Report

Organ Size Control Is Dominant over Rb Family Inactivation to Restrict Proliferation In Vivo

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SUMMARY

In mammals, a cell's decision to divide is thought to be under the control of the Rb/E2F pathway. We previously found that inactivation of the Rb family of cell cycle inhibitors (Rb, p107, and p130) in quiescent liver progenitors leads to uncontrolled division and cancer initiation. Here, we show that, in contrast, deletion of the entire *Rb* gene family in mature hepatocytes is not sufficient for their long-term proliferation. The cell cycle block in Rb family mutant hepatocytes is independent of the Arf/p53/p21 checkpoint but can be abrogated upon decreasing liver size. At the molecular level, we identify YAP, a transcriptional regulator involved in organ size control, as a factor required for the sustained expression of cell cycle genes in hepatocytes. These experiments identify a higher level of regulation of the cell cycle in vivo in which signals regulating organ size are dominant regulators of the core cell cycle machinery.

INTRODUCTION

A cell's decision to continue cycling, exit the cell cycle, or start proliferating is paramount to embryonic development, homeostasis in the adult organism, and cancer development. Extra- and intracellular signals that govern the cell cycle are integrated and relayed by the Rb pathway. Under cytostatic conditions, Rb and its family members p107 and p130 block cell cycle progression in G0/G1, in large part by interacting with E2F transcription factors to repress the expression of cell cycle genes. Phosphorylation by Cyclin/Cdk complexes in response to mitogens functionally inactivates Rb family members and results in increased E2F activity and cell cycle progression. Rb pathway members have also been involved in many other biological processes, including differentiation, survival, chromosomal stability, metabolism, and senescence. Alterations in members of the Rb

pathway are found in the vast majority of human tumors, underscoring the central role of this pathway in the biology of mammalian cells and the maintenance of homeostasis (reviewed in Dick and Rubin, 2013; Manning and Dyson, 2012; Nicolay and Dyson, 2013).

A notable feature of the cell cycle machinery in mammals is a high level of functional redundancy. Accordingly, while overexpression of one Rb family member is often sufficient to promote cell cycle arrest (or cell death), inactivation of only one family protein often results in limited long-term cell cycle defects. For instance, acute loss of Rb function can only partly abrogate quiescence, in part because of a compensatory transcriptional upregulation of p107 (Sage et al., 2003). In contrast, combined inactivation of Rb family members leads to increased proliferation and, eventually, complete loss of the G1 checkpoint in culture (see for example Dannenberg et al., 2000; Sage et al., 2000). Similarly, while *Rb* mutant mice have a limited tumor spectrum, inactivation of the Rb family results in long-term ectopic proliferation and is often followed by neoplastic transformation in mice (see for example Dannenberg et al., 2004; McEvoy et al., 2011; Robanus-Maandag et al., 1998; Viatour et al., 2011).

The liver of mammals has a remarkable regenerative ability compared to other adult organs and tissues. Hepatocytes are capable of proliferation and growth in response to liver damage, including partial hepatectomy. Under certain conditions, liver progenitor cells with the capacity of generating both hepatocytes and bile duct cells are believed to contribute to regeneration in the adult liver (reviewed in Duncan et al., 2009). Thus, with a large number of cells having retained the capacity to divide, the adult liver is a system of choice to explore the mechanisms regulating cell cycle progression in vivo.

Here, we investigated the consequences of in vivo abrogation of the Rb pathway in the liver of adult mice. We found that the control of cell cycle is different in hepatocytes and their progenitors and identified interactions between the Rb/E2F pathway and organ size sensing mechanisms that are critical for the long-term proliferative potential of hepatocytes in vivo.
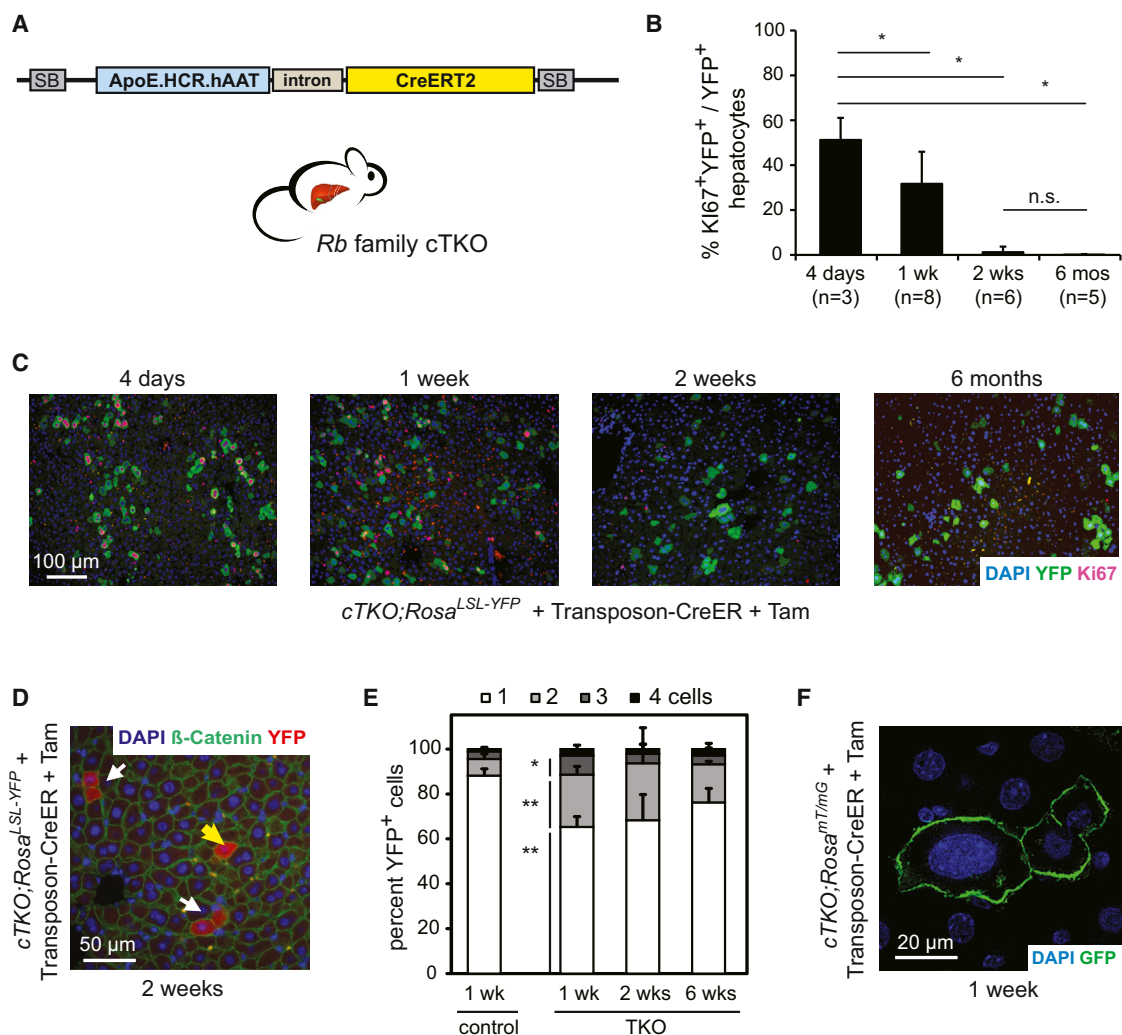
Figure 1. Rb Pathway Inactivation Leads to Transient Cell Cycle Entry in Hepatocytes

