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Single-Cell Analysis of the Liver Epithelium Reveals Dynamic Heterogeneity and an Essential Role for YAP in Homeostasis and Regeneration

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SUMMARY

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AUTHOR CONTRIBUTIONS

B.J.P.-M. and F.D.C. conceived the study; B.J.P.-M., and M.T.D. designed, performed and analyzed experiments, and prepared figures. A.A. aided in single-cell figure generation, performed all scRNA-seq computational analysis, and additional statistical analysis with direction from B.J.P.-M. and M.T.D. under the supervision of A.v.O.; J.O.-M. performed "Seq-Well" hepatocyte capture, library preparation, sequencing, and pre-processed sequencing data under the supervision of A.K.S.; Y.M., A.R. and A.S. performed experiments on collaborative mouse models under the supervision of N.O., P.A.D., and S.A.; M.M. performed experiments on collaborative mouse models; F.D.C. supervised the study. B.J.P.-M. and M.T.D. wrote the manuscript, which was edited by all co-authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

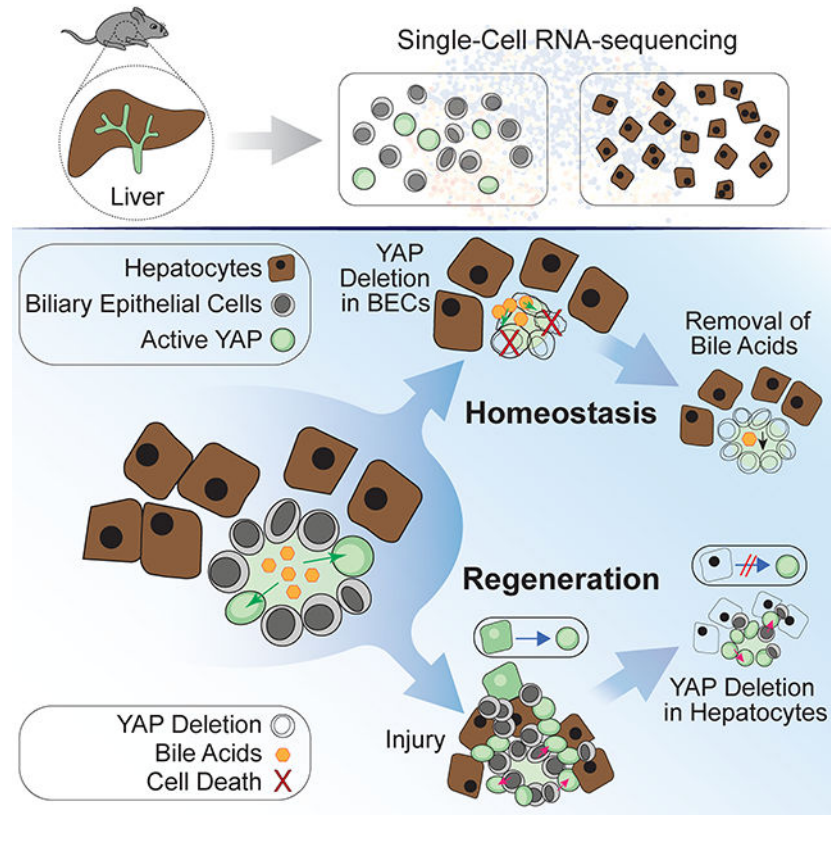
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The liver can substantially regenerate after injury, with both main epithelial cell types, hepatocytes and biliary epithelial cells (BECs), playing important roles in parenchymal regeneration. Beyond metabolic functions, BECs exhibit substantial plasticity and in some contexts can drive hepatic repopulation. Here we performed single-cell RNA sequencing to examine BEC and hepatocyte heterogeneity during homeostasis and after injury. Instead of evidence for a transcriptionally-defined progenitor-like BEC cell, we found significant homeostatic BEC heterogeneity that reflects fluctuating activation of a YAP-dependent program. This transcriptional signature defines a dynamic cellular state during homeostasis and is highly responsive to injury. YAP signaling is induced by physiological bile acids (BA), is required for BEC survival in response to BA exposure, and is necessary for hepatocyte reprogramming into biliary progenitors upon injury. Together, these findings uncover molecular heterogeneity within the ductal epithelium and reveal YAP as a protective rheostat and regenerative regulator in the mammalian liver.

eTOC

The transcriptional landscape of the epithelium in healthy and regenerating murine livers was investigated, revealing a dynamically fluctuating and heterogeneous YAP transcriptional program. Further analysis uncovered YAP signaling dualism: It is essential in biliary epithelial cells for homeostatic maintenance and in hepatocytes for the regenerative response to injury.

Graphical Abstract



INTRODUCTION

The liver is an indispensable organ with compartmentalized metabolic function and significant regenerative capabilities. While the two main epithelial cell types within the liver, hepatocytes and biliary epithelial cells (BECs), play a vital role in both of these processes, recent work has illuminated a more prominent functional role for BECs. Indeed, while BECs are essential for the modification and transport of toxic canalicular bile (Jones et al., 2015; Tabibian et al., 2013), they also exhibit remarkable plasticity and can even serve as the major cell source for regenerative cellular expansion after damage (Jörs et al., 2015; Rodrigo-Torres et al., 2014; Schaub et al., 2014; Yanger et al., 2014). Furthermore, recent lineage tracing studies have shown that BECs can act as facultative liver stem cells to repopulate the liver parenchyma in fulminant or chronic liver injury (Deng et al., 2018; Huch et al., 2015; Lu et al., 2015; Raven et al., 2017).

Despite the emerging significance of BECs as liver progenitor cells, debate remains as to whether all BECs have regenerative capacity. Studying BEC heterogeneity has proven arduous, due to low cell abundance and difficulties with their isolation and purification. As a result, understanding has remained cursory with subset evaluations restricted to isolation techniques that account for differences in cell size (Kanno et al., 2000) or by *a priori* choices of variably expressed biliary markers, such as ST14 or CD133 (Bin Li et al., 2017; Kamimoto et al., 2016; Kanno et al., 2000; Lu et al., 2015; Okabe et al., 2009). Despite these advances, a detailed and unbiased approach to understanding heterogeneity of the liver epithelium, in homeostasis and upon regeneration, has yet to be realized.

Here, we utilize high-throughput single-cell RNA-sequencing (scRNA-seq) to uncover and dissect the transcriptional heterogeneity of adult homeostatic and injured hepatocytes and BECs. Upon combination, our data uncover a YAP transcriptional module driving dynamic heterogeneity in BECs. Additional lineage-tracing and hepatocyte- and BEC-specific *Yap* knockout (KO) mouse models further reveal that YAP in the liver epithelium is an essential transcriptional rheostat, regulating the dynamic and regenerative response to environmental stimuli.

RESULTS

ScRNA-seq Analysis Reveals Transcriptional Heterogeneity in the Biliary Epithelium

To probe heterogeneity in an unbiased manner, intrahepatic BECs from adult mice were isolated and purified by fluorescence-activated cell sorting (FACS) using EpCAM, a well-established marker for BECs (Lu et al., 2015), and processed by inDrop (Klein et al., 2015) for scRNA-seq (Figure 1A, Figure S1A). A total of 2,344 BECs from three independent adult mice were analyzed and showed strong transcriptomic overlap by t-distributed stochastic neighbor embedding (t-SNE), as well as similar transcript and gene counts per cell (Figure 1B, Figure S1B–C). Notably, while the common BEC markers *Sox9* and *Epcam* were uniformly highly-expressed, several other well-known biliary markers, such as *Krt19*, and *Hnf1b*, showed variable or low expression levels (Figure 1C, Figure S1D), supporting previously observed differences in labeling or recombination efficiency (Yanger et al., 2013). Previously proposed progenitor markers, such as *Prom1* (Lu et al., 2015), *St14* (Bin Li

et al., 2017), or *Foxj1* (Dorrell et al., 2011), did not typify any subpopulation in our clustering analysis (Figure S1E), nor did they show a unique set of shared genes by correlation analysis. While positive correlations with receptor/transporter genes commonly known to associate with larger, distal BECs, such as *Cftr* and *Sctr* (SR), revealed other interesting transporters, these did not define a unique BEC subset in the scRNA-seq analysis and therefore further evaluation was not pursued (Figure S1F, Table S1). Finally, analysis identified a small subpopulation of cells (N=5), expressing *Dmbt1* and *Ly6d*, suggestive for extrahepatic BECs (De Lisle et al., 1997). Immunofluorescence (IF) stains confirmed these genes to be exclusively expressed extrahepatically (Figure S1G–H, Table S2).

While the isolated BECs maintain a generally congruous transcriptional landscape, an unsupervised clustering analysis with RaceID3 revealed that heterogeneity was primarily defined by a set of genes including *Cyr61*, *Ankrd1*, and *Gadd45b* (Figure 1C). Hierarchical clustering of the filtered 286 genes, based on z-scored expression revealed two cell groupings separated across t-SNE2 (y-axis): subset A (26% of the cells), which express these genes, as opposed to subset B (Figure 1D). Upon examination of the full list of differentially expressed genes between the two populations (Table S3), we recognized many *bona fide* targets of the central downstream transducer of the Hippo signaling pathway, YAP (Galli et al., 2015; Stein et al., 2015). Performing a Gene Set Enrichment Analysis of our gene list over a well-established active-YAP signature (Dong et al., 2007), we confirmed that these differentially expressed genes were indeed significantly enriched for YAP targets (Figure 1E). Identification of YAP-peaks at, or within the vicinity of, promoters of *Cyr61*, *Klf6*, *Ankrd1*, and *Gadd45b* in previously acquired chromatin immunoprecipitation sequencing (ChIP-seq) data from human biliary cells (Galli et al., 2015) further suggests these genes are direct targets of YAP (Figure S2A). Gene ontology (GO) analysis revealed other enriched GO terms, such as MAPK signaling, transcription factor AP-1 complex, and mechanical stimulus/cell adhesion (Figure 1F) which have been associated with active YAP signaling (Dubois et al., 2016; Reddy and Irvine, 2013; Yang et al., 2016; Zanconato et al., 2015). Taken together, we find that BEC transcriptional heterogeneity in homeostasis is defined by a YAP target gene signature. Previously, YAP has been shown to be critical for BEC maturation during embryonic development (Zhang et al., 2010) and can lead to expansion of ductal-like cells (Benhamouche et al., 2010; Lee et al., 2010; Lu et al., 2010). Yet, while there is evidence that supports a role for YAP in establishing biliary fate, the role of YAP in ductal heterogeneity, and its overall function in adult BECs has not been previously defined.

YAP Transcriptional Activity Distinguishes BEC Heterogeneity and is Dynamic

To validate heterogeneous YAP activity *in vivo*, we utilized IF as well as RNA single molecule *in situ* hybridization (RNA-ISH) for *Cyr61*, *Klf6* and *Hes1*, another gene identified by our scRNA-seq analysis (Figure S1I). Our data demonstrate a range of expression of these genes within individual ducts, including BECs that display an absence of such transcripts (Figure 2A). Significant correlation of gene expression, observed in the scRNA-seq, was confirmed between *Cyr61* and *Klf6* or *Hes1* (Figure 2B). Similarly, IF for JUNB protein and phosphorylated ERK (pERK), a surrogate for active MAPK signaling, highlighted clear intra-ductal molecular heterogeneity and small groupings of cells (2–3

cells) with positive signal (Figure 2C). Using a knock-in BAC-transgenic YAP-reporter mouse, which expresses eGFP under the control of the *Cyr61* regulatory sequences (Cyr61eGFP), we further demonstrated heterogeneous expression *in situ* and by FACS (Figure 2D–F). We confirmed that this Cyr61eGFP reporter mouse is responsive to YAP activity *in vivo* (Figure S2B–C).

