UNIQUE IMPACT OF RB LOSS ON HEPATIC PROLIFERATION:
TUMORIGENIC STRESSES UNCOVER DISTINCT PATHWAYS OF CELL CYCLE CONTROL

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ABSTRACT

The retinoblastoma (RB) tumor suppressor pathway is disrupted at high frequency in hepatocellular carcinoma. However, the mechanisms through which RB modulates physiological responses in the liver remain poorly defined. Despite the well-established role of RB in cell cycle control, the deletion of RB had no impact on the kinetics of cell cycle entry or the restoration of quiescence during the course of liver regeneration. While these findings indicated compensatory effects from the RB-related proteins p107 and p130, even the dual deletion of RB with p107 or p130 failed to deregulate hepatic proliferation. While these findings suggested a modest role for the RB-pathway in the context of proliferative control, RB loss had striking effects on response to the genotoxic hepatocarcinogen diethylnitrosamine. With diethylnitrosamine, RB deletion resulted in inappropriate cell cycle entry that facilitated secondary genetic damage and further uncoupling of DNA replication with mitotic entry. Analysis of the mechanism underlying the differential impact of RB-status on liver biology revealed that, while liver regeneration is associated with the conventional induction of cyclin D1 expression, the RB-dependent cell cycle entry, occurring with diethylnitrosamine treatment, was independent of cyclin D1 levels and associated with the specific induction of E2F1. Combined, these studies demonstrate that RB loss has disparate effects on the response to unique tumorigenic stresses, which is reflective of distinct mechanisms of cell cycle entry.

INTRODUCTION

Liver cancer is a major worldwide health concern. In excess of 500,000 cases are diagnosed throughout the world annually and nearly 680,000 deaths was attributed to liver cancer in 2007. In addition, there is a significant mortality rate
associated with the disease, with a 5-year survival rate of only 11% in developed countries. Furthermore, there are few successful interventions for advanced disease and while new therapeutics are being deployed in the clinic their overall efficacy has been limited. Most cases of liver cancer can be traced to specific etiological events. For example, the majority of liver cancer cases in Eastern Asia are associated with hepatitis B infection, while in Western Europe hepatitis C infection represents a likely primary causal event. In addition to viral infection, it is well appreciated that certain environmental agents can strongly predispose to the development of liver cancer. Such toxins can represent genotoxic agents such as Aflatoxin B1 or vinylchloride exposures. Conversely, agents that are not primarily associated with DNA damage have also been implicated in liver tumorigenesis. Such non-genotoxic carcinogens can lead to chronic liver damage, steatosis, or deregulation of hepatic proliferation. The causal impact of these agents has been directly addressed utilizing animal models. Critically, the progression of liver cancer likely represents a complex relationship between environmental stresses and genetic events associated with tumor development.

Loss of the Retinoblastoma (RB) tumor suppressor pathway represents a relatively common event in liver cancer. Specifically, the region of the Rb locus (13q14) is subject to loss of heterozygosity at relatively high frequency in liver cancer and histological loss of RB protein expression occurs in a subset of tumors. RB can be functionally inactivated through a variety of mechanisms including deregulated phosphorylation and direct sequestration by oncoproteins. In liver cancer, cyclin D1 deregulation or loss of the p16ink4a tumor suppressor is hypothesized to contribute to RB inactivation. In addition, the oncoprotein gankyrin and specific hepatitis virus encoded proteins (e.g. NS5B, NS5A) have been shown to promote the functional inactivation of RB. Thus, through these combined mechanisms, it has been postulated that the loss of RB function is a relatively common event in liver cancer and has been associated with tumor development in addition to overall poor prognosis of the disease.