(A) Schematic representation of the *Sleeping Beauty* (SB) transposon-CreER system. ApoE.HCR.hAAT drives the expression of CreERT2 specifically in hepatocytes.

(B and C) Acute Cre-mediated *Rb* gene family deletion in adult hepatocytes leads to cell cycle entry followed by cell cycle arrest. Quantification (B) and representative images (C) of Ki67+ cells (red) after Cre activation in transfected hepatocytes (YFP+, green) in *cTKO;Rosa^LSL-YFP* mice. Nuclei are visualized by DNA staining (DAPI, blue). *p < 0.05; n.s., not significant.

(D and E) Quantification of single (yellow arrow) and adjacent (white arrows) YFP+ (red) hepatocytes in transposon-CreER-injected *Rosa^LSL-YFP* (control) or *cTKO;Rosa^LSL-YFP* (TKO) mice at different time points after Tam. Cell membranes are visualized by immunostaining for β-catenin (green). Significances are shown between control and TKO cells 1 week after Tam. *p < 0.05; **p < 0.01.

(F) Representative example of enlarged nuclei (DAPI, blue) in TKO hepatocytes (GFP+, green) in transposon-CreER-injected *cTKO;Rosa^mTmG* mice.

Data in (B) and (E) are presented as mean ± SD.

RESULTS

Acute Loss of the Rb Family Results in Transient Proliferation in Mature Hepatocytes

To delete *Rb* family genes in mature hepatocytes, we constructed a *Sleeping Beauty* transposon that harbors a tamoxifen (Tam)-inducible Cre recombinase (CreER) under the control of a hepatocyte-specific promoter (Figure 1A). This transposon stably integrates into a low percentage of liver cells following hydrodynamic tail vein injection. We transfected *Rb* family conditional triple-knockout hepatocytes (cTKO, *Rb^lox/lox;p130^lox/lox; p107^−/−*) with this transposon-CreER vector. After 2 weeks of recovery, mice were injected with Tam and triple-knockout (TKO) hepatocytes were tracked using a Cre-inducible fluorescent reporter. Deletion of *Rb* family genes initially resulted in cell cycle entry, but TKO hepatocytes rapidly and stably exited the cell cycle (Figures 1B and 1C). cTKO hepatocytes remained quiescent after Tam injection (YFP− cells in Figure 1C).

We developed three other systems to delete the *Rb* gene family in adult hepatocytes: intrasplenic injections of Ad-CMV-Cre (Ad-Cre) in cTKO mice and Tam injections in $Rosa26^{CreER/+}$ cTKO mice or $Alb^{CreERT2/+}$ cTKO mice. Figure S1A summarizes the advantages and limitations of the four approaches. Importantly, cell cycle re-entry (7–10 days after Cre induction) and exit (2–4 weeks after Cre induction) were similarly observed in all systems tested (Figures S1B–S1E and data not shown), indicating that these phenotypes are independent of the approach used to delete *Rb* family genes.

Strikingly, less than one-third of TKO hepatocytes underwent one or two cell divisions (Figures 1D and 1E). In all the systems used, a number of TKO hepatocytes had abnormally high ploidy (Figures 1F and S1F and data not shown). We did not observe apoptotic cell death (data not shown). These experiments indicate that, in response to loss of Rb family function, adult hepatocytes divide once or twice and/or undergo endoreduplication, as has been shown for *Rb*-only mutant hepatocytes (Mayhew et al., 2005) and is commonly observed in wild-type hepatocytes (Duncan, 2013). Thus, the Rb family is required to maintain the cell cycle arrest of adult hepatocytes, but loss of Rb family function is not sufficient for their prolonged proliferation.

The Cell Cycle Arrest in Rb Family Mutant Hepatocytes Is Independent of the p53 Pathway

The Rb/E2F and p53 pathways intersect at many levels, including shared targets between E2F and p53, such as the p21^{Cdkn1a} and E2F7 cell cycle inhibitors (Aksoy et al., 2012; Hiyama et al., 1998). Activation of the p53 pathway can serve as a tumor-suppressive mechanism in Rb-deficient cells, including in the liver (McClendon et al., 2011; Symonds et al., 1994). We examined the expression of E2F targets and members of the p53 pathway when TKO hepatocytes are exiting the cell cycle 10 days after Cre induction. As expected, loss of Rb family function led to increased expression of cell cycle genes (Figure 2A and data not shown). We also observed induction of a number of cell cycle inhibitors, including high levels of p21^{Cdkn1a} as well as other known p53 targets (Figures 2A and S2A and data not shown).

