RESEARCH ARTICLE

Figures and figure supplements

Intermittent fasting induces rapid hepatocyte proliferation to restore the hepatostat in the mouse liver

Abby Sarkar *et al.*

Figure 1. Intermittent fasting (IF) induces rapid hepatocyte proliferation. (**A**) Ki67 immunofluorescence for the detection of proliferating cells in ad libitum (AL), 1- and 3-week IF-treated livers. IF-treated livers were analyzed 30 min after re-feeding cycle. (**B, C**) Quantification of spatial distribution and percentage of Ki67+ hepatocytes in AL- and IF-treated livers. One-way analysis of variance (ANOVA), *N* = 4.(**D**) Dox inducible Axin2-rtTA; Teto-H2BGFP system to label Axin2+ pericentral hepatocytes and trace cell proliferation. Mice were pulsed with dox for 7 days, cleared of dox for 3 days and AL-fed or intermittently fasted for 6 days. (**E**) GFP immunofluorescent images showing increased hepatocyte expansion in AL and IF compared to T0. (**F**) Percentage of GFP+ hepatocyte nuclei in AL, IF livers from A. One-way ANOVA, *N* = 3 (T0), 5 (AL), 5 (IF). **p < 0.01; *p < 0.05. Error bars indicate standard deviation. Scale bar, 100 µm. wk, weeks.

Figure 1—figure supplement 1. Hepatocyte proliferation kinetics in ad libitum (AL) fed and intermittent fasted animals. (**A**) Schematic of unbiased system to trace cell proliferation during AL feeding and intermittent fasting (IF). *R26-CreER* mice were crossed to *R26-Confetti* mice enabling permanent cell labeling and lineage tracing by four fluorescent reporters after tamoxifen administration. (**B**) At T0, mostly single, HNF4A+ hepatocytes were labeled throughout the lobule. At 1 and 3 weeks, multicellular hepatocytes clones (dotted circles) grew in AL and IF livers, with increased pericentral

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

growth in IF. (**C**) The number of hepatocytes per 3D clone at each collection in A. One-way analysis of variance (ANOVA), 439–602 clones analyzed at T0, 430–1085 clones at 1 wk AL, 625–904 clones at 1 wk IF, 523–615 clones at 3 wk AL, and 442–615 at 3 wk IF. $N = 3$. (**D**) Percentage of 3D clones consisting of different cell sizes, from C, in different liver lobule locations. PC, pericentral; Mid, midlobular; PP, periportal. Differences between 1-, 2-, and >3-cell clones in periportal and pericentral zones are indicated by p values above bars. Two-way ANOVA. $N = 3$. (**E**) Schematic of system to trace hepatocyte proliferation over 3 months of IF or AL treatment. Axin2-rtTA; Teto-Cre; *Rosa26-mTmG* mice were induced with tamoxifen enabling permanent cell labeling and lineage tracing by reporter GFP. (**F**) GFP immunofluorescent images highlighting Axin2+ hepatocyte tracing from T0, 1 week, 3 weeks, and 3 months after AL or IF treatment. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Error bars indicate standard deviation. Scale bar, 100 μm.