RB functions as a cell cycle regulatory factor to modulate proliferation in response to mitogenic and anti-mitogenic signals. In quiescent cells RB is hypophosphorylated and assembles protein complexes that repress the activity of genes regulated by the E2F-family of transcription factors. Mitogenic signaling induces the expression of cyclin D1, which leads to the activation of CDK4/6 complexes that mediate RB phosphorylation. This phosphorylation disrupts the association of RB with E2F proteins, thereby stimulating the expression of E2F-target genes that encompass a wide range of factors critical for progression through S-phase and mitosis. In contrast with such mitogenic signaling, agents that inhibit proliferation lead to the dephosphorylation of RB and the repression of E2F-mediated gene expression. Thus, RB is a critical integrator of proliferative and anti-proliferative stresses.

Typically, RB loss is viewed as promoting unchecked proliferation in tissue and contributing specifically to the initiation of tumor development. However, studies in multiple tumor tissues and mouse models have indicated that the functional consequence of RB loss is strongly modulated by both the genetic and tissue context. In part, this context specificity is mediated by the RB-related proteins p107 and p130 that can compensate for RB loss and serve to restrict E2F activity. In the liver, deletion of RB leads to a relatively modest effect on cell cycle control, but demonstrates a significant effect on tumor susceptibility. Here we explored the requirement for RB function in multiple facets of liver biology and define both critical RB-independent and RB-dependent aspects of liver physiology that are of profound importance in considering the mechanisms through which RB loss predisposes to tumor development.

**MATERIALS AND METHODS**

**Animals:** The generation and genotyping of the Rb<sup>fr</sup> and Rb<sup>fr</sup>; albcre<sup>+</sup> mice have been previously discussed. To directly address the function of the other pocket protein members, RB/p107 and RB/p130, double null mice were created. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Thomas Jefferson University.

**Animal treatments:** 8-week old Rb<sup>fr</sup> and Rb<sup>fr</sup>; albcre<sup>+</sup> and double null RB/p107 or RB/p130 littermates
were administered a single interperitoneal (i.p) injection of a 10% solution of carbon tetrachloride (CCl₄) dissolved in corn oil (Sigma). Mice were then sacrificed at 0, 24, 48, 60 and 168 hours post-CCl₄ for Rbf⁵/⁶ and Rbf⁵/⁶; albcre+ mice. Double null RB/p107 or p130 mice were sacrificed 0, 48 and 168 hours post-treatment. For acute diethylnitrosamine (DEN; Sigma) studies, 14-day old littermates were given a single i.p injection, dissolved in a 0.9% saline at a dose of 20mg/kg body weight. Mice were then euthanized at 24 and 72 hours post-DEN injection. All mice were given a single i.p. injection of 150mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma) dissolved in 0.9% saline 1 hour before sacrifice to label S-phase hepatocytes.

**PCR genotyping and detection of recombination:** DNA extraction and genotyping was performed as previously described for Rbf⁵/⁶ and Rbf⁵/⁶; albcre+ mice. Primers used to detect p107 and p130 were commercially available. Genomic PCR steps were configured to manufacture's protocol. Extraction of RNA and RT-PCR analyses were performed using standard approaches.

**Preparation of liver nuclei, protein analysis, ploidy determination and kinase activity:** Liver nuclei, protein extraction, immunoprecipitation and immunoblotting procedures were performed exactly as previously described. Immunoblotting was performed using commercially available antibodies, PCNA (P-10, Santa Cruz) and MCM7 (141.2, Santa Cruz). Polyclonal antibodies included, E2F1 (C-20, Santa Cruz), Cyclin D1 (Ab-3, neo-markers), Cyclin A (C-19, Santa Cruz), p130 (C-10, Santa Cruz), p107 (C-20, Santa Cruz), γH2AX (upstate CSS) and Lamin B (M-20, Santa Cruz). The CDK2 complex was immunoprecipitated using anti-CDK2 (H-298, Santa Cruz) and RB was immunoprecipitated using anti-RB (BD pharmingen). Cyclin B1 was detected using a privately produced monoclonal antibody, a generous gift from laboratory of Philipp Kaldis.