To test whether activation of p53 and/or p21 may be in part responsible for the cell cycle exit in TKO hepatocytes, we crossed cTKO mice to $p53^{lox/lox}$ mice or $p21^{-/-}$ mice (and to $Rosa^{mTmG/+}$ reporter mice). In both cases, the kinetics of cell cycle re-entry were similar in quadruple-mutant mice compared to TKO mice (Figures 2B and 2C). We also observed cell cycle exit in quadruple-mutant hepatocytes 2 weeks after Cre (Figures 2D and 2E), despite efficient deletion of *p53* (Figure S2B). We detected slightly elevated mRNA levels of the cell cycle inhibitor *p19^{Arf}* in both TKO and TKO;$p21^{-/-}$ mutant livers compared to controls (Figures 2A and S2C) and generated $cTKO;p19^{Arf-/-}$ mice to investigate p53/p21-independent functions of p19^{Arf}. However, loss of p19^{Arf} did not prevent cell cycle exit (Figures 2G). Similarly, pentuple-mutant hepatocytes in $cTKO;p21^{-/-};p19^{Arf-/-}$ mice still exited the cell cycle (Figure 2H). Therefore, the cell cycle arrest in Rb family mutant hepatocytes is independent of the p19^{Arf}/p53/p21 module.

The Cell Cycle Arrest in Rb Family Mutant Hepatocytes Correlates with an Inhibition of E2F Transcriptional Activity

To gain insight into the mechanisms underlying the cell cycle exit in TKO hepatocytes, we extracted RNA and proteins from adult cTKO livers (quiescent controls), cTKO liver 36 hr after partial hepatectomy or 48 hr after treatment with CCl$_4$ (both conditions induce wild-type hepatocytes to cycle), $cTKO;Alb^{CreERT2/+}$ mice 1 week after Tam (TKO cycling), and $cTKO;Alb^{CreERT2/+}$ mice 2 weeks after Tam (TKO arrested). As expected, Rb/E2F cell cycle targets were expressed at low levels in quiescent control livers and at higher levels in cycling cells. These genes were also downregulated in arrested TKO hepatocytes (Figures 3A, 3B, and 3C), which was confirmed in an RNA sequencing (RNA-seq) analysis on two samples from each of the four groups (Figures 3D, 3E, and S3A; Table S1). These data suggested that mechanisms exist in adult hepatocytes to inhibit the prolonged transcription of E2F target genes even in absence of the Rb family.

Arrested Rb Family Mutant Hepatocytes Display Low YAP/TEAD Levels and Activity

We reasoned that the mechanism enforcing cell cycle exit in TKO hepatocytes may exist in both wild-type and mutant livers but may be even more active in the TKO context to counteract the propensity of the mutant cells to divide. Thus, we sought to identify transcription factors that may regulate the genes whose expression is specifically altered in arrested TKO cells compared to quiescent wild-type cells. A computational analysis of the RNA-seq data identified candidates with known roles in proliferation and tumorigenesis (Figure S3B; Tables S2 and S3), including enrichment for TEF1 binding sites in genes downregulated in arrested TKO cells. TEF1/TEAD1 is a major partner of the transcriptional coactivators YAP and TAZ downstream of Hippo signaling, one of the key regulators of hepatic growth, liver size control, and liver cancer (Dong et al., 2007; Lu et al., 2010; Zender et al., 2005). Hippo/YAP signaling has also been shown to regulate Wnt pathway activity, another pathway highly enriched in the analysis that plays a central role in liver biology (Rosenbluh et al., 2012). Gene set enrichment analysis (GSEA) showed a strong enrichment for hepatic YAP targets (Dong et al., 2007) in genes significantly downregulated in arrested TKO livers (Figure 4A), which was confirmed by RT-PCR analysis of known YAP targets (Figure 4B). Furthermore, we observed increased YAP phosphorylation as well as reduced YAP and TEAD1 protein levels in arrested TKO livers (Figures 4C and S4A) suggestive of an active downregulation of YAP and TEAD1 in TKO arrested cells.

Based on these observations, we wondered whether E2F and YAP may regulate similar sets of genes. We used two independent studies where YAP targets were defined in gene expression profiling analyses (Dong et al., 2007; Zhao et al., 2007). For each study, we performed an enrichment analysis using transcription factor target sites identified by ENCODE chromatin immunoprecipitation sequencing (ChIP-seq) experiments (Auerbach et al., 2013). In both cases, we found a strong enrichment for E2F binding in YAP targets, with the vast majority of YAP targets being also bound by E2F (Table S4; Figure S4C). ChIP-PCR
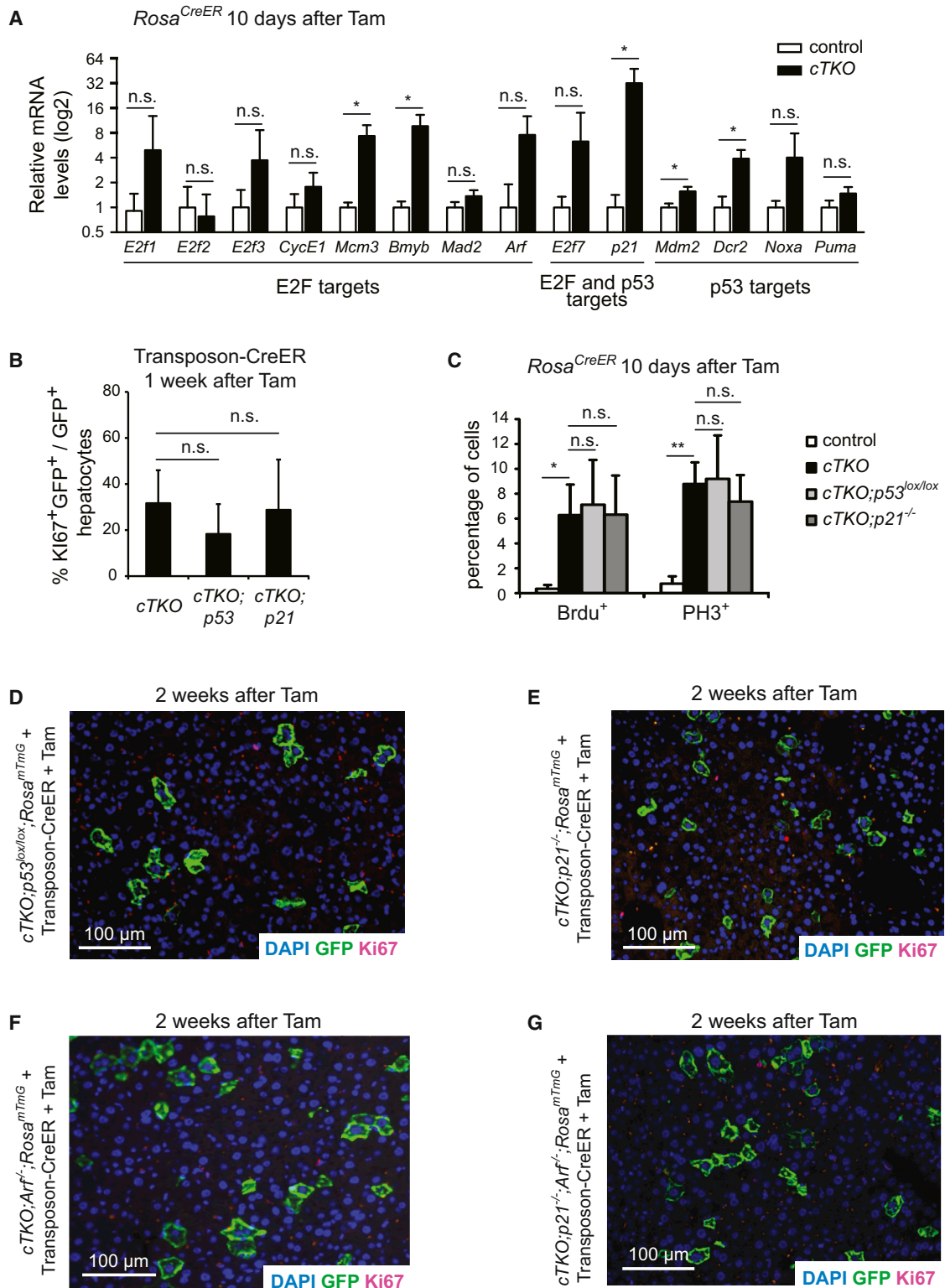
Figure 2. The Cell Cycle Arrest in *Rb* Family Mutant Hepatocytes Is Independent of the Arf/p53/p21 Axis
(A) RT-PCR analysis of E2F and p53 target genes in control and TKO livers 10 days after Cre activation in *cTKO;RosaCreER* mice (n ≥ 3) (n.s., not significant; *p < 0.05, paired t test).