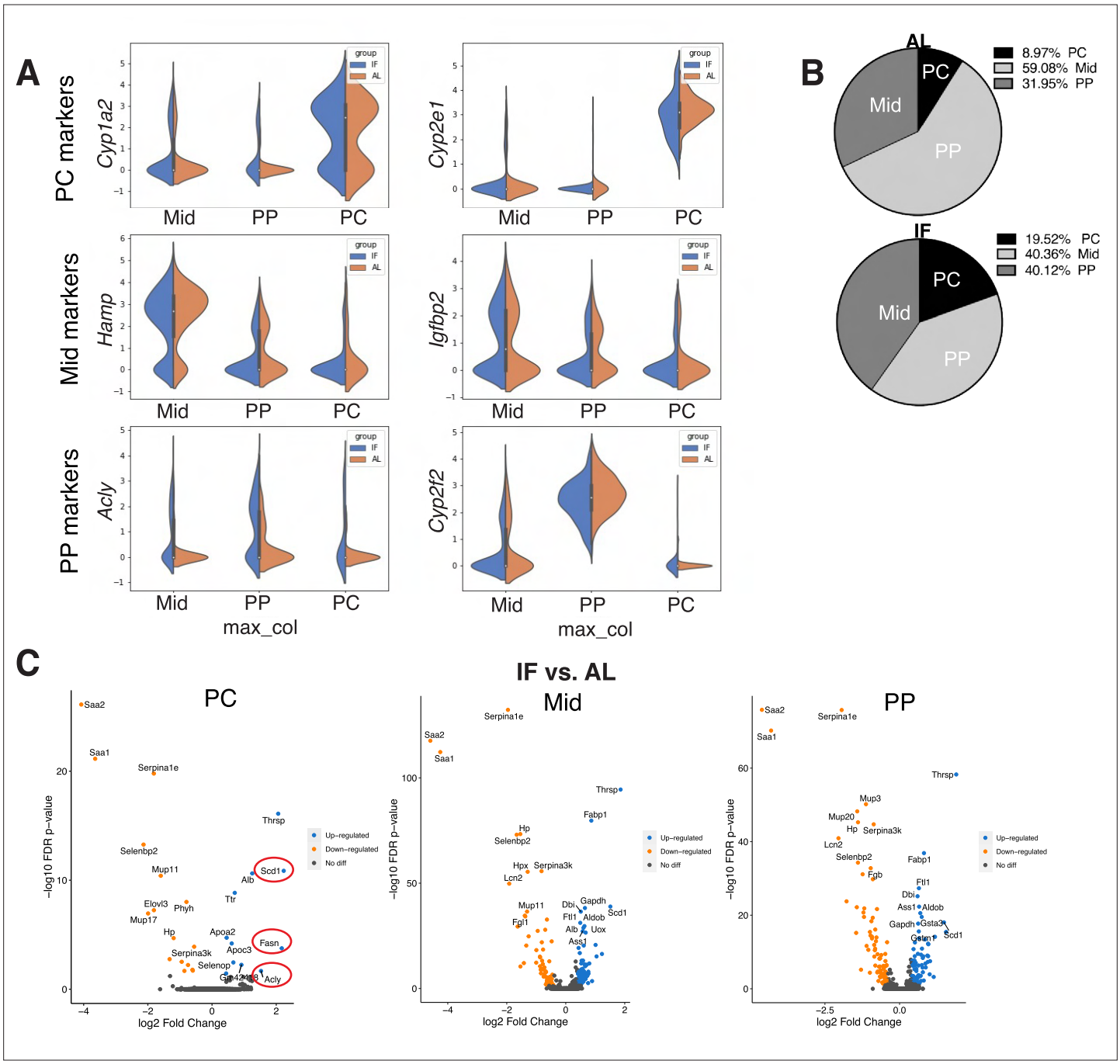

Figure 1—figure supplement 2. Single-cell RNA-seq comparing hepatocytes in ad libitum (AL) fed and intermittent fasted livers. (**A**) Violin plots, from scRNA-seq, demonstrating zonal marker gene expression used to classify single hepatocytes from AL and intermittent fasting (IF) livers as pericentral (PC), midlobular (Mid), and periportal (PP) hepatocytes. (**B**) Pie charts of hepatocyte zonal populations identified in scRNA-seq highlighting increase in PC hepatocytes in IF compared to AL livers. (**C**) Volcano plots highlighting differentially expressed transcripts in IF versus AL livers in PC, Mid, and PP hepatocytes. Red circles highlighting transcripts involved in de novo lipogenesis.