**Histological and Immunohistochemistry and Immunofluorescence:** Liver tissue embedded in paraffin was sectioned at 4µm, slides were then deparaffinized and stained with hematoxylin and eosin. Brdu incorporation was measured in liver sections as previously described. Commercically available monoclonal and polyclonal antibodies against, Ki67 (Rat monoclonal anti-kî67, 1:100 dilution; DAKO), γH2AX (Rabbit polyclonal anti-γH2AX, 1:500; upstate CSS) and phospho-histone H3 serine10 (Rabbit polyclonal anti-phospho-histone H3 serine 10, 1:1000 dilution; Upstate Biotechnology) were utilized for immunohistochemistry. All stained slides were stained as previously described.

**Statistical analysis and quantitation data:** Slides were scored in a blind fashion for at least 600 hepatocytes from several different fields for each staining. For histological assessment, a veterinary pathologist scored slides in blind fashion (A.W.). Statistical significance between groups was calculated by using a 2-tailed unpaired t-test. Differences were classified as statistically significant p<0.05.

**RESULTS**

Based on the central role of the RB tumor suppressor in cell cycle control, it was anticipated that RB loss would have a significant impact on the kinetics of liver regeneration. To directly investigate the impact of RB loss on regeneration, we utilized Rbf⁵/⁶ and Rbf⁵/⁶; albcre+ mouse strains. These mice harbor liver-specific deletion at the Rb locus, which can be clearly visualized in liver tissue by genomic PCR. Furthermore, RB protein levels were ablated in livers of Rbf⁵/⁶; albcre+ mice, as assessed by immunoprecipitation analyses (Figure 1A). In this context, CCl₄ administration leads to massive liver damage that precipitates a highly synchronous regenerative process to replace liver mass within 168 hours. Mice were treated then sacrificed at 0, 24, 48, 60 and 168 hours. Livers were weighed and the relative liver weight was expressed as a percentage of total body weight. The regenerative process is associated with a transient increase in liver weight. While there was no significant difference in weight between RB-proficient and RB-deficient livers treated with CCl₄, the RB-deficient livers trended toward larger weight (Figure 1A). Such a finding suggested that RB might have a relevant impact on liver proliferation in response to CCl₄ induced damage. Furthermore, qualitative histological examination of the livers showed that there was minimal difference in the kinetics of repair to liver injury. Specifically, at 24 hours both Rb⁵/⁶ and Rb⁵/⁶; albcre+ livers exhibit necrotic regions in proximity to portal veins, while at 48 hours, necrotic regions
encompass approximately 50% of liver area; however by 168 hours a complete recovery was observed in both Rb\(^{f/f}\) and Rb\(^{f/f\text{; albcre}}\) livers (Figure 1B). Kinetic analyses of DNA synthesis, as determined by BrdU incorporation, indicated that there was an equivalent entry of RB-proficient and deficient cells into S-phase. Moreover, the kinetics of re-entry into quiescence was virtually identical (Figure 1C). Under these conditions, mitotic figures were readily apparent at 48 hours and mitotic progression was further noted (Figure 1D). Again, RB status had minimal effect on these markers during regeneration. Together, these data indicate that regeneration as a result of CCl\(_4\) induced damage in the mouse liver occurs independently of RB status as well as the re-establishment of quiescence.

In conjunction with these findings, we investigated the control of RB/E2F target genes and possible compensatory mechanisms that would mask the effect of RB-deficiency. In untreated, RB-proficient and RB-deficient livers E2F target genes (MCM7 and PCNA) are largely undetectable at the RNA and protein level (Figures 2A and 2B, lanes 1, 2 and 7, 8). This finding indicates that the adult liver is largely quiescent and that compensatory mechanisms are mediating repression of these genes in the absence of RB. At 48 hours post-CCl\(_4\) administration, there is an induction of cyclin D1 expression and CDK2 activity (Figure 2B, lanes 3, 4 and 9, 10). In relation to these findings RB related proteins were analyzed. It is believed that p130 is a key mediator of compensation with RB loss, however in this context p130 RNA and protein levels remain relatively constant. In contrast, p107 RNA and protein levels accumulate and dissipate similar to other E2F-target genes (Figure 2C and 2D, lanes 3, 4 and 9, 10). This finding is consistent with the established regulation of p107 expression via E2F\(^{26}\). Together, these data suggest that modulation of proliferation in this context is not dependent on RB status, rather Cyclin D1 accumulation in conjunction with CDK2 suppress the activity of all pocket proteins inducing proliferation, with attenuation of this CDK-activity and p107/p130 mediating cell cycle exit.