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assays confirmed binding of YAP to *Mcm3* and *Mcm6*, two canonical E2F targets (Figure S4D). Together, these experiments suggested that decreased YAP/TEAD1 levels and transcriptional activity may affect the long-term expression of key cell cycle E2F targets (Table S5).

YAP was detectable by immunostaining in the nucleus of cycling wild-type and TKO hepatocytes and absent in arrested wild-type and TKO cells (Figure 4D). We recently reported that loss of Rb family function in liver progenitor cells drives these cells out of quiescence and leads to the development of hepatocellular carcinoma (Figure S4E) (Viatour et al., 2011). Cycling TKO progenitors also displayed nuclear YAP (Figure 4E), which correlated with increased expression of YAP target genes (Figure 4F). The expression of nuclear YAP and elevated expression of YAP target genes were conserved in TKO liver tumors (Figures S4F and S4G). Thus, in the adult liver, hepatocyte progenitors and hepatocytes respond differently to loss of Rb family function and YAP/TEAD1 do not become inactivated in progenitor populations.

Reduced Liver Size and Ectopic Activation of YAP Are Sufficient to Revert the Cell Cycle Arrest Observed in Rb Family Mutant Hepatocytes

These experiments revealed a striking correlation between E2F activity, proliferation, and YAP activity in liver cells in the hepatocytic lineage, which led us to test the hypothesis that downregulation of YAP/TEAD activity in TKO hepatocytes was limiting for proliferation. YAP activity has been linked to the regulation of organ size (reviewed in Avruch et al., 2011). To test if changes in liver size may affect the proliferation of TKO hepatocytes, we performed partial hepatectomy (PH) assays. PH was associated with nuclear translocation of endogenous YAP, expression of E2F targets such as MCM6, and proliferation in both quiescent wild-type and arrested TKO hepatocytes (Figures 5A–5C, and S5A). Remarkably, once the livers reached their normal size 5 days after PH, YAP molecules were again excluded from the nucleus (Figure S5B) and TKO hepatocytes re-exited the cell cycle (Figures 5D). Thus, the cell cycle potential of both wild-type and TKO hepatocytes is under the control of the signals involved in PH response.

To directly test if low YAP activity was a limiting factor specifically in TKO hepatocytes, we transfected hepatocytes with transposon vectors expressing wild-type or constitutively active forms of YAP in which phosphorylation sites are mutated to inhibit YAP nuclear exclusion and degradation (YAPS127A, YAP3SA, or YAP5SA) (Dong et al., 2007). Wild-type YAP and the partially active S127A mutant were primarily cytoplasmic in both control and TKO cells, which correlated with low cell cycle activity (Figures 5E and 5F). Strong activation of YAP has been shown to force hepatocytes out of quiescence (Dong et al., 2007; Lu et al., 2010; Zhou et al., 2009). Accordingly, expression

of the nuclear YAP5SA mutant led to proliferation of both control and TKO hepatocytes (Figure 5E). We also observed the recruitment of immune cells around the transfected cells with this YAP isoform and the elimination of the transfected cells after a few weeks, although the basis for this observation remains unclear (data not shown). Importantly, expression of YAP3SA, a form of YAP with presumed intermediate potency, led to a higher cell cycle activity specifically in TKO cells compared to control cells (Figures 5E and 5F). Additionally, the E2F target MCM6 was significantly expressed only in TKO cells transfected with YAP3SA and not in TKO cells with a control transposon or wild-type hepatocytes transfected with YAP3SA (Figures 5G and S5C). Thus, *Rb* family mutant hepatocytes are more sensitive to YAP activation than control hepatocytes, indicating that YAP activity is a key limiting factor in the capacity of TKO hepatocytes to proliferate.

DISCUSSION

Experiments in the past three decades have identified the Rb/E2F pathway as an essential regulator of a cell's decision to enter or exit the cell cycle. Here, we show that even in the absence of the Rb family, hepatocytes in the liver of adult mice possess a mechanism that can enforce quiescence in vivo.