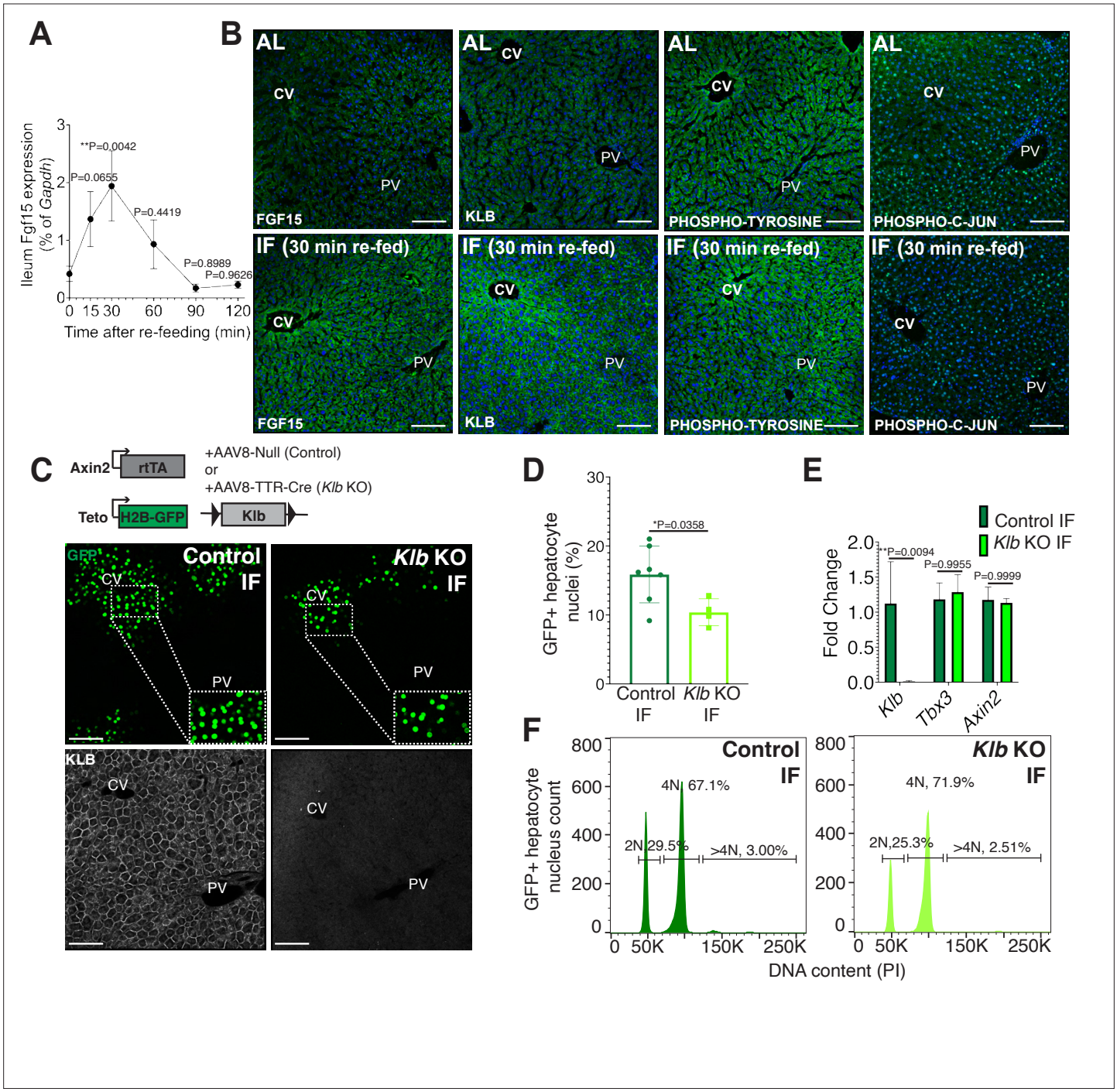

Figure 2. Endocrine FGF15-β-KLOTHO (KLB) signaling is required for hepatocyte proliferation during intermittent fasting (IF). (**A**) Quantitative real-time PCR analysis highlighting rapid increase in Fgf15 expression in ileum 30 min after re-feeding in 1-week IF-treated livers. One-way analysis of variance (ANOVA), comparison with time 0, $N = 3$. (**B**) Immunofluorescence for endocrine FGF pathway components highlighting pathway activation in 1-week IF-treated livers 30 min after re-feeding (ZT12). (**C**) Schematic of method to deplete hepatocytes of *Klb*. Axin2-rtTA; Teto-H2BGFP; *Klb flox/flox* mice were injected with AAV8-TTR-Cre (*Klb* KO). GFP and KLOTHO immunofluorescent images showing decrease in hepatocyte expansion and loss of KLOTHO in *Klb* KO compared to control livers. (**D**) Percentage of GFP+ hepatocyte nuclei in *Klb* KO and control livers. (**E**) Quantitative real-time PCR analysis confirming loss of *Klb* but not WNT target genes, *Tbx3* and *Axin2*, in *Klb* KO livers. Two-way ANOVA, $N = 3$. (**F**) Ploidy distribution of GFP+ hepatocyte nuclei incontrol IF and *Klb* KO IF livers. Unpaired *t*-test. $N = 7$ (control), 4 (*Klb* KO). $^{**}p < 0.01$, $^{*}p < 0.05$. Error bars indicate standard deviation. Scale bar, 100 μm.