In multiple systems, the deletion of p107 and/or p130 will synergize with RB loss to deregulate cell cycle control\(^{27}\). Therefore, to uncover potential roles of the other pocket proteins (p107 and p130) in the re-establishment of quiescence, mice harboring the combined loss of Rb/p107 and Rb/p130 were created. As shown in figure 3A and 3B, these mice exhibited p107 or p130 deletion in addition to liver specific RB-deletion, as noted by Cre-recombinase. At 8-weeks of age mice were administered CCl\(_4\) to induce liver injury. Immediately after sacrifice, livers were excised and weighed. No apparent difference in liver weight at any time point was detected between the double null mice and all mouse livers had returned to control weights by 168 hours (data not shown). Additionally, induction of DNA synthesis was analyzed by BrdU incorporation. At 48 hours an induction of BrdU incorporation was observed regardless of pocket protein status; however, by 168 hours, BrdU levels had returned to control levels in all conditions (Figure 3C). In conjunction with the establishment of quiescence, RB/E2F targets gene expression returned to control levels by 168 hours post-injection as well (Figure 3D). Combined, these data suggest that loss of p107 or p130 in conjunction with RB ablation does not influence the regeneration process in the mouse liver. Thus, highly redundant mechanisms can constrain E2F activity in the liver and mediate quiescence in vivo.

RB has been implicated in mediating DNA damage checkpoints in cell culture\(^{28,30}\), and has been shown to function as an important inhibitor of DEN-mediated liver tumorigenesis\(^{23}\). In order to investigate the general effect of RB loss on the response of the liver to the genotoxic hepatocarcinogens, the acute response of DEN were monitored. 14-day old mice received a single i.p. injection of DEN and were sacrificed at 24 and 72 hours post-injection. Immediately after sacrifice, liver weight was determined and expressed as a percent of total body weight. DEN exposure had little overall influence on liver weight in RB-proficient and RB-deficient mice (data not shown). Furthermore, histological analyses revealed little qualitative difference between conditions (data not shown). Since there is proliferation occurring in neonatal liver tissue, potential impacts on checkpoint responses in liver tissue were identified by BrdU incorporation. Interestingly, RB-proficient livers exhibited minimal BrdU incorporation (~3%) in either the absence or presence of DEN. However, RB deficient livers displayed a greater than 4-fold increase in BrdU incorporation (Figure 4A). At 72 hours post-DEN, BrdU levels in all conditions had
decreased to the levels of control animals. Since these effects on BrdU incorporation could reflect repair synthesis associated with DNA damage, Ki67-staining was performed. These data showed that consistent with the BrdU incorporation, an aberrant proliferative response was occurring (Figure 4A). In keeping with these findings, the induction of a number of RB/E2F target genes was also observed (Figure 4C, compare lanes 3 and 4 to 7 and 8). To determine whether the liver cells were capable of progressing into mitosis, phospho-histone H3 serine10 reactivity was examined. Consistent with such genotoxic agents inducing a “checkpoint”, the percent of phospho-histone H3 serine10 positive cells in RB-proficient livers was diminished; however, RB-deficient livers exhibited an increase in positive cells (Figure 4D). Interestingly, in contrast with CCl4 treatment, we observed a total lack of mitotic figures in DEN treated livers sections irrespective of RB status. In conjunction with these findings livers deficient for RB and DEN treated whilst accumulating CDK1 show a lack of Cyclin B1 induction (Figure 4C and 4D). Thus, RB-deficiency yields a unique impact on genotoxic carcinogens by facilitating cell cycle entry, yet yielding a failure to produce a productive mitosis, due at least in part to the lack of cyclin B1 accumulation. It has been shown that checkpoint deregulation can elicit additional secondary damage (e.g. strand breaks) driven by inappropriate DNA replication. Thus, γH2AX reactivity was determined by immunofluorescence analyses of tissues sections and immunoblot analyses of liver extracts, to determine the impact of RB status on this marker of DNA strand breaks. These analyses demonstrated that RB-deficient livers exhibited a significant increased in γH2AX vs. RB-proficient littermates (Figure 4B). Combined, these findings indicate that loss of RB has a profound impact on the cell cycle response to the genotoxic hepatocarcinogen DEN that influences DNA damage burden.