We next assessed potential functional differences between these cells, similarly to previous studies (Bin Li et al., 2017; Lu et al., 2015), based on whether cells with higher levels of *Cyr61* expression would have higher primary biliary organoid forming capacities, a surrogate for stemness (Huch et al., 2015). Cyr61eGFP⁺ BECs showed a minimal enrichment in their propensity to form colonies *in vitro* in comparison to GFP⁻ BECs (Figure S2D–E) suggesting that YAP transcriptional activity is not a major determinant of organoid forming capacity. Interestingly, imaging and FACS analysis of sorted GFP⁻ BECs cultured as organoids revealed dramatic upregulation of GFP expression in culture over time (Figure 2G, Figure S2F). This suggests that the YAP-driven gene signature can be induced in previously YAP-naïve cells, likely for survival in these culture conditions. Taken together, these data imply that the identified biliary YAP gene signature reflects a dynamic cell state, as opposed to a specialized cell type.

scRNA-seq of Regenerating BECs Reveals a Broadened YAP Transcriptional Response and the Compartmentalized Emergence of a Wnt-Associated Signaling Module

To address the heterogeneity of transcriptional changes in response to chronic injury *in vivo*, we next performed scRNA-seq of BECs from mice which had been administered, for 1 week, a diet supplemented with 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Kaneko et al., 2015). As expected, DDC injury resulted in increased biliary proliferation as determined by IF for PCNA (Figure S3A). Upon scRNA-seq analysis, 1,268 BECs from a DDC-treated mouse occupied a separate transcriptional space in the t-SNE compared to control BECs (Figure S3B–C). This could be well explained by expression changes in a variety of typically upregulated genes, such as *Cxcl2* and *Tacstd2* (Rodrigo-Torres et al., 2014) (Figure S3C, Table S4).

Intriguingly, we also determined that the number of YAP-active BECs increased upon DDC administration but no longer occupied a separate spatial location by t-SNE (Figure 3A–B). IF for GFP and pERK, FACS analysis of Cyr61eGFP mice, and RNA-ISH for *Cyr61* and *Klf6* all confirmed increased YAP activity in BECs upon DDC injury (Figure 3C–E). Furthermore, the same changes were also observed in bile duct ligated mice (Figure 3F). These data demonstrate that BECs utilize a YAP transcriptional program to dynamically respond to chronic liver injury *in vivo*. The upregulation of this transcriptional program likely reflects naïve BECs undergoing a change of state as opposed to the expansion of a subpopulation of YAP-active cells.

Exclusively in the DDC sample, we also identified a unique subset of BECs with abundant expression of Wnt-related genes: *Wnt7a*, *Wnt7b*, *Wnt10a*, and *Cd44* (Figure 3G–H). These genes were previously found to be generally upregulated in injured BECs and were suggested to mark a progenitor-like state (Okabe et al., 2016). Interestingly, the cells expressing this Wnt-associated signaling module also showed an anti-correlative expression

pattern with certain mature hepatocyte markers, such as *Apoc1*, *Alb*, *Ttr*, but also the biliary marker *Spp1* (Figure 3G–H). These cells had a distinct transcriptional profile and could be identified, using CD44 as a surrogate marker, in a subset of BECs within intact luminal bile duct structures (Figure 3G–I). As we were unable to detect *Lgr5*- or *Axin2*- induction in BECs in DDC treated livers by scRNA-seq or by RNA-ISH (data not shown), the activation of these Wnt-related genes appears to be non-canonically regulated, which has been suggested previously (Okabe et al., 2016).

YAP is Essential in Hepatocytes for the Regenerative Ductular Response to Injury

To investigate if the identified YAP signature was unique in BECs, we performed scRNA-seq analysis on isolated hepatocytes from untreated (5087 cells) and DDC-injured (2596 cells) livers using Seq-Well (Gierahn et al., 2017) (Figure S3D–F). Our data confirmed previously established hepatic zonation gene differences based on spatial positioning in the hepatic lobule in control hepatocytes (Halpern et al., 2017) (Figure S3G–H). Interestingly, while we could not detect homeostatic hepatocytes expressing high levels of genes associated with active YAP, a fraction of hepatocytes was found to upregulate *Cyr61* and *Klf6*, as well as the biliary marker *Spp1*, upon one week of DDC-injury, suggesting the emergence of the YAP transcriptional program in injured hepatocytes as well (Figure 4A, Figure S3I). Strikingly, RNA-ISH for *Cyr61* and *Klf6* showed robust co-localization and increased expression in an average of 9.23% of DDC-injured hepatocytes primarily in the periportal zone compared to controls (Figure 4B). Our results speak to the activation of YAP upon regeneration in a subset of hepatocytes. As shown previously in the literature, a population of hepatocytes can undergo reprogramming to atypical duct cells during multiple types of injury, generating cells thought to have progenitor-like capacities in a process considered to be critical for normal regenerative response (Yanger et al., 2013). To test whether upregulation of the YAP-signature in hepatocytes was important for this process, we induced ablation of YAP and concurrent expression of TdTomato (Tom) selectively in hepatocytes, using *Yap^{fl/fl}*; *R26^{LSL-TdTomato/+}* mice administered with AAV8-TBG-Cre (*Yap^{HEP}*), followed by injury (Figure 4C, Figure S3J). We found that loss of YAP specifically in hepatocytes resulted in a significant attenuation of the ductular reaction post DDC, whereas control mice typically showed increased YAP in some periportal hepatocytes (Figure 4D–F). Additionally, we observed that while lineage-traced hepatocytes can form duct-like structures under prolonged DDC injury, this response was abrogated in mice who had YAP deleted from the hepatic parenchyma (Figure 4G–H). Considering the extent of the diminished ductular reaction in *Yap^{HEP}* mice, additional cell non-autonomous mechanisms might also be in play. Taken together, we find that activation of a YAP-driven transcriptional program is crucial for the process of liver regeneration after DDC injury and specifically for hepatocyte reprogramming towards a progenitor, biliary-like fate.

YAP is Required for the Maintenance of Adult Homeostatic BECs

To better understand the specific role of YAP in BECs, we utilized *Yap^{fl/fl}*; *Krt19^{CreER/+}*; *R26^{LSL-TdTomato/+}* (*Yap^{BEC}*) mice, in which tamoxifen (TAM) administration induces the deletion of *Yap* and concurrent expression of Tom in *Krt19*-expressing biliary cells (Means et al., 2008). *Krt19^{CreER/+}*; *R26^{LSL-TdTomato/+}* (Control^{BEC}) mice were used as controls (Figure 5A). Loss of *Yap* was observed in about 40% of BECs 3 days after TAM, which correlated

with the percentage of Tom expressing cells (Figure S4A). Using Tom expression as a surrogate for *Yap* KO, we found a dramatic decrease of Tom⁺ BECs in *Yap*^{BEC} mice over time, compared to a constant labeling percentage in Control^{BEC} mice (Figure 5B–C). YAP IF on *Yap*^{BEC} mice confirmed loss of *Yap* KO cells, while the persistence of YAP⁺ BECs that were also Tom⁺ demonstrated the ability of YAP⁺ escaper BECs to survive (Figure S4B–C). Further analysis in *Yap*^{BEC} mice compared to Control^{BEC} mice, 7 days after TAM, revealed that remaining YAP⁺ BECs in *Yap*^{BEC} mice proliferate significantly more than control or YAP⁻ BECs (Figure 5D).

As escaper BECs are able to compensate for *Yap* KO-induced BEC loss, in the *Yap*^{BEC} mouse, we wanted to challenge the biliary epithelium by using *Yap*^{fl/fl}; tetO-Cre; CAG-rTA3 (*Yap*) mice, which allow for complete, albeit not BEC-specific, KO of *Yap* upon doxycycline administration (Figure 5E). In addition to the widespread loss of *Yap* throughout the liver, *Yap* mice showed extensive disruption of bile duct integrity and morphology with a significant decrease in the number of pCK⁺ cells per portal area 2 weeks after KO (Figure 5F–G). Long-term analysis 12 weeks after *Yap* KO, revealed dramatic bile duct paucity throughout the entire parenchyma in the *Yap* livers compared to controls, concurrent with patches of hepatic necrosis and elevated ALT and bilirubin levels (Figure 5H, Figure S4D–E). Long-term hepatocyte-specific KO of *Yap* did not result in appreciable changes in morphology or blood chemistry, suggesting against hepatocyte-induced phenotypic effects in the *Yap* model (Figure S4F–G). Surprisingly, while *Yap* deletion efficiency was robust in the entire organism, we did not observe any gross morphological difference upon KO in other solid tissues (Figure S4H). This remarkable observation suggests a unique role for YAP within BECs. Of note, we observed some intact ductal structures, including in the extrahepatic ducts and gallbladder epithelium, at 12 weeks; however, these ducts were YAP⁺, thus highly suggestive for reconstitution of ducts from the few escaper YAP⁺ BECs (Figure S4I–J). Taken together, these findings demonstrate that under homeostatic conditions, YAP is acutely and uniquely required for BEC maintenance and the integrity of the liver compared to other tissues in the body.

To identify transcriptional differences, BECs from *Yap* and control mice were FACS-sorted for bulk RNA-seq. Transcriptome analysis revealed 1,426 genes >1.5-fold differentially expressed (Figure 5I, Table S5) with a mean reduction in normalized *Yap* transcript counts of >90%. Interestingly, among the top enriched downregulated GO terms we could identify “MAPK signaling” and “focal adhesion”, similar to the scRNA-seq data of the YAP signature subset of BECs in homeostasis (Figure 5J and Figure 1F). As anticipated, downregulation of YAP target genes was also observed upon KO (Figure 5I, Figure S5A–C). We also observed almost complete disappearance of pERK in BECs upon *Yap* loss, strengthening the evidence that YAP has effects on MAPK/ERK signaling in adult BECs (Figure S5D–E). For upregulated genes upon YAP deletion, the enriched GO terms were mainly associated with inflammation and cell death. Specifically, several pro-apoptotic genes were identified (Figure 5J, Figure S5F), suggesting that the deletion of *Yap* in BECs may lead to cell loss through an apoptotic mechanism. Indeed, IF for cleaved Caspase-3, as well as TUNEL analysis, in intrahepatic BECs, revealed increased cell death in *Yap* compared to controls (Figure 5K–L and Figure S5G–H). To capture the act of apoptosis in a

low abundant cell type, such as BECs, is particularly challenging and we posit that as *Yap* loss leads to downregulation of cell adhesion genes, these cells are most likely extruded quickly into the bile. In support of this hypothesis, IF for β -Actin reveals the typical basal actin condensing in the process of extrusion (Gu et al., 2011) upon *Yap* KO (Figure S5I–J).

Homeostatic YAP Activity in BECs is Regulated by Bile Acids

The observation that YAP is essential in BECs, even under homeostatic conditions, is particularly remarkable given that YAP is dispensable in several adult homeostatic tissues studied so far (Barry et al., 2012; Chen et al., 2014; Zanconato et al., 2015), and as also suggested from our own initial histological assessment of the *Yap* mouse (Figure S5E). This notable requirement for YAP, along with the observations that BECs can dynamically upregulate the YAP transcriptional program, led us to the question, what might be a unique, causal property of the BEC environment that so distinctly requires YAP? BECs are constantly exposed to bile, containing high levels of hydrophobic bile acids (BA), such as deoxycholic acid (DCA), which have been shown to be important regulatory molecules and can be toxic in high concentrations (Higuchi et al., 2003). We therefore hypothesized that BA might induce cell toxicity, causing BECs to upregulate the YAP transcriptional program as a protective mechanism. Therefore, we examined the effect of chronically administering DCA-supplemented feed to *Cyr61eGFP* mice. This resulted in a dramatic increase in the number of BECs expressing the YAP transcriptional program (Figure 6A–F and Figure S6A–B).

We next aimed to determine if the endogenous activity of YAP, perceived in a subset of homeostatic BECs, was correlated to inputs from physiological concentrations of BAs. Hypothesizing that restricting physiological BA exposure would limit active YAP signaling in BECs, *Cyr61eGFP* mice were fed a diet containing 2% of the resin cholestyramine (resin), which is a BA sequestrant (Zhang and Klaassen, 2010), for 1 week. Remarkably, we found that limiting the physiological levels of BAs in mice led to a significant mitigation of the number of BECs expressing the YAP transcriptional program (Figure 6A–F and Figure S6A–B). We also observed a small number of hepatocytes which co-upregulated *Cyr61* and *Klf6* upon DCA administration, suggesting that a non-physiological increase in BAs can induce YAP activity in hepatocytes as well (Figure 6G). The gallbladder epithelium, physiologically exposed to very high levels of BAs, did not show an increased induction of YAP activity compared to intrahepatic BECs, suggesting additional protective mechanisms in these cells (Figure S6C–D). Together, our findings indicate that physiological BA exposure in intrahepatic BECs is sufficient to activate the YAP-driven transcriptional program.