Figure 3. Paracrine WNT and WNT target gene *Tbx3* promote hepatocyte proliferation during intermittent fasting (IF). (**A**) Method to constitutively activate WNT signaling in midlobular, periportal cells. AAV8-U6-sgAPC was injected into the tail vein of *Rosa26-Cas9* mice. Animals were IF treated for 1 week before analysis. GS immunofluorescent images for detection of Apc mutant clones in ad libitum (AL) and IF livers. (**B**) The number of Apc mutant hepatocytes per 3D clone expand in IF compared to AL livers. Mann–Whitney test. 130 clones analyzed in AL. 74 clones analyzed in IF. *N* = 3. White

Figure 3 continued on next page

Figure 3 continued

dashed lines demarcate multicellular non-pericentral GS+ clones. (**C**) Schematic of method to deplete hepatocytes of the WNT target, *Tbx3*. Axin2-rtTA; Teto-H2BGFP; *Tbx3flox/flox* mice were intraperitoneally injected with AAV8-TTR-Cre (*Tbx3* KO). GFP and TBX3 immunofluorescent images to show IF-induced proliferation and *Tbx3* depletion, respectively, in control and *Tbx3* KO livers. (**D**) Percentage of GFP + hepatocyte nuclei decreased in Tbx3 KO IF compared to control IF livers. Unpaired *t*-test, (N = 7 control IF), N = 4 (*Tbx3* KO IF). (**E, F**) Nuclear ploidy distribution of GFP+ hepatocytes highlighting hyper-polyploidization in *Tbx3* KO IF compared to control IF livers. Two-way analysis of variance (ANOVA), N = 3. (**G**) Schematic for Fgf15 overexpression. Axin2-rtTA; Teto-H2BGFP; *Tbx3 flox/flox* mice were injected with AAV-TTR-FGF15 (Fgf15 OE) and AAV8-Null (control) or AAV-TTR-CRE (*Tbx3* KO). (**H**) GFP immunofluorescent images from c AL, Fgf15 OE AL, *Tbx3* KO AL, Fgf15 OE; *Tbx3* KO AL livers. (**I**) Percentage of GFP+ hepatocyte nuclei highlighting lack of hepatocyte proliferation in *Tbx3* KO livers. Unpaired *t*-test, N = 4. (**J**) Dot plot highlighting increase in nuclear area with Fgf15 overexpression both with and without *Tbx3*. Unpaired *t*-test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Error bars indicate standard deviation. Scale bar, 100 μm.

Figure 4. Hepatocyte proliferation or compensatory polyploidization maintains the hepatostat during intermittent fasting (IF). (**A**) Liver-to-body weight ratio in wild-type livers during 2 days, 1 week, and 3 weeks of IF and ad libitum (AL) feeding. (**B–K**) Liver analyses after 3 weeks of IF treatment in control, *Klb* KO, and *Tbx3* KO livers. (**B**) Liver-to-body weight ratio. (**C**) Hepatocyte nuclear area. (**D**) Nuclear ploidy distribution of pericentral hepatocytes with hyper-polyploidization in *Tbx3* KO IF livers. (**E**) Immunofluorescence images for β-CATENIN and HNF4A highlighting hepatocyte cell and nuclear area

Figure 4 continued on next page

Figure 4 continued

during IF. (**F**) AST and ALT liver injury marker presence in serum. (**G**) Quantification and representative images of senescence-associated β-galactosidase stains. (**H**) RNAscope images and quantification for pericentral marker Cyp2e1 and periportal marker Cyp2f2. (**I**) Metabolomics PCA plot comparing control IF, *Klb* KO IF, and *Tbx3* KO IF livers. (**J**) Volcano plots comparing metabolites between control AL and control IF livers and control AL and *Klb* KO IF livers. The top 3 most significantly changed bile metabolites are labeled in blue. (**K**) Expression of bile acid pathway enzymes genes in livers. Quantitative PCR for genes for critical enzymes in bile acid pathway. All statistics were performed on N=3-5 animals using one-way analysis of variance (ANOVA). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Error bars indicate standard deviation. Scale bar, 100 μm.