To define how RB status impacts the response to diverse stresses, a detailed analyses of the mechanisms through which DEN and CCl4 impinge on cell cycle control was determined. In mice exposed to CCl4 cell cycle entry was associated with the induction Cyclin D1 levels, the corresponding loss of p130, and accumulation of E2F target genes (Figure 5A, lanes 5, 6, 11 and 12). These facets of signaling occurred irrespective of RB-status, and are consistent with the well-proscribed mechanisms of cell cycle control. In contrast, DEN treated livers displayed a response that was highly dependent on RB-status. While DEN treatment had little impact on the levels of cyclin D1 or p130, there was a significant increase in E2F1 protein specifically in RB-deficient livers (Figure 5A, compare lanes 3 and 4 to 9 and 10). Correspondingly, the induction of E2F-target gene expression was specifically stimulated in RB-deficient livers treated with DEN (Figure 5B, compare lanes 3 and 4 to 9 and 10). Thus, these data indicate that discrete mechanisms are engaged by DNA damage versus regeneration signals to mediate cell cycle entry in the liver.

DISCUSSION

It is well established that RB status impacts the interplay between mitogenic and anti-mitogenic signals 16, 17, 29. Thus, it was suspected that loss of RB would yield highly significant effects on all facets of proliferative signaling in liver tissue. However, there was a striking specificity to the impact of RB status in response to exposure to diverse agents, suggesting that loss of RB may be particularly relevant in the context of specific etiological agents for HCC.

The capacity and appropriate regulation of liver regenerative processes is a critical facet in the liver and is associated with liver failure in the face of chronic damage. Numerous studies have suggested that the RB pathway plays a crucial role in regulating the kinetics of liver regeneration and the re-establishment of quiescence 31, 32. However, the results described here clearly show that loss of the RB gene has a relatively modest effect on the regeneration process induced by CCl4. Particularly, the kinetics of entry and exit from the cell cycle are indistinguishable with loss of RB. Presumably, this observation is due to the potent mitogenic effect of Cyclin D1 to drive the cell cycle irrespective of RB status or the presence of other pocket proteins. In addition, it has been previously shown that deficiency of Cyclin D1 abrogates the proliferation response following exposure to hepatic mitogens 33. Interestingly, since quiescence occurred regardless of RB status, it suggests those mechanisms that restrain proliferation in the liver are also RB-independent. While p107 or p130 deletion can illustrate that these factors compensate for RB loss the dual loss of
RB/p107 or RB/p130 did not deregulate these cell cycle transitions. These findings suggest that either a single pocket protein can mediate quiescence, or that alternative mechanisms are suppressing cell cycle progression in these models. Unfortunately, the simultaneous ablation of p107 and p130 in the mouse leads to neonatal lethality precluding the analyses of complete loss of pocket protein function. However, such findings suggest that the specific role of RB in liver cancer is likely not associated with the suppression of aberrant proliferation.