BA-Induced YAP Activity is ASBT-Dependent and Dynamically Fluctuates in BECs

Our data provide evidence that during homeostasis only a subpopulation of BECs displays YAP transcriptional activity. Thus, it remains perplexing as to why YAP is required in most, if not all, BECs for survival. We considered the possibility that YAP activity would fluctuate among BECs so that, over a certain timeframe, most BECs would have traversed through a YAP activated state. This scenario would explain the observed chronic requirement for YAP in the biliary epithelium. It also would suggest that, if required, most BECs should be able to

rapidly turn on YAP transcriptional activity. Indeed, following only 24h after DCA injection, Cyr61eGFP expression can be detected in up to 40% of BECs (Figure 7A). To provide further evidence for these transcriptional dynamics, we utilized a lineage tracing model to label the subset of cells that at a given time displayed YAP transcriptional activity. As *Hes1* mRNA expression significantly correlated with YAP target genes in YAP-active BECs, we used *Hes1*^{CreER/+}; *R26*^{LSL-TdTomato/+} reporter mice (Tom^{Hes1}) (Kopinke et al., 2011) (Figure 7B). In support of our initial observations regarding the number of YAP-active BECs, we found that 5 days after TAM ~13% of BECs were labelled, similar to Cyr61eGFP stains (Figure S7A and Figure 6D). We reasoned that if the YAP-active cell state is variable between BECs over time, a higher overlap of YAP target genes and the Tom label would be observed 1 day after induction when compared to 5 days after. Indeed, RNA-ISH analyses demonstrated a clear correlation between *TdTomato* and *Cyr61* at 1 day post TAM that was not present after 5 days (Figure 7C–D). We interpret this finding as highly supportive of the idea that quiescent BECs dynamically change their YAP activity state over time.

To provide insight into, why only a subset of BECs are YAP-active at a given time, we next assessed whether the expression of genes involved in regulating BA exposure to BECs, such as Mucins or BA transporters, correlate with YAP activity. Although we evaluated several candidates by targeted amplification in our scRNAseq libraries, we could not identify any significantly correlated genes (Figure S7B–C, Table S6). We posited that the lack of transcriptional correlation might be because these genes are regulated post-transcriptionally. To further functionally assess a potential link between YAP activity and several of the main proteins in BA physiology, we decided to evaluate several candidate genes *in vivo*. We focused on: 1. TGR5 (also known as *Gpbar1*), a G-protein coupled receptor known to transmit BA-mediated signaling (Deutschmann et al., 2018); 2. IQGAP1, a scaffolding protein identified to mediate BA-induced YAP-response in hepatocytes (Anakk et al., 2013); and, 3. ASBT (also known as *Slc10a2*), a central apical BA transporter in BECs (Lazaridis et al., 1997). Of the three KO mouse models examined, only ASBT showed a significant reduction in the number and distribution of YAP-active BECs during homeostasis, as measured by RNA-ISH for *Cyr61* (Figure 7E, Figure S7D–E). Remarkably, ASBT deficiency drastically abrogated the activation of YAP transcription in BECs following DCA challenge, as compared to controls (Figure 7E). These data provide evidence that BAs not only activate YAP signaling in BECs in a direct manner, but that, to do so, they also need to be transported intracellularly.

BA Sequestration Rescues *Yap* Deletion-Induced BEC Loss

As YAP activity in BECs is regulated by homeostatic levels of BA exposure, this led us to inquire if the phenotype associated with *Yap* KO in BECs might be due to the inability of BECs to respond to BA toxicity through YAP activation. To test this idea, we evaluated whether BA sequestration *in vivo* could partially abrogate the BEC-loss phenotype associated with YAP deletion. Intriguingly, we found that a resin diet almost completely rescued the *Yap* KO phenotype in *Yap* mice. *Yap*-deficient BECs in this diet retained a normal bile duct morphology and their loss was significantly suppressed (Figure 7F–H). This finding further supports the important physiological role for YAP as a vital signaling regulator to protect against cell death in BECs.

DISCUSSION

Understanding signaling dynamics and heterogeneity in the liver in homeostasis and regeneration has important implications for advancing treatment of liver disease. As an increasing focus has shifted towards understanding the role of BECs and their regenerative abilities in the liver (Deng et al., 2018; Jörs et al., 2015; Kamimoto et al., 2016; Raven et al., 2017), there has been a vibrant search to identify unique subpopulations of liver cells. Using scRNA-seq we provide the first unbiased evaluation of the transcriptional landscape of BECs in homeostasis and regeneration. Although we could distinguish rare, transcriptionally distinct, extrahepatic BECs, we were unable to identify a cluster of BECs that could define a potential long-lived stem cell population. Although a limitation of high-throughput scRNA-seq is capture efficiency and sequencing depth per cell, it would seem surprising that a potential progenitor cell would be defined only by a small number of correlated modules comprised of a few low-abundance genes that do not define a distinct cluster of cells in the t-SNE. Our work, instead, uncovers heterogeneity in the biliary epithelium defined by active YAP signaling which can be induced in response to injury or BA exposure *in vivo*. These results discourage the idea of a transcriptionally distinct progenitor-like cell among BECs, rather they project the hypothesis that these cells have a dynamic ability to respond to environmental cues. The emergence of a subset of BECs expressing Wnt associated genes upon chronic injury further highlights the plasticity of BECs to respond to environmental signaling inputs.

Regeneration is an essential ability of the liver, which is exposed daily to a multitude of potentially toxic xenobiotics. Several studies have revealed that liver regeneration, typically after partial hepatectomy, does not involve a single pathway, but rather engages several different signaling pathways, suggesting redundancy in signaling networks (Michalopoulos, 2010). In chronic liver injury, Yanger et al. identified Notch signaling as required for hepatocytes to biliary-progenitor-cell reprogramming (Yanger et al., 2013). Our work expands the list of crucially involved pathways, as YAP KO completely inhibits the hepatocytes' reprogramming capacity. As we have previously demonstrated that overexpression of active YAP in hepatocytes drives reprogramming via *Notch2* transcriptional regulation (Yimlamai et al., 2014), the combined evidence is suggestive that YAP-NOTCH is indeed a crucial axis for this process. Interestingly, our single-cell analysis demonstrated that only a fraction of hepatocytes induced YAP activity. Further analyses will be required to determine if these hepatocytes have a pre-determined ability to reprogram, and if hepatic paracrine factors downstream of YAP influence the ductular reaction by BECs.

In addition to the dynamic response of YAP signaling in the liver epithelium upon injury, the potent BEC loss phenotype observed upon YAP KO is particularly remarkable. Indeed, Hippo signaling had previously appeared to be essential in embryonic development and for injury response but dispensable under homeostatic conditions (Patel et al., 2017). Previous work using an *Mx1^{Cre}* model, has suggested that Yap KO in the liver without injury does not result in an appreciable phenotype (Bai et al., 2012). Using two different conditional *in vivo* models, however, we show a vital and distinct requirement for YAP in homeostatic BECs. This finding is particularly relevant as inhibition of YAP has been proposed as a cancer

therapeutic strategy (Kim and Myung, 2018; Zanconato et al., 2016). Unless appropriately addressed, hepatotoxicity may therefore become a main limitation to their future use.

Our work resonates with previous observations that YAP and associated pathways are responsive to BAs (Anakk et al., 2013; Centuori et al., 2016) but, in contrast, shows that even physiological levels of BA are sufficient for that response. Interestingly, this BA-triggered YAP activity in BECs seems to dynamically fluctuate between cells. Our short-term BA treatments, coupled with our YAP deletion and *Hes1* lineage tracing data suggests that most, if not all, homeostatic BECs can, and will eventually need to induce YAP activity. Our tracing data reveal that, even at 5 days, a different set of BECs express *Hes1/Cyr61*, suggestive of relatively rapid fluctuation. Further studies should provide insight into the timescale of this process, whose dynamics probably explain the lack of consistent correlation between pERK levels and YAP target gene expression in single BECs. Furthermore, we have also identified that ASBT is vital for this transcriptional response, suggesting that the intracellular presence of BA is required. The heterogenic and dynamic transcriptional response in BECs is most likely influenced by a multitude of factors known to alter cellular exposure to BAs, such as the apical glycocalyx, bicarbonate secretion and the presence of exporting BA transporters which might cumulatively lower the threshold for a trigger of YAP activity in the individual cell. Additionally, an alternative explanation for the observed heterogeneity is that BECs could become refractory for a certain time to YAP activity. As oscillatory gene expression behaviors have been described in multiple contexts (Halpern et al., 2015), whether this pattern of YAP activation in BECs is random or follows a defined pattern is unclear and therefore is worth future exploration.

Our findings add to the nuance, complexity, and variability of YAP signaling depending on environmental conditions and tissue type. For the biliary epithelium, as well as for hepatocytes, this work has determined that YAP signaling is acutely positioned to drive a dynamic response to environmental cues for continued survival and to regulate regeneration. Further studies evaluating the vital properties of YAP signaling in the liver will lead to a deeper understanding about the nature of our ability to respond to this tempest of environmental toxins and signaling inputs that we encounter daily.

STAR METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Fernando Camargo (fernando.camargo@childrens.harvard.edu).

Experimental Model and Subject Details

Mice—All animal protocols and procedures were approved by the respective local animal institutional committees. Mice were housed in specific pathogen-free facilities on a 12h light/dark cycle and were given food and water ad libitum. All animals used in this study were 7–12 weeks of age unless otherwise specified and were on a C57Bl/6 background, except *Tgr5* KO and their controls (FVB) and *Iqgap1* KO and their controls (129/SVJ); both

female and male mice were used for experiments. No influence of sex was observed in any of the performed experiments. Whenever possible, littermates with negative genotypes were used as controls. Only experimentally naïve mice were used for experiments. The following mouse lines were utilized or generated:

1. *Cyr61eGFP* BAC-transgenic mice were derived from GENSAT and C57BL/6J were obtained from Jackson laboratories.
2. *R26^{lox-stop-lox-rtTA/+}; Coll1a1^{Teto-YapS127A/+}; Cyr61eGFP* mice were generated by mating *Cyr61eGFP* mice with *R26^{lox-stop-lox-rtTA/+}; Coll1a1^{Teto-YapS127A/+}* mice that were previously described (Yimlamai et al., 2014).
3. *Krt19^{CreER/+}* mice (Jackson Laboratories) were mated with *R26^{LSL-TdTomato/+} Ai9* mice (Jackson Laboratories) and *Yap^{fl/fl}* mice (Schlegelmilch et al., 2011) to obtain *Yap^{fl/fl}; Krt19^{CreER/+}; R26^{LSL-TdTomato/+} (Yap^{BEC})* mice and control *Krt19^{CreER/+}; R26^{LSL-TdTomato/+}*
4. CAG-rtTA3 (Jackson Laboratories) mice were mated with (tetO)7-Cre (Jackson Laboratories) and *Yap^{fl/fl}* mice (Schlegelmilch et al., 2011) to obtain the following genotypes *Yap^{fl/fl}; tetO-Cre; CAG-rtTA3 (Yap)* and control *Yap^{fl/fl}; tetO-Cre*.
5. For KO of *Yap* in hepatocytes we generated *Yap^{fl/fl}; R26^{LSL-TdTomato/+} Ai9 (Yap^{HEP})*.
6. *Hes1^{CreER/+}* mice (Kopinke et al., 2011) were mated with *R26^{LSL-TdTomato/+} Ai14* mice (Jackson Laboratories) (*Tom^{Hes1}*).
7. *Asbt*, *Tgr5* and *Iqgap1* KO mice have previously been described (Dawson et al., 2003; Li et al., 2000; Vassileva et al., 2006).

