigation has shown that the YAP paralogue TAZ shares similar functions with YAP in mediating cell proliferation and is regulated by the Hippo pathway in a similar manner (6). Zhou et al. (20) found that acute inactivation of Mst1/2 in the liver resulted in decreased levels of both p-TAZ and TAZ and, therefore, concluded that TAZ does not likely play a role in mediating hepatocyte proliferation following Mst1/2 inactivation. Interestingly, our investigation of TAZ activity during liver regeneration revealed no significant alterations in total TAZ or inactive p-TAZ levels during the first 7 days following PH. These findings suggest that YAP and TAZ may not share similar functions in mediating hepatocyte proliferation during liver regeneration. Importantly, our findings indicate a clear correlation between the rate of hepatocyte proliferation and the presence of active YAP. By day 21 post-PH, the levels of phosphorylated YAP returned to baseline levels at which point the rate of liver regeneration is decreased tremendously and the liver has reached its near final mass. Interestingly, although the prehepatectomy liver weight was reached by 7 days post-PH, p-YAP levels remained reduced through 14 days following PH. Previous studies have shown that during liver regeneration the liver architecture is initially disorganized and undergoes a period of reorganization (13). Our finding of persistently decreased p-YAP levels following the liver reaching its near final

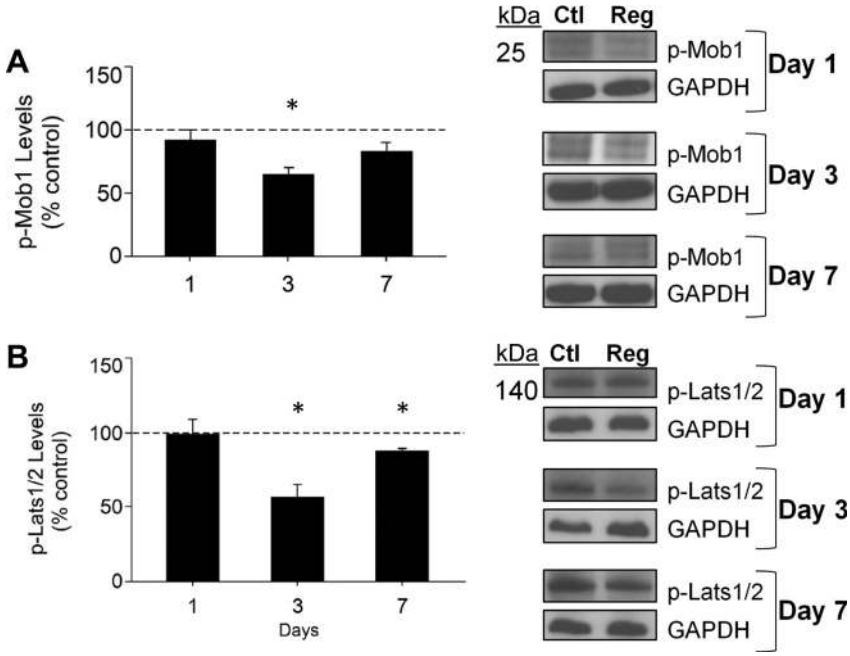

Fig. 7. Lats1/2 kinase and Mob1 activation is decreased during liver regeneration. *A*: Western blot analysis demonstrating decreased active p-Mob1 levels in regenerating (Reg) liver at 3 days post-PH. *B*: Western blot analysis demonstrating decreased active p-Lats1/2 levels in Reg liver at 3 and 7 days post-PH. Dashed line represents p-Mob1 and p-Lats1/2 levels in Ctl group ($n = 4$/group; $^*P < 0.05$).

mass may suggest that YAP continues to play a role in the reorganization of liver architecture.

Phosphorylation of YAP inhibits its function as a transcriptional coactivator through decreased nuclear localization. Therefore, decreased p-YAP levels suggest decreased cytoplasmic retention, which is expected to result in increased YAP nuclear localization. We confirmed this using immunohistochemical studies showing significantly increased YAP nuclear localization at 1 and 7 days following PH (Fig. 2*C*). In addition, as expected, we noted significant hepatocyte proliferation 1 day following PH by using immunohistochemical staining for the cell cycle marker Ki-67. Coimmunostaining demonstrated that the majority of Ki-67-positive cells expressed increased nuclear levels of YAP; however, YAP nuclear localization was not clearly present in a subset of Ki-67-positive cells. In addition, there were numerous cells that demonstrated increased YAP nuclear localization without concomitant Ki-67 expression. These findings may suggest that increased YAP nuclear localization precedes entry into the cell cycle and its nuclear localization may resolve before cell cycle exit. Interestingly, recent investigation has revealed that in addition to the direct role of YAP in mediating cell autonomous proliferation, a non-cell autonomous mechanism has been demonstrated through the secretion of factors under YAP transcriptional regulation that stimulate proliferation of neighboring cells. Specifically, the YAP target gene amphiregulin, an epidermal growth factor receptor ligand, has been demonstrated to stimulate proliferation in a non-cell autonomous manner in mammary epithelial cells (17). This non-cell autonomous function of YAP may be another mechanism of enhanced hepatocyte proliferation during liver regeneration; however, further investigation is needed to fully elucidate this mechanism of YAP-mediated hepatocyte proliferation.