Loss of Rb family function in adult stem/progenitor cells often results in the development of preneoplastic or neoplastic growth, as was shown for example in the hematopoietic system (Viatour et al., 2008), the mammary gland (Jiang et al., 2010), and the liver (Viatour et al., 2011). In postmitotic differentiated cells, loss of the Rb family has either no effect or often leads to cell death, which can be triggered by p53 (Symonds et al., 1994; Viatour et al., 2008). Loss of Rb family function can also lead to dedifferentiation, proliferation, and tumor formation (Ajioka et al., 2007) or to prolonged noncancerous growth (Garfin et al., 2013). In hepatocytes, however, the transient proliferative state triggered by inactivation of the Rb family is followed by a stable cell cycle arrest. Together, these observations indicate that the Rb family is a primary regulator of the cell cycle in stem/progenitor cell populations. In contrast, additional mechanisms may regulate the cell cycle of differentiated cells, even within the same organ or tissue.

The cell cycle entry observed in TKO hepatocytes occurs in the context of a liver of normal size. We propose that this initial proliferative phase, which is associated with an increase in liver size (Figure S5D), triggers a compensatory mechanism connected to organ size sensing in the liver, instructing TKO hepatocytes to stop cycling. At the molecular level, loss of Rb family function initially results in activation of E2F and transcription of cell cycle genes. As this ectopic proliferative phase takes place, compensatory signals eventually lead to the downregulation of YAP/TEAD transcriptional activity, in part through cytoplasmic

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(B) Quantification of cell cycle entry in triple- and quadruple-knockout hepatocytes 1 week after induction of Cre in transposon-CreER-injected mice. (n.s., not significant).

(C) Quantification of cell proliferation in triple- and quadruple-knockout hepatocytes 10 days after Cre induction (n.s., not significant; *p < 0.05; **p < 0.01). Data in (A)–(C) are presented as mean ± SD.

(D–G) Immunofluorescence analysis of Ki67 (red) expression in mutant (GFP+, green) hepatocytes 2 weeks after Cre induction in the liver of transposon-CreER-injected mice.
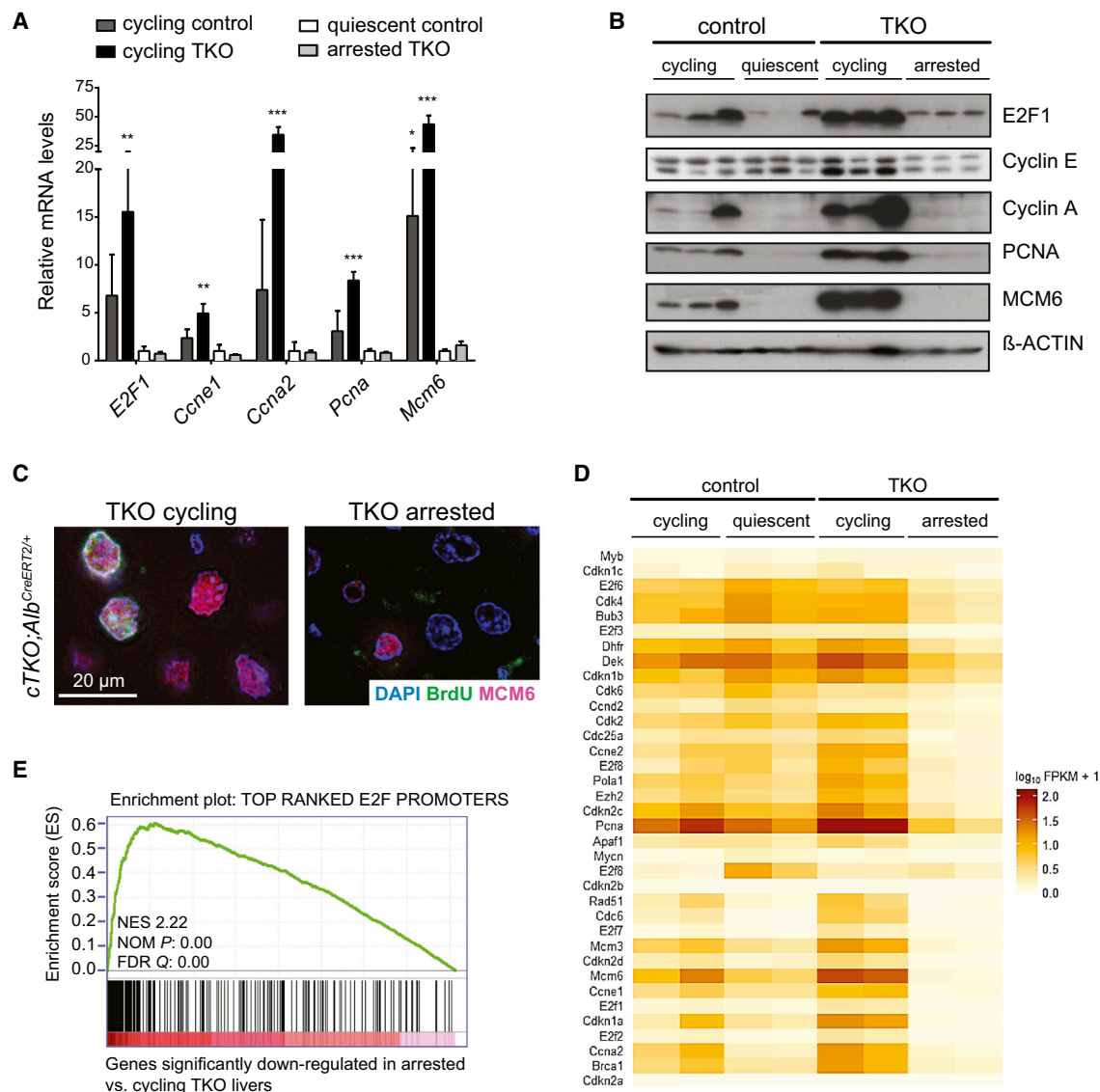
Figure 3. Downregulation of E2F Targets in Arrested *Rb* Family Mutant Hepatocytes

(A) RT-PCR analysis of E2F target genes in control and TKO livers (cycling control, 48 hr after CCl$_4$ treatment or 36 hr after partial hepatectomy; quiescent control, Tam-treated *cTKO;Alb$^{+/+}$* mice; cycling TKO, *cTKO;Alb$^{CreER/+}$* mice 1 week after Tam; arrested TKO, *cTKO;Alb$^{CreER/+}$* mice 2 weeks after Tam) (n = 3) (*p < 0.05; **p < 0.01; ***p < 0.001). Data are presented as mean ± SD.