Mouse genotyping was performed with GoTaq Green Master Mix (Promega) using the primers listed in Table S7. For injury models, mice were given feed supplemented with 0.1% 3,5-diethoxycarbonyl-1,4-dihydroxycholellidine (DDC) (Bio-Serv) as previously established (Yanger et al., 2014), for the duration specified. Bile duct ligations were performed on adult mice as described previously (Tag et al., 2015) and controlled with sham-operated mice. Gamma irradiated rodent diet supplemented with 2% cholestyramine (resin) or 0.3% deoxycholic acid (DCA) (purchased from Bio-Serv) was administered to mice as previously described (Zhang and Klaassen, 2010). For 24h BA administration, mice were injected intraperitoneally with 24,4 mg/kg of deoxycholic acid (DCA) in Ethanol (1 ml/kg) once (Paolini et al., 2002). To induce Cre expression in the *Krt19^{CreER}* models described above, mice were administered 2 doses of 4mg tamoxifen (TAM) (Sigma-Aldrich) dissolved in corn oil intraperitoneally. *Tom^{Hes1}* mice were administered 1mg TAM once intraperitoneally. Doxycycline was administered to mice in drinking water at a concentration of 1mg/ml unless otherwise specified. For AAV virus administration, AAV8.TBG.PI.eGFP.WPRE.bGH (University of Pennsylvania Vector Core) and AAV8.TBG.PI.Cre.rBG (University of Pennsylvania Vector Core) were administered via retro-orbital injection at the specified dosages at a volume of 100µl. For clinical chemistry analysis, 80 µl of blood was obtained via capillary retro-orbitally and analyzed utilizing chemistry rotors for Vetscan VS2 (Abaxis).

Cell lines—L-WRN cells were used as described to generate conditioned media for organoid culture (secreting Wnt3a, R-spondin, Noggin) (Miyoshi and Stappenbeck, 2013). Cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

Method Details

BEC Isolation—Optimal isolation of a single-cell suspension of biliary cells was obtained by modifying previously established, two-step liver perfusion protocols (Dorrell et al., 2008; Li et al., 2010). Mice were euthanized, the heart and liver were surgically exposed, and a cannula was immediately inserted through an incision in the right atrium into the suprahepatic vena cava inferior. Mouse livers were then perfused by a pre-warmed (37°C) perfusion buffer (50 mM EDTA, 10 mM HEPES in 1X HBSS [Gibco]) at an initial flow rate of 3 ml/min for 2.5 minutes, followed by an increase in flow rate to 4 ml/min for an additional 2.5 minutes. This was then followed by perfusion with a pre-warmed (37°C) Collagenase Type IV (150 U/mL; Invitrogen) digestion buffer solution (1.25 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES in 1x HBSS) for 8–10 minutes at a flow rate of 4 ml/min. For the duration of the two-step liver perfusion, the infrahepatic inferior vena cava was digitally occluded every 30 seconds for 10 seconds.

After isolating the liver and surgically removing the gallbladder, the capsule was disrupted and the liver was gently and mechanically agitated to release dissociated single cells. The remaining liver was then subjected to serial digestions in Collagenase Type IV (150 U/mL), Accutase (EMD Millipore), and trypsin (0.25%) for 30 minutes, 30 minutes, and 20 minutes, respectively, at 37°C. Dissociated cells were collected after each step and filtered through a 100 µm cell strainer, washed, and re-suspended in ice-cold resuspension buffer (2% FBS, 1.25 mM CaCl₂, 4 mM MgCl₂, 10mM HEPES, 5 mM glucose in 1X HBSS). The remaining procedure was at 4°C. Hepatocytes were pelleted by a 30×g spin for 5 minutes and discarded, and the remaining cells in the supernatant were spun at 300×g for 5 minutes. The pelleted cells were resuspended in 5 ml red blood cell lysis buffer (Qiagen), treated on ice for 7 mins and washed with 10 ml resuspension buffer at 300×g for 5 minutes. The cells were then re-suspended in cold resuspension buffer for further processing or analysis.

Fluorescence Activated Cell Sorting—BECs were stained by a fluorescent antibody for the positive selection biliary marker EpCAM (Biolegend, 1:100) and negatively selected for by the mesenchymal/hematopoietic lineage markers CD45 (BD Biosciences, 1:100), CD11b (BD Biosciences, 1:100), and TER-119 (Biolegend, 1:100) for 30 minutes at 4°C. After a wash with resuspension buffer at 300×g for minutes, BECs were then sorted or analyzed by FACS utilizing a BD FACSAria II, using the 100µm nozzle.

BEC Single-Cell RNA Sequencing—Isolated single BECs were encapsulated, and libraries were generated using inDrops (Zilionis et al., 2017) performed at the Single Cell Core at Harvard Medical School. Approximately 1500 cells were encapsulated for each sample. Libraries were sequenced on an Illumina NextSeq 500 sequencer using a NextSeq 500/500 High Output v2 kit, 75 cycles: 35 cycles for read 1, 6 cycles for index i7 read, and 51 cycles for read 2.

Targeted Amplification of scRNA-seq library—For targeted amplification the post-in vitro transcription back-up of the three control BEC scRNA-seq libraries were used with an adaptation of the original inDrops protocol (Zilionis et al., 2017). The samples were directly reverse transcribed according to protocol but without prior fragmentation and using random hexamers without PE2-adaptor. After clean-up with SPRI (Agencourt AMPure XP) beads at a 1.2X ratio, targeted PCR amplification was performed with 5% of the purified cDNA per each individual reaction using Kapa HiFi Hot Start Mix (KAPA Biosystems) with the respective forward primers and a general reverse primer (Table S7). After clean-up with SPRI beads at a 0.8X ratio and evaluation of amplification by gel-electrophoresis, 10 cycles of indexing PCR were performed of the pooled PCR products (15% of the purified PCR product mixed in equal volumes), according to the inDrops protocol. Libraries were sequenced on an Illumina NextSeq 500 sequencer using a NextSeq 500/550 Mid Output v2.5 kit, 150 cycles: 100 cycles for read 1, 8 cycles for index i7 read, 8 cycles for index i5 read, and 14 cycles for read 2.

Hepatocyte Isolation—For hepatocyte isolation, a similar perfusion protocol as described for BEC isolation was used with adaptation of the digestion step, by using liver digest medium (Thermo Fisher) and increasing the digestion time to 16–20 minutes while maintaining identical flow rates. After excision of the liver, the whole procedure was performed at 4°C. The liver capsule removed, and the liver gently swirled in resuspension buffer to yield a cell suspension. After filtration through a 100 µm cell strainer, the hepatocytes were selectively pelleted by centrifugation with 30×g for 5 minutes, which removes the non-parenchymal cell fraction. The resuspended hepatocytes were then mixed 1:2.2 with 40% iodixanol (Optiprep, Axis-Shield) solution (diluted in resuspension buffer) for a volume of 4.8ml, overlaid with 3 ml 18% iodixanol and 0.5 ml resuspension buffer and spun at 500×g for 25 minutes at 4°C with reduced deceleration. The top cell layer was collected, mixed with resuspension buffer supplemented with 3% BSA and washed once at 30×g for 5 min at 4°C. The cells were then re-suspended in ice-cold resuspension buffer (containing 2% BSA and 9% Optiprep), quantified and assessed for viability by Trypan blue stain. Routinely, viability of over 98% was obtained.

Hepatocyte Single-cell RNA-Sequencing—We utilized the Seq-Well platform for massively parallel scRNA-seq to capture transcriptomes of single hepatocytes on barcoded mRNA capture beads. Full methods on implementation of this platform are available in Gierahn et al. (Gierahn et al., 2017). In brief, 20,000 cells from one *in vivo* hepatocyte condition were loaded onto one array containing 86,000 barcoded mRNA capture beads. The loaded arrays containing cells and beads were then sealed using a polycarbonate membrane with a pore size of 0.01 µm, which allows for exchange of buffers but retains biological molecules confined within each microwell. Subsequent exchange of buffers allows for cell lysis, transcript hybridization, and bead recovery before performing reverse transcription *en masse*. Following reverse transcription and exonuclease treatment to remove excess primers, PCR amplification was carried out using KAPA HiFi PCR Mastermix with 2,000 beads per 50 µL reaction volume. Six libraries (totaling 12,000 beads) were then pooled and purified using Agencourt AMPure XP beads by a 0.6X SPRI followed by a 0.7X SPRI and quantified using Qubit hsDNA Assay (Thermo Fisher). Libraries were constructed using the

Nextera Tagmentation method on a total of 800 pg of pooled cDNA library from 12,000 recovered beads. Tagmented and amplified sequences were purified at a 0.6X SPRI ratio yielding library sizes with an average distribution of 650–750 base pairs in length as determined using the Agilent hsD1000 Screen Tape System (Agilent Genomics). Arrays were sequenced with an Illumina 75 Cycle NextSeq500/550 High Output v2 kit at a final concentration of 2.8 pM. The read structure was paired end with Read 1 starting from a custom read 1 primer containing 20 bases with a 12bp cell barcode and 8bp unique molecular identifier (UMI) and Read 2 being 50 bases containing transcript information.

Immunohistochemistry / Immunofluorescence—If mice were harvested for BEC isolation, the right-lateral lobe of the liver was ligated and resected before perfusion. If mice were not used for BEC isolation, the whole liver was isolated. Livers and other organs were fixed in 4% Paraformaldehyde for 24–48 hours at room temperature, washed with PBS and 70% ethanol, and embedded in paraffin. 5 µm sections were rehydrated and treated with citric-acid based pH 6.0 Antigen Unmasking Solution (Vector Labs,) at 95°C in a cooker for 1h for antigen retrieval. Slides were then treated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity. To prevent non-specific antibody binding, slides were incubated in protein blocking solution (5% donkey Serum [Sigma-Aldrich] in 1% BSA/PBS/0.1% Tween) for 1 hour before overnight incubation with primary antibody at 4°C. The slides were washed three times in PBS for 5 minutes and then for immunofluorescence (IF) incubated with appropriate fluorescent secondary antibodies for 45 minutes in blocking buffer. For IF stains for GFP and JUNB, tyramide amplification was used (AF488 Tyramide SuperBoost Kit, Thermo Fisher Scientific) according to kit specifications. Slides were then washed and incubated with 1 µg/ml DAPI for 10 minutes in PBS and then mounted with Prolong Gold antifade with DAPI (Invitrogen), for IF.

To process immunohistochemistry (IHC) samples, appropriate biotinylated secondary antibodies (Vector Labs) were used with the Vectastain Elite ABC-HRP reagent (Vector Labs) and Diaminobenzidine-reaction kit (Vector Labs) to develop the IHC signal. Harris modified haematoxylin (Fisher Scientific) was used to counterstain nuclei. Finally, slides were washed, dehydrated, and mounted using Vectamount (Vector Labs). TUNEL assay was performed using the *In Situ* Cell Detection Kit, TMR red (Sigma Aldrich) according to manufacturer's instructions.

RNA In Situ Hybridization—RNAscope Multiplex Fluorescent Detection kit v2 (ACDbio) in combination with TSA Fluorescein and Cyanine3 Plus Evaluation kits (Perkin Elmer) were used for RNA *in situ* hybridization according to manufacturer's instructions. Before mounting, the slides were counterstained with a primary antibody against pCK or KRT19 for 1h at RT, and then processed as described for the IF methods. Probes for *TdTomato* and murine *Cyr61*, *Klf6* and *Hes1* were used (ACDbio).

Microscopy—IHC images were taken on a Zeiss Axio scope. IF images were obtained on a Zeiss AxioObserver Z1 or on a Zeiss LSM 700 Laser Scanning Confocal microscope. Organoids were visualized by a Nikon SMZ18 and a Zeiss AxioObserver Z1 with Apotome. Composite IF images are indicated by colored text that corresponds to each respective overlaid channel within the image.