Microarray analysis of tissue from transgenic mice with liver-specific YAP overexpression has provided insight into the transcriptional activity of YAP (2). YAP regulates the transcription of several genes involved in hepatocyte proliferation as well as inhibitors of apoptosis. Specifically, known YAP target genes CTGF, Cyr61, and AmotL2 have been shown to play a role in cellular proliferation, extracellular matrix production, angiogenesis, and cell migration (15, 19). Our results demonstrated a significant increase in the Cyr61 and AmotL2 transcript levels at 1 and 3 days post-PH. In addition, single gene ChIP analysis demonstrated an increased association of YAP with both gene promoters (Cyr61 and AmotL2) at 1 day post-PH, strengthening the hypothesis that increased YAP binding at the gene promoters leads to activation of their expression. However, our finding of increased YAP nuclear localization and YAP target gene expression at 1 day following PH without significant alteration in YAP phosphorylation suggests that YAP activation at this early phase of liver regeneration may be driven by a mechanism other than decreased phosphorylation. Our results demonstrated a decrease in active p-Mst1/2 levels and increase in total YAP levels by 1 day post-PH. Wu et al. (16) demonstrated that Mst1/2 activity in the liver regulates YAP mRNA levels. Using a liver-specific Mst1/2 knockout mouse model, they demonstrated that acute inactivation of Mst1/2 resulted in increased YAP mRNA levels and, subsequently, increased YAP protein levels (16). These findings suggest that YAP activation during the early phase of liver regeneration may be mediated by increased expression secondary to decreased Mst1/2 activation. In addition, our data contradict previous studies that have demonstrated an increase in CTGF transcription (16, 19). This discrepancy may be explained by the different experimental models as prior investigations of YAP transcriptional regulation were performed using knockout models of Hippo pathway components (20) or transgenic overexpression of YAP (2), which are fundamentally different than our liver regeneration model. Our investigation of YAP transcriptional activity during the natural process of liver regeneration may represent a more physiologic process of YAP transcriptional regulation and could explain the observed differences with these previous findings.

Given our findings of increased YAP activation and nuclear localization, we sought to examine changes in activity of core Hippo kinases during liver regeneration. Recent in vivo studies of the Hippo pathway have demonstrated that the core Hippo kinases Mst1/2 play a critical role in regulating the phosphorylation state and activation of YAP (20). The majority of activated Mst1/2 kinase in quiescent liver is present as a phosphorylated amino-terminal fragment with absence of a nuclear export signal, resulting in enhanced nuclear translocation to target its nuclear downstream effector, YAP (14, 20). In addition, deletion of Mst1/2 kinase in the liver results in YAP-induced hepatocyte proliferation (7, 20). Our results confirm that Mst1/2 kinase is constitutively activated in quiescent liver tissue as a phosphorylated 28-kDa fragment (Fig. 6*C*). Additionally, we found that Mst1/2 kinase activation is attenuated following PH. This is supported by the observed decrease in active p-Mst1/2 levels during the first 3 days post-PH (Fig. 6*A*). Comparison of active p-Mst1/2 to inactive Mst1 indicated that the relative proportion of active p-Mst1/2 decreased at 3 and 7 days post-PH (Fig. 6*B*). Importantly, the significant decrease in p-Mst1/2 activity at 1 day post-PH preceded the significant decrease in p-YAP levels observed by day 3. These findings suggest a temporal sequence of Mst1/2 inactivation with subsequent decreased YAP phosphorylation during the early phase of liver regeneration.

In the canonical Hippo pathway, the core kinases Mst1/2 in association with scaffold protein Sav1 function to phosphorylate Lats1/2 and the noncatalytic protein Mob1 (2). Phosphorylated Mob1 then binds to and facilitates the activation of Lats1/2, which in turn phosphorylates YAP. However, in vivo investigation of Lats1/2 function in liver-specific Mst1/2 knockout mice has demonstrated inconsistent findings. Zhou et al. (20) observed that acute Mst1/2 inactivation in the liver did not significantly alter Lats1/2 phosphorylation in vivo. Additionally, following transfection of Mst1 in an Mst1/2 null HCC cell line, YAP phosphorylation increased and YAP nuclear localization decreased; however, Lats1/2 phosphorylation was not significantly altered. These results suggest that Mst1/2 regulates YAP in hepatocytes and HCC cells independently of Lats1/2 kinase. However, Lu et al. (7) observed a significant decrease in Lats1/2 phosphorylation following inactivation of Mst1/2 in the liver, suggesting that Mst1/2 and Lats1/2 kinases function in a cascade to regulate YAP phosphorylation. Our investigation of Mob1 and Lats1/2 kinase activity revealed a significant decrease in Mob1 and Lats1/2 phosphorylation following PH, suggesting that Mst1/2 regulates YAP through Mob1 and Lats1/2 during liver regeneration. These observations are consistent with the findings by Lu et al. (7) and in vitro studies demonstrating that Mst1/2 kinases directly phosphorylate and regulate Mob1 and Lats1/2 kinase activity (9).

In summary, our findings demonstrate that the transcriptional coactivator YAP is activated during liver regeneration, resulting in its nuclear localization and activation of a proliferative transcriptional program. The pattern of activation and inactivation of YAP and associated Mst1/2 and Lats1/2 kinases correlates with liver growth following PH. Future studies will aim at determining if the Hippo signaling cascade and YAP activation are sufficient and necessary for liver regeneration as well as the mechanisms responsible for the reactivation of the Hippo signaling pathway during the termination phase of liver regeneration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.L.G., F.C., G.S.-V., and K.V. conception and design of research; J.L.G., M.H., K.M., S.R., and J.B. performed experiments; J.L.G., M.H., K.M., S.R., G.S.-V., and K.V. analyzed data; J.L.G., M.H., K.M., S.R., F.C., G.S.-V., and K.V. interpreted results of experiments; J.L.G. prepared figures; J.L.G. drafted manuscript; J.L.G., F.C., G.S.-V., and K.V. edited and revised manuscript; J.L.G., G.S.-V., and K.V. approved final version of manuscript.

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