(B) Immunoblot analysis of control and TKO livers (samples as in A).

(C) Immunofluorescence analysis of bromodeoxyuridine (BrdU, green) and the replication factor MCM6 (red), 1 (cycling) and 2 (arrested) weeks after Cre activation in *cTKO;Alb$^{CreER/+}$* mice.

(D) CummeRbund heatmap showing differential expression of curated canonical E2F target genes in cycling and noncycling TKO and control livers (RNA-seq; n = 2).

(E) Gene set enrichment analysis of genes significantly downregulated in arrested versus cycling TKO livers using a gene set generated from top E2F targets (Xu et al., 2007).

relocalization of YAP. While low YAP levels suggest that kinases in the Hippo pathway may be involved in this response, the lower levels of TEAD, which is not currently known to be regulated by phosphorylation, suggest that additional mechanisms may be at play. One intriguing possibility would be if inhibitors of YAP and TEAD were E2F targets, creating a negative feedback loop. The molecular basis for the differential response to

loss of the Rb family in progenitors and mature liver cells remains unknown.

YAP/TEAD complexes bind to many key cell cycle genes, and we propose that the long-term transcription of these E2F targets requires YAP/TEAD as cofactors. In support of this idea, evidence in *Drosophila* has linked E2F and YAP activity at the promoter of target genes. For instance, E2F activity is important for
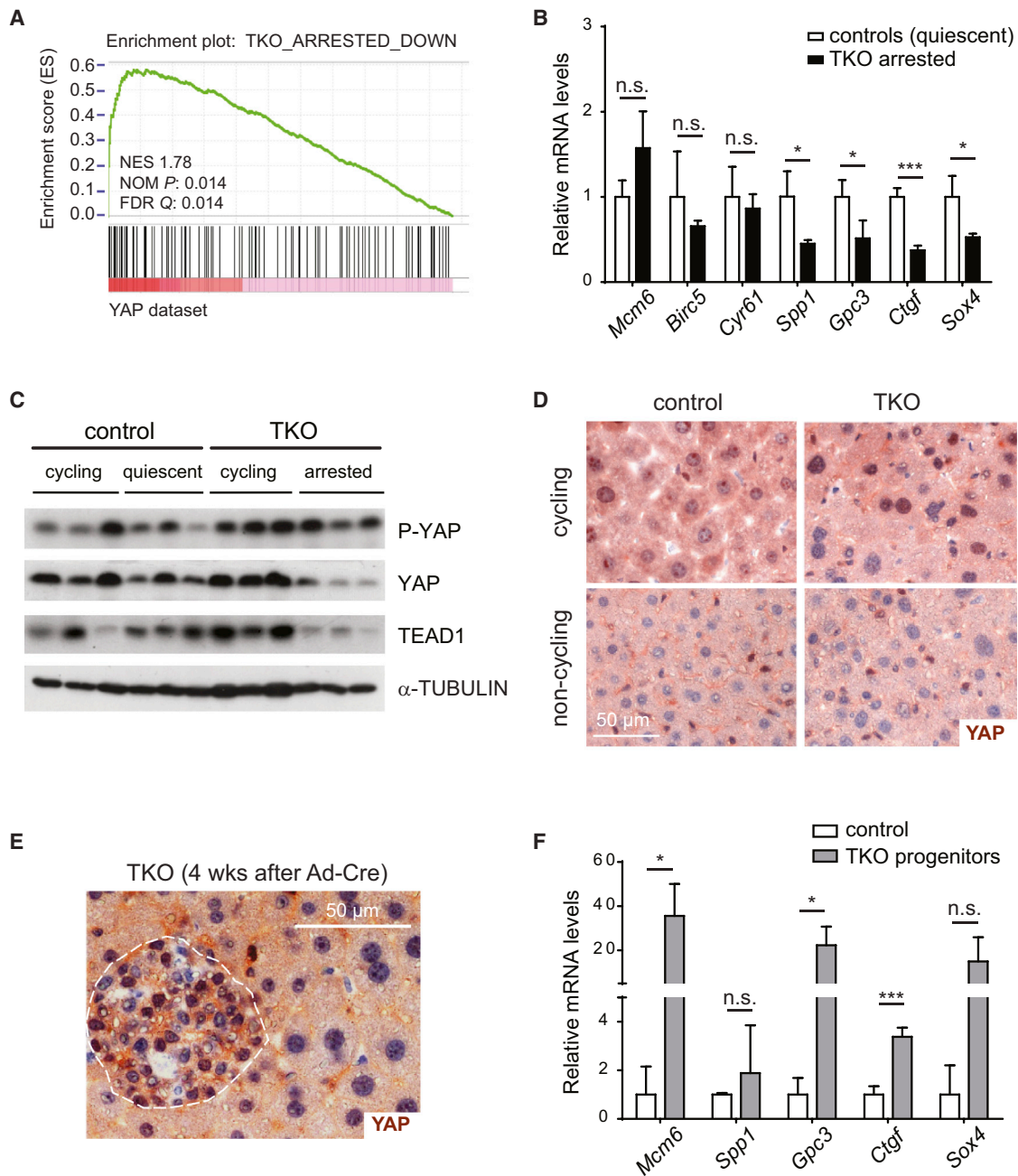
Figure 4. YAP Inactivation in Arrested TKO Livers

(A) Gene set enrichment analysis of genes upregulated upon hepatic YAP overexpression (Dong et al., 2007) in a gene set of significantly downregulated genes in arrested TKO livers.

(B) mRNA analysis of E2F- (*Mcm6, Birc5*) and YAP-regulated genes in noncycling TKO and control livers (n = 3; *p < 0.05; ***p < 0.001).

(C) Immunoblot analysis of YAP and TEAD1 in livers of control and *cTKO;Alb^CreER/+* mice.

(D) Immunohistochemistry for YAP (brown, counterstain: hematoxylin, blue) in cycling control (36 hr after partial hepatectomy) and TKO (*cTKO;Alb^CreERT2/+* 1 week after Tam) livers (top) and noncycling control and TKO (*cTKO;Alb^CreERT2/+* 2 weeks after Tam) livers (bottom).