Single-Cell Organoid Forming Assay—Single BECs were sorted directly into a 96-well plate (1 cell/well) containing organoid media with 5% Geltrex (Thermo Fisher Scientific, LDEV-Free, hESC-Qualified, reduced growth factor basement membrane matrix) as previously described (Bin Li et al., 2017). Organoid forming percentage was determined 14 days after plating. Organoid media consisted of a 1:1 mixture of L-WRN conditioned media generated as described previously (Miyoshi and Stappenbeck, 2013) and fresh 2X-Media (Advanced Dulbecco's modified Eagle's medium /F12 medium [Invitrogen], 10^3 U/ml; 10^3 µg/ml Penicillin/Streptomycin (Invitrogen), 2 mM L-Glutamine, 2x N2-supplement [Invitrogen], 2x B27 without vitamin A supplement [Invitrogen], 20 mM nicotinamide [Sigma-Aldrich], 0.002 mM dexamethasone [Sigma-Aldrich], 10 mM HEPES [Invitrogen], 20 µM Y27632 [Sigma-Aldrich] (only upon initial derivation and upon passage), 50 ng/ml rmEGF [R&D Systems], 40 ng/ml rmHGF [Peprotech], and 1:500 Primocin [Invivogen]).

RNA Isolation and quantitative RT-PCR—RNA was isolated using Trizol Reagent (Life Technologies) or NucleoSpin® RNA XS (Macherey-Nagel) according to manufacturers' instructions. Isolated RNA was reverse transcribed for real-time quantitative PCR (qRT-PCR) by an iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed on One Step plus Sequence Detection System (Applied Biosystems) using Fast SYBR® Green Master Mix (Life Technologies). Gene expression data was normalized to *Gapdh*. qRT-PCR primer sequences are listed in Table S7.

Bulk RNA-Sequencing—RNA was isolated from FACs sorted BECs using the NucleoSpin® RNA XS Kit (Macherey-Nagel) according to the manufacturer's instructions. Libraries were prepared on the same day using TruSeq RNA Library Preparation Kit v2, Set B (Illumina). Quantitative PCR and TapeStation to assess library concentration were run by the Biopolymers facility at Harvard Medical School. Samples were sequenced using an Illumina NextSeq 500 with Mid Output kit v2 for 56 cycles.

Quantification and Statistical Analysis

BEC scRNA-Seq Computational Analysis—Reads from BEC scRNA-seq were mapped using bwa mem-0.7.10 with default parameters to the reference transcriptome (*Mus musculus* 10) after extraction of the corresponding cell-specific barcode and unique molecular identifier (UMI). The number of unique transcripts per gene for each cell-specific barcode was extracted from the UMI count as previously described in Grün et al. (Grün et al., 2016). We hence refer to transcripts as unique molecules based on UMI correction. In a first filtering step, only cellular barcodes with more than 1000 mapped reads were kept (1228, 1249 and 1304 barcodes in homeostatic mice 1, 2, and 3 respectively; 1517 barcodes in DDC). Next, cellular barcodes with more than 20% mitochondrial transcripts were discarded. We ran RaceID3 (Herman et al., 2018) to downsample transcript count per cell to 700, and we subsequently removed genes that are present only in 2 cells or less that have 5 transcripts or less. The remaining 286 genes (from originally 14,193 genes found in the raw data) were used to build a t-distributed stochastic neighbor-embedding (t-SNE) map based on Pearson-correlation distances between cells. Non-BEC contaminants, and highly stressed cells (identified as those where heat-shock proteins represent > 1.25% of the whole

transcriptome), were identified and removed. The robustness of the data-set was tested with two batch correction algorithms, scran and the one included in RaceID3(Haghverdi et al., 2018; Herman et al., 2018). Unsupervised clustering (k-medoids) was performed using RaceID to identify subpopulations. To generate the heatmap in Figure 1D, the cells in the x-axis were ordered left to right according to their y-coordinate in the t-SNE from Figure 1C (bottom to top). This strategy, to order the cells in that way, was confirmed by performing hierarchical clustering and unsupervised k-medoids clustering (using RaceID3) of the cells based on their gene expression. Both independent clustering approaches identified cellular populations that were ordered along the y-axis of the t-SNE. The 286 genes on the y-axis were hierarchically clustered by their z-scores (with maximum value set to 3). Genes in clusters annotated with numbers 1, 2 and 5, which show opposing gradients in the average gene expression, were used to define a distinct BEC subset A (26% of cells) and subset B (74%). These gene selections were consistent with the ones defining the cellular subpopulations obtained by k-medoid-based and hierarchical-based clustering. For Figure 1E, a differential gene expression analysis between cell subsets A and B was performed (Table S3) to rank the significant differentially expressed genes based on \log_2 fold-change and to compare with the top 600 expressed genes from *Yap* overexpression in the liver(Dong et al., 2007) by GSEA3.0. In Figure 1F, gene ontology analysis of these differentially-expressed genes was performed using EnrichR(Chen et al., 2013). In Figure S3B–C for the construction of the t-SNE map, the DDC data set was combined with the BEC control data.

The sequencing data of the targeted amplification was mapped in the same way. For Figure S7B, cells were stratified according to their YAP status (subset A vs. subset B from Figure 1D), and the distribution of number of detected UMIs per cell was computed for each of the targeted genes. Significance was assessed using Kolmogorov-Smirnov test.

Hepatocyte scRNA-Seq Computational Analysis—Read alignment was performed as in (Macosko et al., 2015). Briefly, for each NextSeq sequencing run, raw sequencing data was converted to demultiplexed FASTQ files using bcl2fastq2 based on Nextera N700 indices corresponding to individual samples/arrays. Reads were then aligned to mm10 genome using the Galaxy portal maintained by the Broad Institute for Drop-Seq alignment using standard settings. Individual reads were tagged according to the 12-bp barcode sequencing and the 8-bp UMI contained in Read 1 of each fragment. Following alignment, reads were binned onto 12-bp cell barcodes and collapsed by their 8-bp UMI. Digital gene expression matrices (e.g. cell by gene tables) for each sample were obtained from quality filtered and mapped reads and UMI-collapsed data and were utilized for further analysis. Barcodes with more than 40% and 80% mitochondrial transcripts in the control and the DDC samples, respectively, were removed.

The number of unique transcripts per gene for each cell-specific barcode was extracted from the UMI count as previously described in Grün et al.(Grün et al., 2016). We hence refer to transcripts as unique molecules based on UMI correction. In a first filtering step, only cellular barcodes with more than 1000 mapped reads were kept (5221 barcodes for adult control mouse and 3192 barcodes for DDC-treated mouse). RaceID3(Herman et al., 2018) was run to downsample transcript count per cell to 700, and subsequently non-hepatocyte contaminants were removed and genes that were present only in 2 cells or less that have 5

transcripts or less. The remaining 277 genes (from originally 16,874 genes found in the raw data) were used to build a t-SNE map based on Pearson-correlation distances between cells. To generate the heatmaps in Figure 4A and Figure S3G, both homeostatic and DDC cells in the x-axis were ordered left to right according to zonation using the algorithm described in (Halpern et al., 2017). In Figures S3F and S3H–I, for the construction of the t-SNE map, the DDC data set was combined with the adult hepatocyte control data.

Bulk RNA-Sequencing Computational Analysis—Raw sequence reads were processed using Trimmomatic-0.33 with the following parameters: TRAILING:25 MINLEN:35. Sequences were aligned using STAR aligner to the mouse genome: mm10. Gene counts were determined using FeatureCounts. Differential analysis between samples was performed by normalizing read counts using DESeq2 in RStudio and examining significantly expressed genes $\text{padj} < 0.05$. Heatmap was generated using pheatmap (v. 1.0.8) in RStudio. Gene ontology analysis of differentially-expressed genes was performed using the DAVID Bioinformatics database (Huang et al., 2009).

ChIP-Seq Data Analysis—ChIP-Seq data was re-analyzed from previously published data (Galli et al., 2015). Data was aligned to the human genome hg19 using the UCSC genome browser.

Quantification / Statistical Analysis—Power calculations were not routinely performed; however, animal numbers were chosen to reflect the expected magnitude of response considering the variability observed in previous experiments. For quantification of sections, in general, 5–10 random portal fields of each liver sample were imaged and then quantified using Fiji software. Data presented are expressed as mean \pm standard deviation (SD) unless otherwise specified. Data other than sequencing data were analyzed and plotted using Prism Software 8.0 (GraphPad). Technical and biological replicates are specified for each experiment in the figure legends. P values are presented as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and were determined by two-tailed Student's t test, unless otherwise specified in the figure legends. When comparing more than two means, significance was assessed by ANOVA, followed by Tukey multiple comparisons test. To compare differences of cellular distribution in regard to mRNA-dots per cell as obtained by RNA-ISH data, the Kullback-Leibler test was performed. FlowJo software was used for all flow-cytometry analysis. Figures were generated using Adobe Illustrator.

Data Availability

The scRNA-seq data (BECs and hepatocytes) and the bulk RNA-seq data (BECs) are deposited in the Gene Expression Omnibus database under accession number GSE125688.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ScRNA-seq of the healthy and injured liver epithelium reveals dynamic heterogeneity
- Transcriptional heterogeneity is driven by fluctuating activation of YAP signaling
- YAP is essential for adult biliary cell survival under homeostatic conditions
- YAP is required in hepatocytes for the ductular response during regeneration

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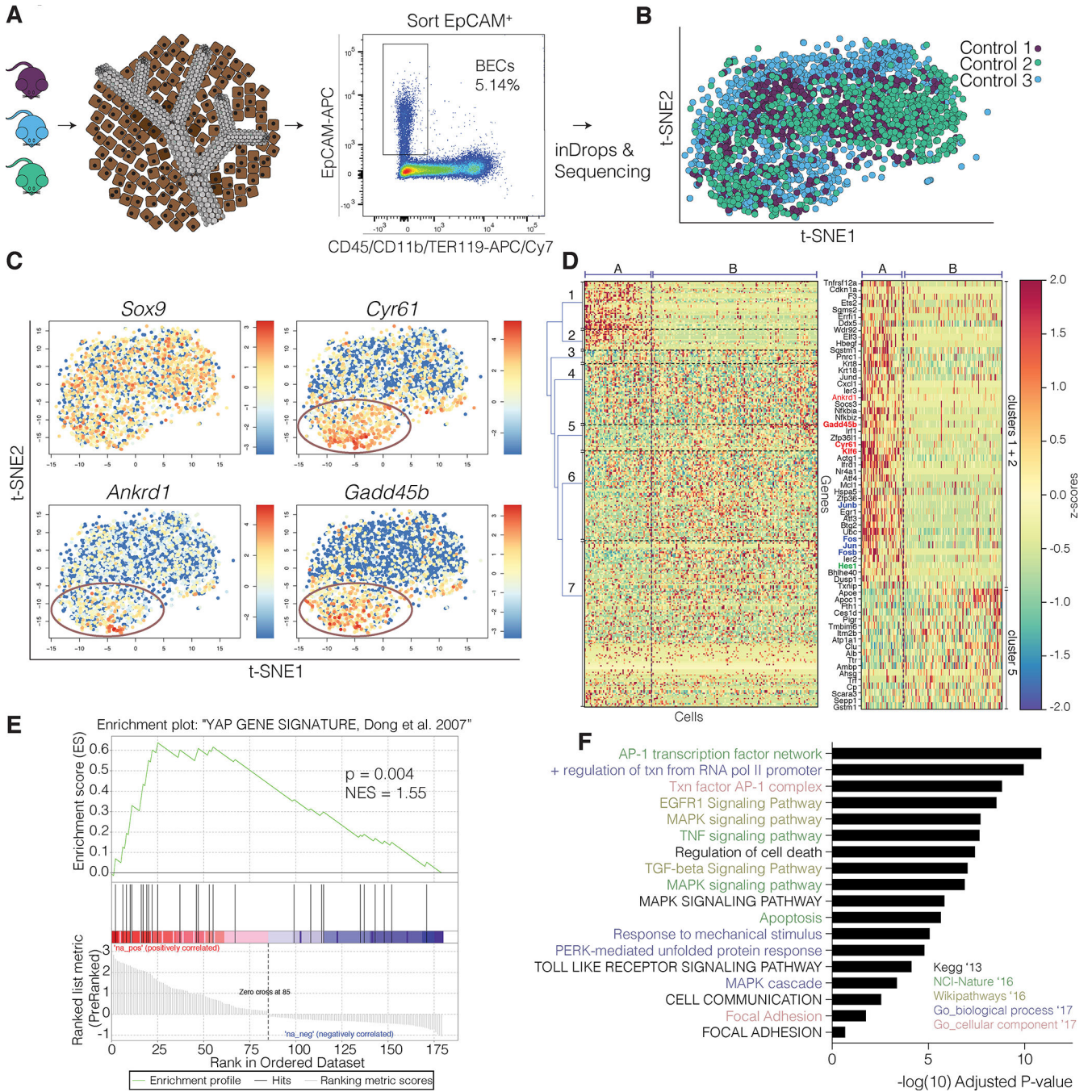


Figure 1. ScRNA-Seq Analysis Reveals Genes Associated With YAP Activity Define Heterogeneity in Adult Homeostatic BECs

(A) Schematic illustrating the isolation procedure for single BECs.
(B) t-SNE plot of the scRNA-seq data of 2,344 adult BECs (n = 3 mice, indicated with different colors).
(C) Relative expression of biliary marker *Sox9* and the YAP targets *Cyr61*, *Ankrd1*, and *Gadd45b*, as represented by t-SNE. Colors denote relative expression of respective gene in each cell (log2). Circles outline cells identified as subset A in panel **(D)**.