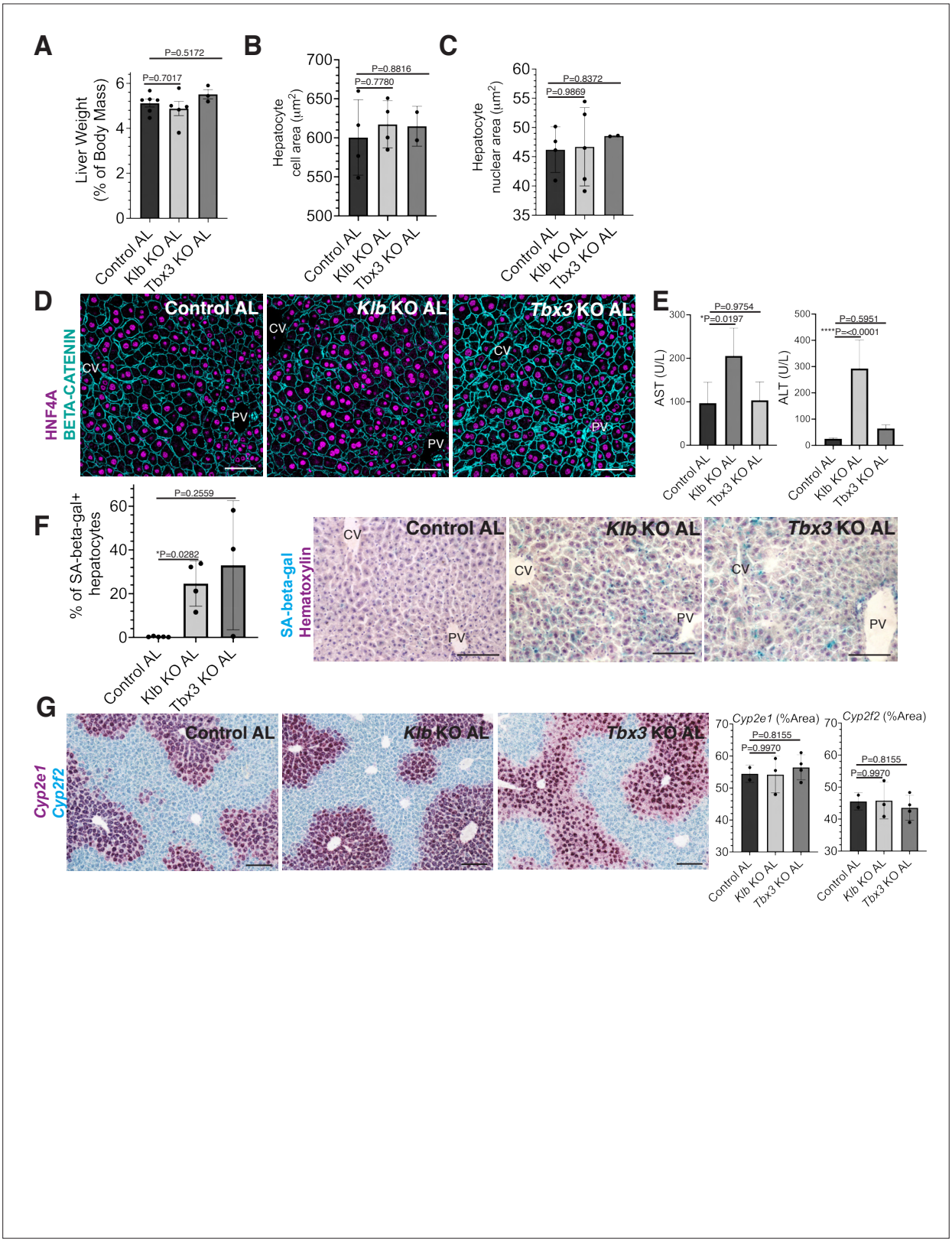

Figure 4—figure supplement 1. Short-term loss of *Tbx3* or *Klb* does not disrupt the hepatostat during ad libitum (AL) feeding. A-H AL *Klb* KO, AL *Tbx3* KO, and control AL livers were assessed at the same time point in ***Figure 4*** (3 weeks after AL feeding). (**A**) Liver-to-body weight ratio. (**B, C**) Hepatocyte cell and nuclear area. (**D**) Immunofluorescence images forβ-CATENIN and HNF4A highlighting hepatocyte cell and nuclear area during AL livers. (**E**) AST and ALT liver injury marker presence in serum. (**F**) Quantification and representative images of senescence-associated β-galactosidase

Figure 4—figure supplement 1 continued on next page

Figure 4—figure supplement 1 continued

stains. (**G**) RNAscope for pericentral marker *Cyp2e1* and periportal marker *Cyp2f2*. All statistics were performed on N=3-5 animals using one-way analysis of variance (ANOVA). ****$p < 0.0001$, *$p < 0.05$. Error bars indicate standard deviation. Scale bar, 100 μm.