(E) YAP staining of early progenitor lesions 4 weeks after intrasplenic injection of Ad-Cre in *cTKO* mice.

(F) mRNA analysis of YAP-regulated genes in TKO progenitor cells (*cTKO;Alb^CreERT2/+* 4 weeks after Tam) and control livers (n = 3; *p < 0.05; **p < 0.01).
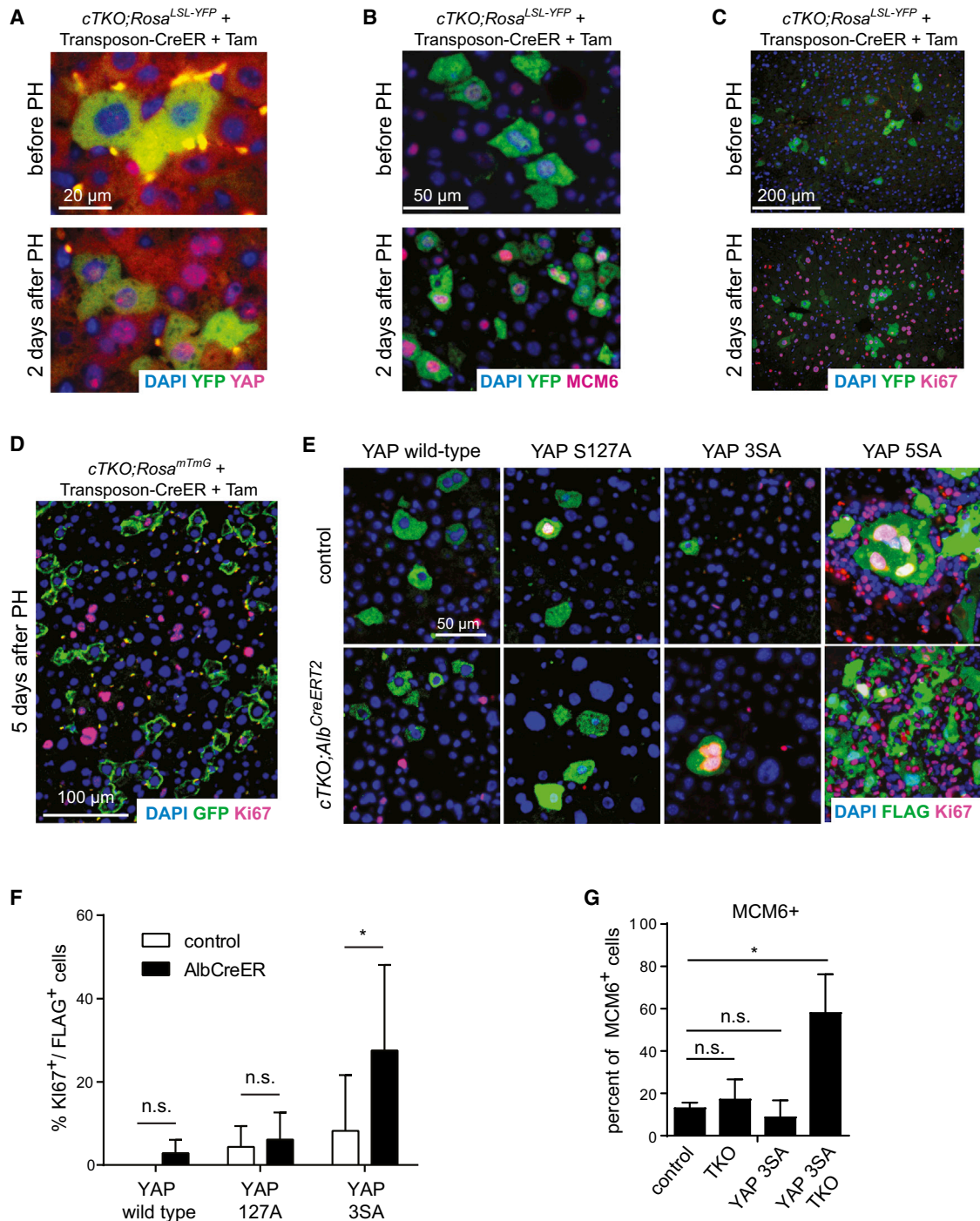
Data in (B) and (F) are presented as mean ± SD.

Figure 5. YAP Activation Is Sufficient to Revert the Cell Cycle Arrest in TKO Livers

(A–C) Immunofluorescence analysis of YFP (green) together with endogenous YAP (A), MCM6 (B), or Ki67 (C), respectively, after two-third partial hepatectomy (PH), 2 weeks after Tam in CreER transposon-injected *cTKO;Rosa^LSL-YFP* mice.

(D) Immunofluorescence staining of GFP (green) and Ki67 (red) 5 days after two-third PH, 2 weeks after Tam in CreER transposon-injected *cTKO;Rosa^mTmG* mice.

(E) Immunofluorescence analysis of YAP (Flag, green) and Ki67 (red) following hydrodynamic tail vein injection of a transposon expressing either wild-type YAP or constitutively active variants of YAP (YAPS127A, YAP3SA, or YAP5SA) into control or arrested TKO livers (transposon injection 1 week after 50 μg Tam, livers analyzed 2 weeks after Tam).

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the proliferation of Hippo mutant cells in flies, and the concomitant loss of Rb and Hippo signaling leads to proliferation and dedifferentiation in flies (Nicolay et al., 2010, 2011). Finally, consensus E2F binding sites are enriched close to YAP binding sites in fly cells (Oh et al., 2013) and human cells (this work and Kapoor et al., 2014; Shao et al., 2014). Thus, a decrease in transcriptional YAP activity in response to the ectopic proliferation induced by loss of Rb family function might prevent the activation of cell cycle genes despite initial high E2F activity in TKO hepatocytes. The molecular basis of the functional interaction between E2F and YAP/TEAD may include direct interactions between transcriptional complexes containing these transcription factors at promoters. In addition, our experiments do not rule out the possibility that other proproliferative factors may be limiting in TKO cells exiting the cell cycle or may be cooperating with low YAP activity. In particular, we found that Myc levels, but not levels of other putative oncogenes such as activated ERK or AKT, rapidly decreased in TKO hepatocytes entering quiescence (Figure S4B and data not shown). Future experiments will further dissect the regulatory networks implicated in cell cycle decisions in adult hepatocytes.