(D) Heatmap with cells ordered along the x-axis (left to right) according to their y-axis position (bottom to top) in the t-SNE, and genes on the y-axis were hierarchically clustered by their z-scores (with maximum value set to 3). Right: The genes identified from clusters 1, 2, and 5 from the left heat-map. A selection of YAP targets is highlighted in red and AP-1 related genes in blue. Dashed lines separate population of interest (population A) from other BECs (population B).

(E) GSEA plot of significant differentially-expressed genes between populations A and B in comparison to a gene list from YAP overexpression in the liver, indicating enrichment in YAP targets for population A.

(F) GO analysis of differentially-expressed genes between populations A and B using EnrichR, listing terms identified, with bars indicating level of significance.
See also Figure S1.

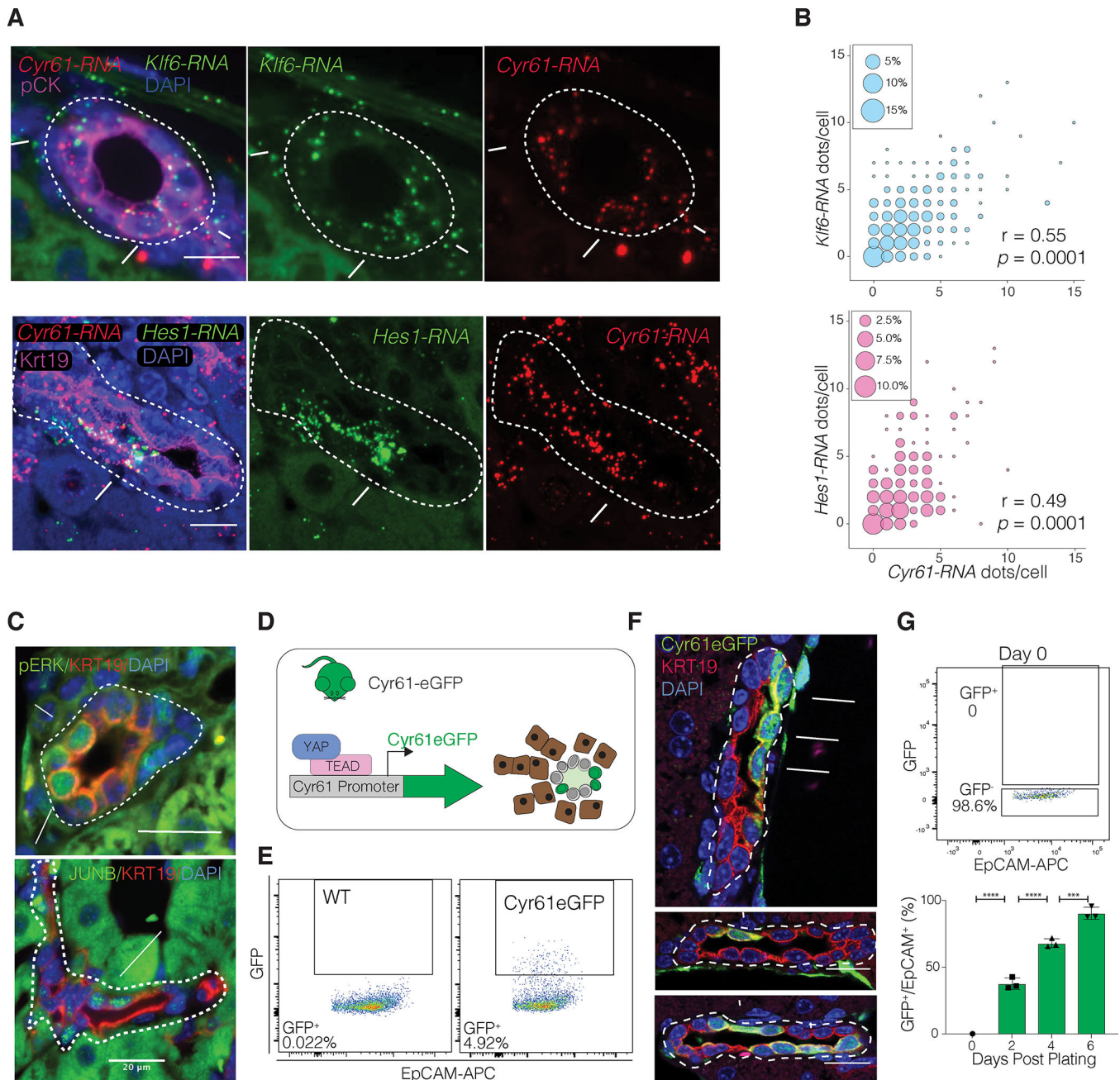


Figure 2. YAP Activity Defines BEC Heterogeneity *In Vivo* and Reflects a Dynamic Cell State *In Vitro*

(A) *Cyr61-Klf6* and *Cyr61-Hes1* RNA-ISH combined with IF stain for pan-Cytokeratin (pCK) of mouse liver sections. Arrows indicate BECs co-expressing *Cyr61* and *Klf6* RNA, and arrowheads RNA-negative molecules.

(B) Bubble plots depicting the correlation of co-localized *Cyr61-Klf6* and *Cyr61-Hes1* RNA molecules per BEC. Size of bubble corresponds to the respective co-expression frequency with inset showing size of bubble corresponding to % of cells with indicated frequency ($n = 4$ mice, BECs from 5 portal fields each, Spearman correlation).

- (C) IF for pERK and JUNB (arrows) demonstrate heterogeneity within murine Cytokeratin19⁺ (KRT19⁺) BECs.
- (D) Schematic for the Cyr61eGFP transgenic allele which expresses eGFP under the *Cyr61* promoter and is used as a reporter for YAP transcriptional activity.
- (E) Representative FACS analysis of GFP expression in BECs of a wildtype (WT) and a Cyr61eGFP mouse, where typically between 3–11% GFP⁺ BECs are seen.
- (F) IF for GFP/KRT19 demonstrating clear intraductal heterogeneity of expression in the liver of a Cyr61eGFP-reporter mice. Arrows designate GFP⁺ cells.
- (G) FACS analysis of freshly isolated GFP⁻ BECs sorted from Cyr61eGFP mice, used for *in vitro* organoid growth assay. Bar plot depicts % of GFP⁺ cells over time, from 5000 initially seeded GFP⁻ cells, showing that >90% of cultured BECs start expressing GFP within 6 days. (Mean ± SD, n = 3 mice, each in triplicate, ANOVA, followed by Tukey multiple comparisons test).
- Dashed lines generally outline biliary structures.
See also Figure S2.

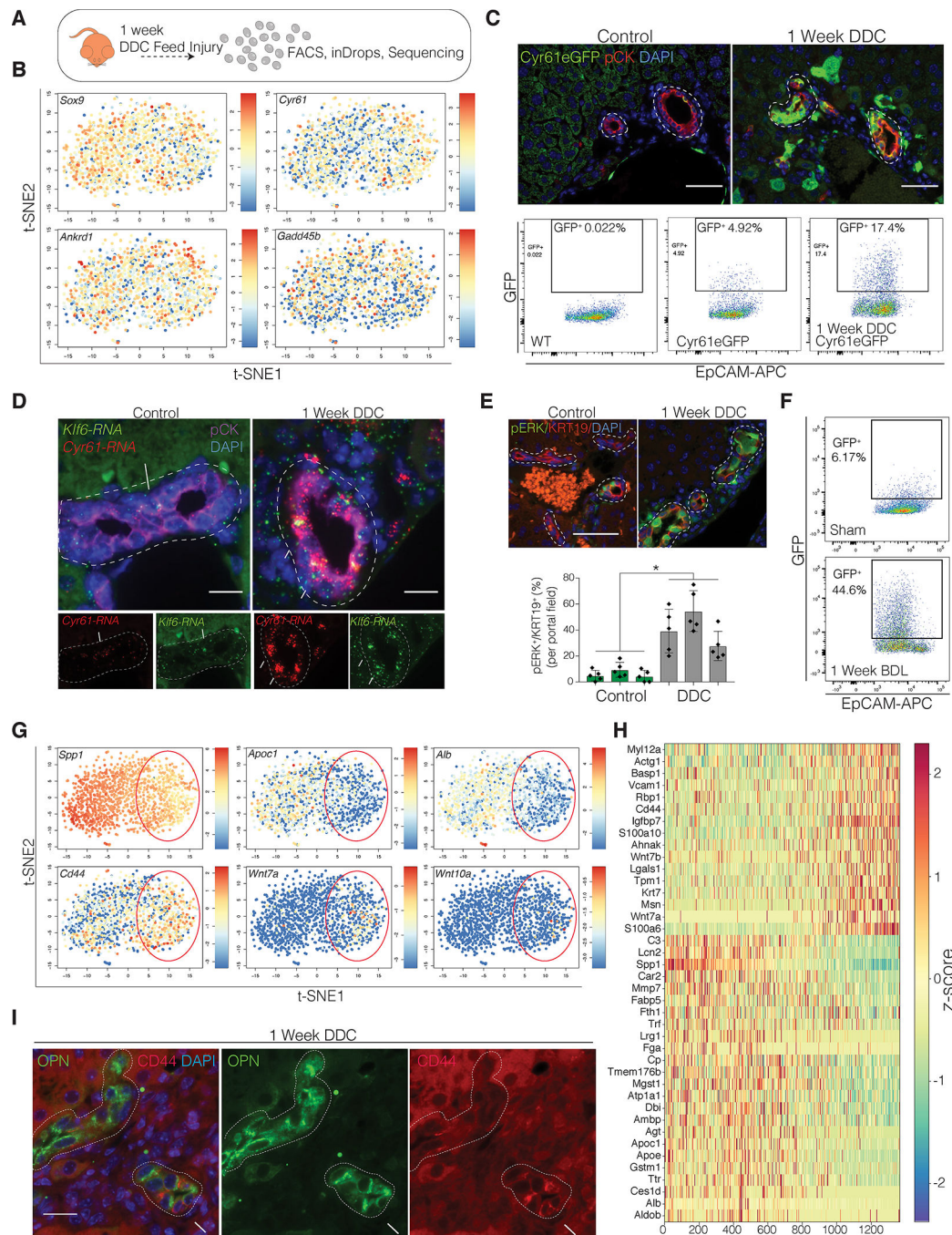


Figure 3. ScRNA-Seq Analysis of DDC-Injured BECs Reveals a Broadened YAP-Signaling Response and the Emergence of a Subset of BECs Expressing a Wnt Transcriptional Module *In Vivo*

(A) Illustration of the experimental design.

(B) t-SNE plot of BEC scRNA-seq data from a mouse fed DDC for 1 week. Colors denote relative expression of *Sox9* (biliary marker) and the YAP-targets *Cyr61*, *Ankrd1* and *Gadd45b* in each BEC (log2).

(C) Top: IF for GFP and pCK on liver sections of Cyr61eGFP mice on regular (Control) or DDC diet for 1 week. Bottom: FACS analysis for GFP on sorted BECs from Control or DDC-fed Cyr61eGFP mice.