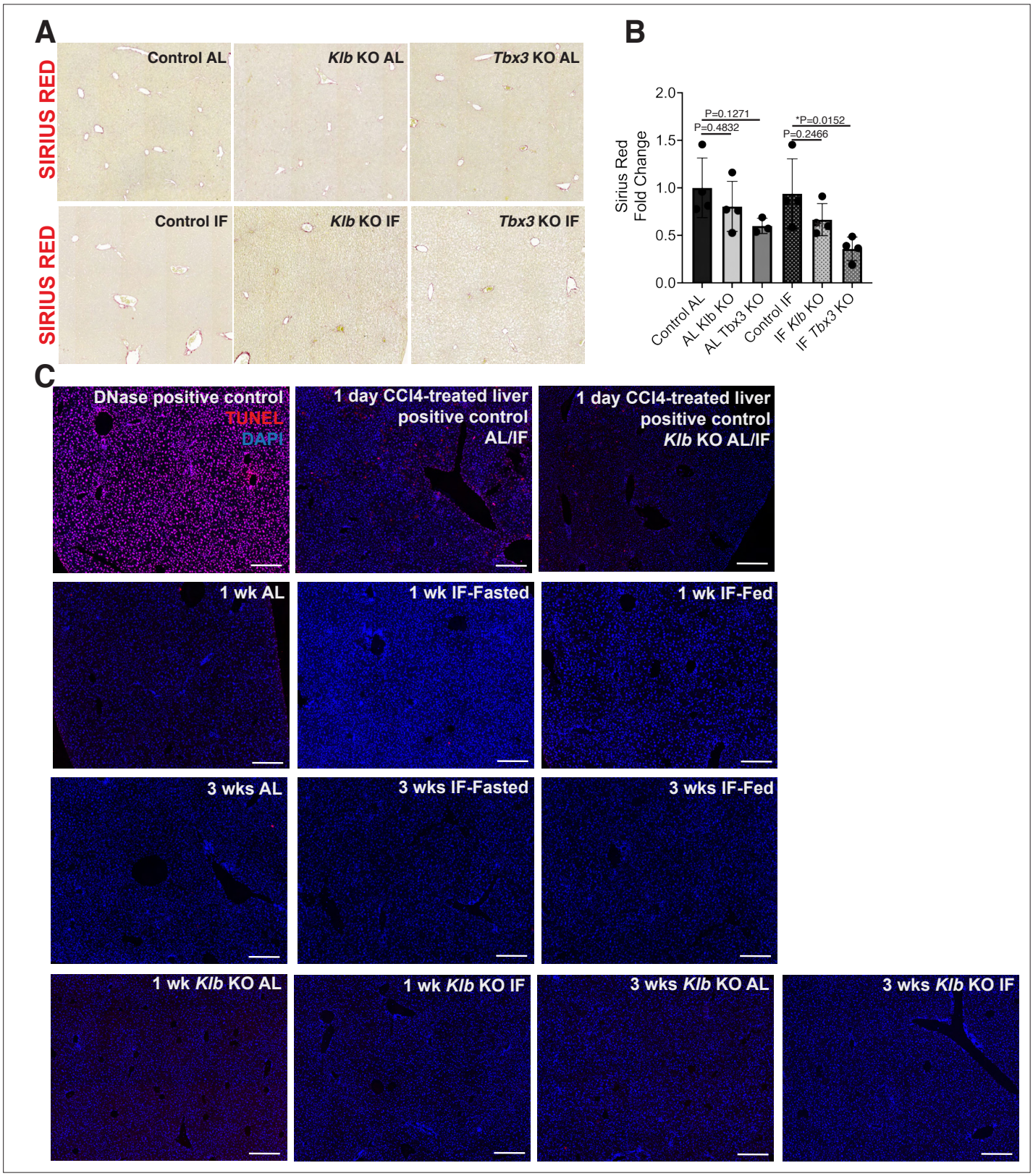

Figure 4—figure supplement 2. Fibrosis and cell death assessment of Control, *Klb* KO, and *Tbx3* KO IF- and ad libitum (AL)-treated livers. (**A, B**) Sirius red staining and quantification to assess for liver fibrosis. (**C**) TUNEL stains on livers. All statistics were performed on N=3-5 animals using one-way analysis of variance (ANOVA). *p < 0.05. Error bars indicate standard deviation. Scale bar, 100 μm.