Recent experiments in mammalian cells indicate that low levels of the LATS2 kinase in the Hippo pathway may weaken the ability of Rb to induce cell cycle arrest in tumor cells (Tschöp et al., 2011). Furthermore, in breast cancer, loss of *RB* may replace amplification of YAP, further connecting these two pathways (Cheng et al., 2010). The dominant effect of organ size control mechanisms and YAP activity in cells with abrogation of the G1/S checkpoint identifies a hierarchy in the regulation of the cell cycle and may have important consequences in regenerative medicine and the development of anticancer strategies.

EXPERIMENTAL PROCEDURES

Mice

Rb family cTKO mice (Viatour et al., 2011) were maintained in a mixed 129Sv/J; C57/BL6 background. *p53*$^{lox/lox}$ and *p19*$^{Arf-/-}$ mice were generous gifts of Drs. Anton Berns and Chuck Sherr, respectively. *p21*$^{-/-}$, *Rosa26*$^{LSL-YFP}$, and *Rosa26*^{mTmG} mice were purchased from The Jackson Laboratory. For Ad-Cre injections, 10- to 12-week-old mice were anesthetized and surgically opened on the upper left quadrant of the abdomen, followed by injection of adenovirus into the spleen. In *cTKO;Rosa*$^{CreERT2/+}$ mice, Cre was induced by three consecutive injections of 1 mg Tam (T-5648; Sigma-Aldrich). In *cTKO; Alb*$^{CreERT2/+}$ mice (a kind gift of Pierre Chambon) (Schuler et al., 2004), Cre was activated by injection of 20 µg to 1 mg Tam. CreER was cloned in the transposon vector (Yant et al., 2000) from the MB80 plasmid (Addgene #12168); YAP, YAPS127A, and YAP5SA were cloned from Addgene plasmids #19045, #17794, and #27371, respectively. YAP3SA (S61A, S109A, S127A) is a swap mutant of YAP and YAP5SA. Samples were 36 hr (for RNA and protein analysis) or 48 hr (for immunostaining and ChIP experiments) after PH. All experiments with mice were approved by the Stanford Institutional Animal Care and Use Committee.

Histology, Immunostaining, and Immunoblot Analysis

Antigen retrieval on paraffin sections was performed using Trilogy (Cell Marque). After blocking, sections were incubated with primary antibodies overnight at 4°C, washed in PBS plus 0.1% Tween 20, and then incubated with secondary antibodies. Quantification was performed using ImageJ software. Primary antibodies used were anti-GFP (Invitrogen), anti-Ki67 (BD Pharmingen), anti-Flag (Sigma), anti-bromodeoxyuridine (BD Pharmingen), anti-YAP, anti-PH3, anti-CC3 (Cell Signaling Technology; data not shown), anti-MCM6, and anti-p21 (Santa Cruz Biotechnology). For immunoblotting, primary antibodies used were anti-YAP, anti-P-ERK, anti-ERK (Cell Signaling), anti-TEF1 (BD Biosciences), anti-p21, anti-E2F1, anti-Cyclin A, anti-PCNA, anti-MCM6 (Santa Cruz Biotechnology), and anti-Cyclin E (eBiosciences).

Flow Cytometry

Hepatic nuclei were isolated from frozen liver tissue (Mayhew et al., 2005), and ploidy was analyzed by flow cytometry using staining for propidium iodide.

RT-PCR, RNA-Seq, and Bioinformatics Analysis

RNAs were processed and quantitative PCR reactions were prepared as previously described (Viatour et al., 2011). RNA-seq, TopHat, and Cufflinks analysis were performed by Centrillion Biosciences. CummeRbund was used for heatmap visualization and clustering of RNA-seq data. E2F1/YAP cotarget genes were defined as genes with a significant ChIP-seq peak for E2F1 (ENCODE data) with direct overlap or within ±2.5 kb from the transcription site of YAP-regulated genes (Dong et al., 2007; Zhao et al., 2007). Curated gene sets for GSEA (version 3.1) (Subramanian et al., 2005) were obtained from http://www.broadinstitute.org/gsea/msigdb/index.jsp. Enrichment was considered significant when the p value was <0.05 and the FDR was <0.25. Gene Ontology analysis was performed using DAVID (GOTERM_BP_FAT, DAVID Bioinformatics Resources 6.7) and transcription factor target analysis using WebGestalt Gene Set Analysis Toolkit V2 (Zhang et al., 2005).

ChIP Analysis

ChIP was performed as described previously (O'Geen et al., 2006). In brief, mouse livers were perfused 5 min with 2% formaldehyde, before homogenization with a Potter-Elvehjem tissue grinder, followed by 10 min crosslinking in 2% formaldehyde. Antibodies used for immunoprecipitation were YAP (Cell Signaling) and p16 (sc-467). Promoter binding was assessed by quantitative PCR using SYBR green (Quanta Biosciences).

Statistical Analysis

Statistical significance was assayed by two-tailed Student's t test (*p < 0.05; **p < 0.01;***p < 0.005). Data are presented as mean ± SD if not stated otherwise.

ACCESSION NUMBERS

Data have been deposited to the Gene Expression Omnibus with the accession number GSE43631.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.025.

AUTHOR CONTRIBUTIONS

U.E. and A.-F.Z. performed and analyzed the experiments; D.V., R.K.A., and A.J.B. performed the bioinformatics analyses; U.E. and M.A.K. designed the transposon construct; and U.E. and J.S. designed the overall research and wrote the manuscript.

(F) Quantification of Ki67-positive YAP-transfected hepatocytes. Data are presented as mean ± SD.
(G) Quantification of MCM6-positive hepatocytes following YAP3SA expression in controls or arrested TKO livers (transposon injection 1 week after 50 µg Tam; livers analyzed 2 weeks after Tam). Controls, Flag-negative cells in control livers; TKO, Flag-negative cells in arrested TKO livers; YAP3SA, Flag-positive cells in control livers; YAP3SA TKO, Flag-positive cells in arrested TKO livers. Data are presented as mean ± SD.

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