(D) RNA-ISH for *Cyr61* and *Klf6* on Control and DDC-fed mice depicting an upregulation of these transcripts within the same pCK+ BECs.

(E) Top: IF for pERK/KRT19 on livers from Control and DDC-fed Cyr61eGFP mice. Bottom: Scatter-bar plot of quantified pERK+ BECs. (Mean \pm SD for five portal fields with each bar representing a mouse).

(F) Representative FACS plots for sorted BECs of Cyr61eGFP mice 1 week after sham operation or bile duct ligation indicating a strong increase of GFP+ BECs upon cholestatic injury.

(G) t-SNE plots of BEC scRNA-seq data from a mouse fed DDC for 1 week. Colors denote relative expression of *Spp1*, *Apoc1*, *Alb*, *Cd44*, *Wnt7a*, and *Wnt10a* (log2). Red oval outlines subset of cells with increased expression of Wnt genes.

(H) Heatmap representing z-scores of gene expression. Cells were ordered on the x-axis (left to right) by their position in the x-axis of the t-SNE. Depicted genes (n=40) were selected based on their correlation coefficients (corr > 0.25 or corr < -0.25) with *Wnt7a*, *Wnt7b*, *Cd44*, *Spp1*, and *ApoE1* and then hierarchically clustered by z-scores (maximum value 2.3).

(I) Representative IF for CD44/OPN (*Spp1*) on livers of mice fed DDC for 1 week. Arrows show CD44^{high} OPN^{low} BECs, whereas arrowheads show CD44^{low} OPN^{high} BECs (n = 3 mice).

Dashed lines generally outline biliary structures.

See also Figure S3.

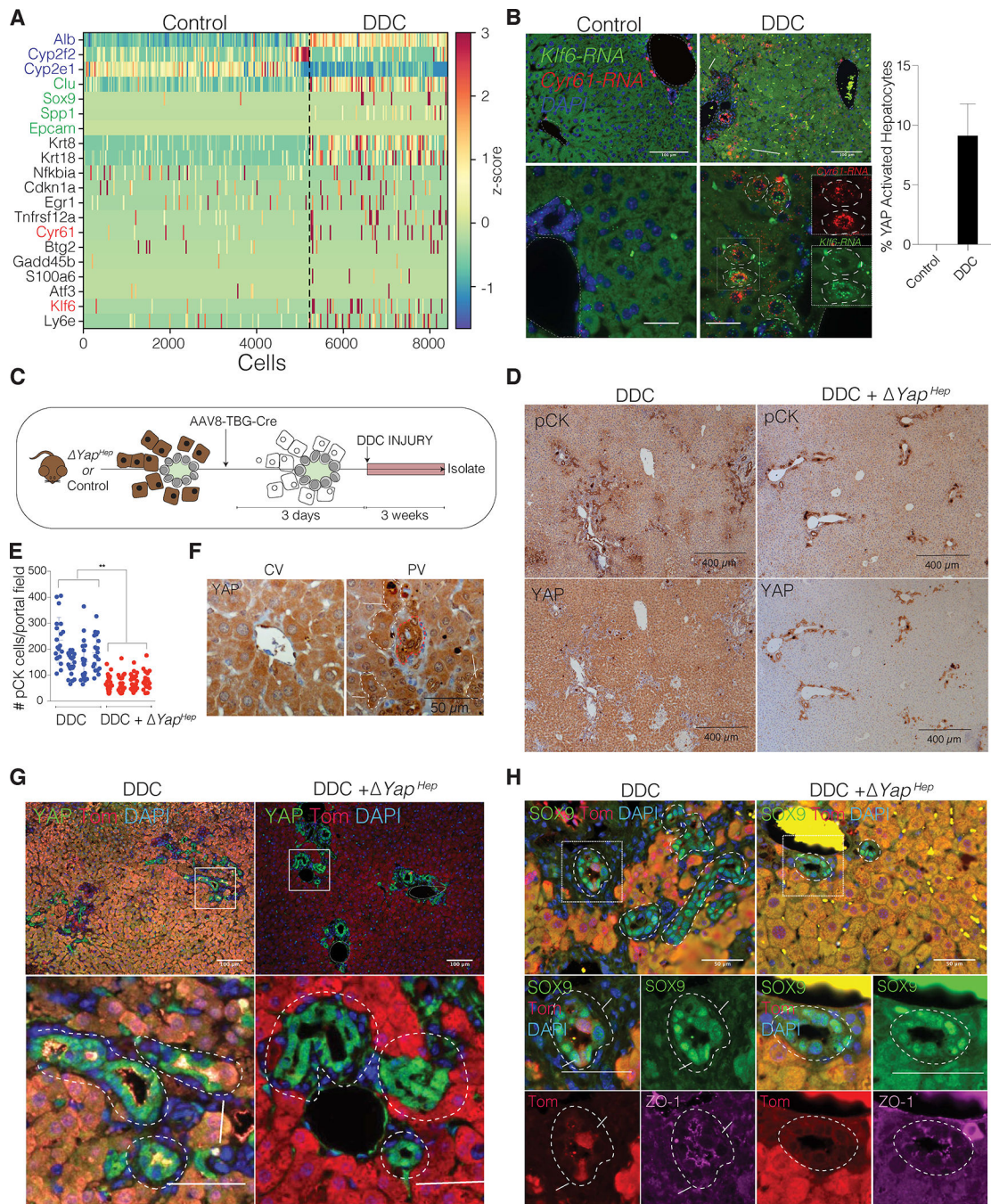


Figure 4. YAP Activity is Upregulated in a Subset of Hepatocytes and is Required for the Hepatocyte Regenerative Ductular Response to Injury

(A) Heatmap of scRNA-seq analysis of hepatocytes isolated from mice either fed a regular (Control) or DDC diet for 1 week, each with cells ordered according to their predicted relative position from the central (left) to the portal vein (right), using landmark genes (exemplary *Alb*, *Cyp2f2* and *Cyp2e1* in blue). Colors denote relative expression for shown candidate genes (highlighted: biliary markers (green) YAP targets from BECs (black), RNA-ISH analyzed genes (red)).

(B) RNA-ISH for *Klf6* and *Cyr61* in livers of mice fed with regular (Control) or DDC diet for 1 week (with magnified insets). Arrows and dashed lines represent YAP-active, periportal hepatocytes that co-express both *Cyr61* and *Klf6* RNA. Bar plot depicts mean \pm SD of YAP-active hepatocytes.

(C) Schematic of study design for panels D-H.

(D) pCK and YAP immunostains of control and *Yap^{Hep}* mice.

(E) Quantification of pCK⁺ cells per portal field (20 per mouse analyzed, mean \pm SD for each mouse (n = 4)).

(F) Representative high magnification images of YAP immunostain for portal and central vein areas highlighting clusters of YAP^{High} hepatocytes (white dashed lines) exclusively periportal. Bile duct is outlined with red dashed line.

(G) IF for YAP/Tom of control and *Yap^{Hep}* DDC mice with magnifications. Presence of Tom+/KRT19+ cells (arrow) are only observed in the biliary structures (dashed lines) of control, DDC only, mice (n = 4 mice per group).

(H) IF for SOX9/Tom/ZO-1 of control and *Yap^{Hep}* DDC mice. Dashed lines outline biliary structures. Magnified insets show SOX9+ ducts with apical ZO-1 expression. Arrows indicate SOX9+/Tom+ cells within the duct, exclusively in DDC control mice.

See also Figure S3.

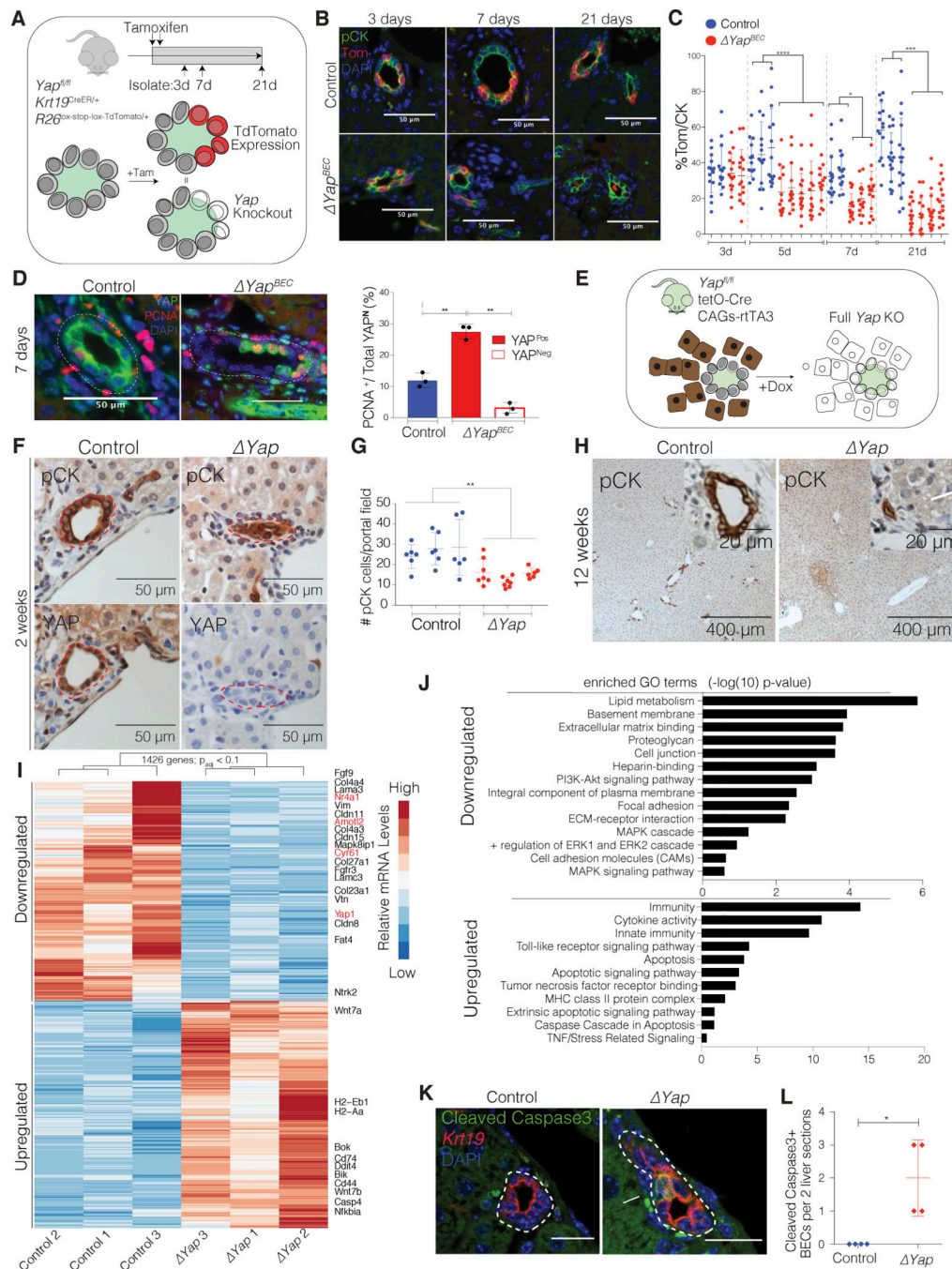


Figure 5. YAP is Required for the Maintenance of Adult Homeostatic BECs

(A) Schematic illustrating experimental design for TAM-inducible biliary-specific *Yap* KO (*Yap*^{BEC}) and control mice and expected correlation of Tom labeling with *Yap* KO.

(B) IF for pCK/Tom in *Yap*^{BEC} and control mice at indicated time points after TAM.

(C) Scatter plot of the % of pCK⁺ BECs that are Tom⁺ at indicated time points after TAM, showing Tom⁺ cell loss in *Yap*^{BEC} mice over time (Mean ± SD for 15 portal fields per mouse).

(D) Left: PCNA and YAP IF on *Yap*^{BEC} and Control mice 7 days after TAM. Right: % of PCNA⁺ BECs stratified according to YAP-positivity, indicating that KO escaper BECs are actively proliferating (Mean \pm SD for 20 portal fields for 3 mice per group, ANOVA, followed by Tukey multiple comparisons test).

(E) Schematic illustrating pan-inducible KO of *Yap* (*Yap* mice).

(F) Representative serial immunostains of pCK and YAP for *Yap* and Control mice 2 weeks after starting Dox, showing efficient *Yap* KO and disintegration of ductal structure in *Yap* mice.

(G) Scatter plot of the number of pCK cells per portal field in *Yap* and Control mice (Mean \pm SD for 6 portal fields per mouse).

(H) Immunostains for pCK of *Yap* and Control 12 weeks after starting Dox. Arrowhead indicates area of intraparenchymal necrosis. Insets depict bile duct at high magnification with arrow indicating few remaining pCK⁺ cells in *Yap* mice.

(I) Heatmap of top 1426 significantly 1.5-fold up- and down-regulated genes ($p_{\text{adj}} < 0.1$) based on RNA-seq data from FACS-sorted *Yap* and Control BECs from mice (n = 3 per group) 2 weeks after start of Dox. YAP-associated genes are highlighted in red.

(J) GO terms of differentially-expressed genes from David Bioinformatics database. Bars indicate level of significance.

(K) IF for cleaved-Caspase 3 (cCasp3) from *Yap* and Control mice 2 weeks after start of Dox. Arrow points out positive signal only observed in *Yap* mice.

(L) Quantification of cCasp3⁺ cells within two histological sections per mouse. (Mean \pm SD, n = 4 mice per group).

Dashed lines generally outline biliary structures.

See also Figures S4 and S5.

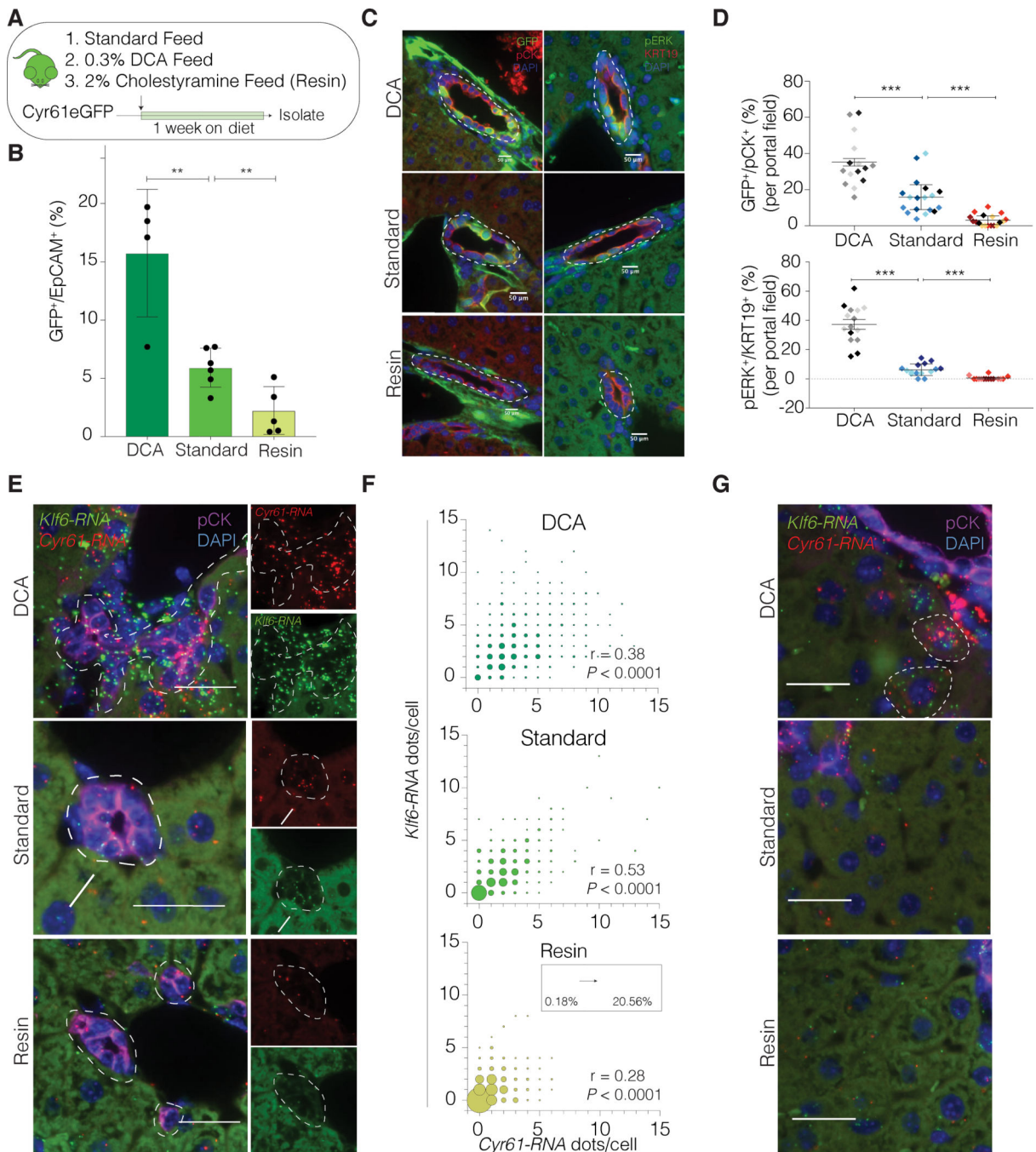


Figure 6. Homeostatic YAP Activity in BECs is Regulated by BAs

(A) Schematic of the experimental design.

(B) Bar-scatter-plot of the % of GFP⁺ BECs from Cyr61eGFP mice on indicated diets by FACS-analysis. Each dot represents a mouse (n = 4–5 per group).

(C) IF for GFP/pCK and pERK/KRT19 in Cyr61eGFP mice fed with the indicated diets. Dashed lines outline bile ducts.

(D) Quantification of GFP⁺ (top) and pERK⁺ (bottom) BECs in IF, depicting significant differences between groups. Each color represents an individual mouse per group (5 portal fields per animal).

(E) Dual RNA-ISH for *Cyr61* and *Klf6* with concurrent IF for pCK on Cyr61eGFP mice administered the indicated diets. Arrow indicates exemplary *Cyr61-Klf6* co-expressing BEC.

(F) Bubble plots for the quantification of co-localized *Cyr61* and *Klf6* RNA molecules per cell. Size of bubble corresponds to the co-expression frequency for *Cyr61* and *Klf6* within each condition and is indicated as % in the inset (n = 4 mice, BECs from 5 portal fields each, Spearman correlation).

(G) Visualization of the hepatic parenchyma using dual RNA-ISH for *Cyr61* and *Klf6* on mice administered the indicated diets. Dashed lines outline hepatocytes with co-expression in the DCA group.

See also Figure S6.

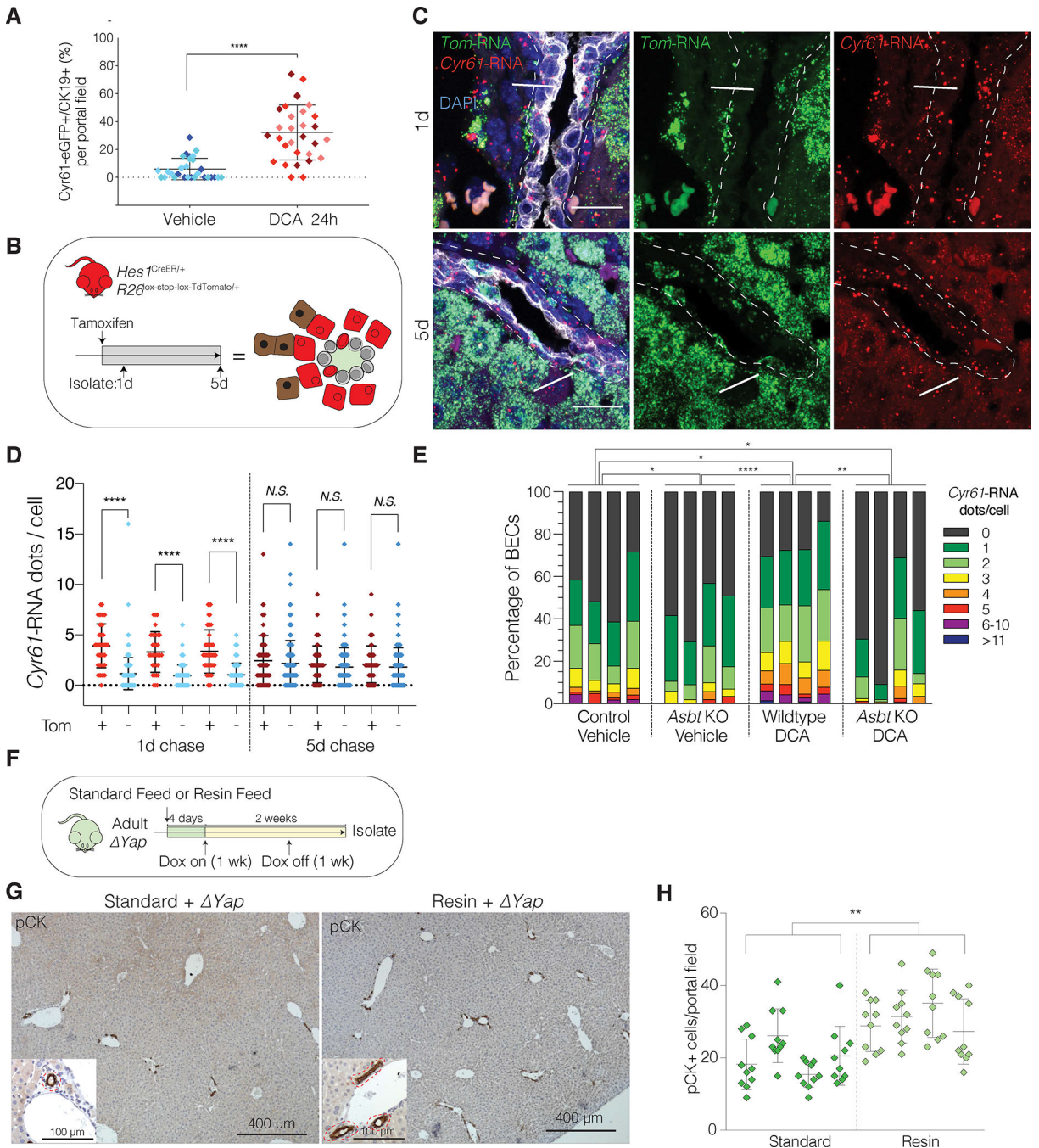


Figure 7. BA-Induced YAP Activity is ASBT-Dependent and Dynamically Fluctuates in BECs Under Physiological Conditions

(A) Scatter plot of the % of GFP⁺ BECs per portal field in IF of Cyr61eGFP mouse livers 24h after i.p. treatment with vehicle or DCA. Each diamond represents a portal field with colors indicating an individual mouse (n = 3 per group).

(B) Schematic illustrating experimental design for TAM-inducible Tom labeling of Hes1-expressing cells with Tom^{Hes1} mice at 1d and 5d chase for panels C-D.

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- (C) Dual RNA-ISH for *Cyr61* and *TdTomato* (*Tom*) with concurrent IF for KRT19 on *Tom^{Hes1}* mice, 1 and 5d after TAM. Arrows indicate *Tom/Cyr61* co-expressing BECs (enriched in 1d group), arrowheads designate exclusively *Tom* expressing BECs.
- (D) Quantification of *Cyr61*-RNA dots per BEC, stratified by *Tom*-positivity indicating a significant positive correlation only at 1d chase (n = 3 mice per group).
- (E) Distribution bar plot of *Cyr61*-RNA ISH quantification for the indicated groups. Each bar represents a mouse. BECs are color-coded according to the number of *Cyr61*-RNA dots and shown as % of cumulative 10 portal fields counted. P-values were computed using the Kullback-Leibler test and indicate significant differences between each group.
- (F) Experimental design.
- (G) Low-magnification immunostains for pCK in *Yap* mice fed with indicated diets. Magnified insets depict portal tracts with bile ducts (red dashed lines).
- (H) Quantification of pCK⁺ cells per portal field (Mean \pm SD of 10 portal fields for the indicated mice (n = 4 per group)).
See also Figure S7.