In contrast with regeneration, it is hypothesized that the initiation of liver tumorigenesis with genotoxic carcinogens is dependent on the mutations induced by such agents. It is well established from cell culture models that loss of RB can affect DNA damage responses leading to an uncoupling of S-phase checkpoints. For the in vivo analyses we utilized the potent hepatocarcinogen DEN. This exposure yielded relatively modest induction of DNA-damage (e.g. γH2AX) and we were incapable of detecting ATM activation in this model. However, we found that DEN exhibits diametrically distinct effects in RB-proficient vs. RB-deficient liver tissue. In RB-deficient cells DEN has a paradoxical mitogenic activity. This effect of DEN is not due to solicitation of a regenerative response, as RB loss does not lead to enhanced cell death following DEN exposure. Furthermore, the resultant DEN-induced cell cycle is both aberrant and associated with molecular events distinct from regeneration. Specifically, the cell cycle initiated by DEN exposure in RB-deficient livers does not progress to a productive mitosis. This finding suggests that certain checkpoints are still operative in the absence of RB, as indicated by the failure to accumulate cyclin B1. The fate of these cells that fail to progress into mitosis remains uncertain, but such hepatocytes could harbor replication induced mutations that contribute to tumorigenesis. The mechanism through which DEN leads to cell cycle entry is distinct from a regenerative response as clearly evidenced by the failure to induce cyclin D1. Rather, DEN treatment results in the stimulation of E2F1 expression, which is most profound in the RB-deficient liver. Studies from multiple laboratories have shown that DNA damage can induce E2F1 by stabilizing the protein. Our findings suggest that RB-status is critical for limiting the accumulation of this protein, and limiting the induction of E2F-mediated transcription following DNA damage. While additional studies will be required to specifically define the requirement of E2F1, our findings demonstrate that in contrast with regenerative signals, DNA damage engages a very specific and novel pathway that is critically dependent on RB.

Combined, these studies illustrate the complexity and context dependence through which RB elaborates cell cycle control in the liver. Furthermore, these analyses reinforce the concept that discrete extrinsic signals will be modified by distinct genetic events in specifying the basis for cell cycle deregulation and tumor susceptibility.
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Figure Legends:

Figure 1. Liver regeneration is not influenced by the status of RB. (A) Cre-mediated recombination of the floxed Rb locus was detected by PCR. In addition, RB was immunoprecipitated from nuclear lysates prepared from the livers of 8-week-old Rb\textsuperscript{fl/fl} and Rb\textsuperscript{fl/fl}; albcre\textsuperscript{+} mice. Livers were weighed and expressed as a percentage of total body weight. (B) FFPE liver tissue sections were stained with H and E. Representative images were taken of necrotic areas of livers. (C) Representative images were taken on BrdU stained tissue at 24, 48 and 168 hours post-CCl\textsubscript{4}. BrdU incorporation was detected and counted then expressed as a percent of total hepatocytes counted. (D) Positive hepatocytes for phospho-histone H3 serine10 expressed as a percent of total hepatocytes counted. Furthermore, images of 0 and 48 hour post-treatment, Rb\textsuperscript{fl/fl}; albcre\textsuperscript{+} livers represent typical mitotic figures.

Figure 2. Modulation of proliferation in the context of regeneration is not dependent on RB status. (A) qRT-PCR analysis of livers 0, 48 and 168 hours post-CCl\textsubscript{4}. RB/ E2F targets (MCM7 and PCNA) were analyzed. In this context β-actin was used as a loading control. (B) Liver tissue samples of equal total protein were separated by SDS-PAGE and levels of RB/E2F target genes were detected by immunoblot analysis at 0, 48 and 168 hours post-treatment. Each condition is represented by 2 samples. Lamin B was used as a loading control. (C) qRT-PCR analysis of livers 0, 48 and 168 hours post-CCl\textsubscript{4}. Other pocket proteins targets (p107 and p130) were analyzed. (D) Liver tissue samples of equal total protein were separated by SDS-PAGE and levels of p107 and p130 were analyzed. Each condition is represented by 2 samples. Lamin B is used as a loading control.

Figure 3. p107 and p130 status have no effect on regeneration in the presence of RB loss. (A) p130 status was determined by PCR analysis in addition to Rb ablation noted by Cre-recombinase. (B) p107 status was determined by PCR analysis in addition to Rb ablation noted by Cre-recombinase. (C) BrdU incorporation was detected in both Rb/p107 and Rb/p130 null livers, counted then expressed as a percent of total hepatocytes. (D) Liver tissue samples of equal total protein were separated by SDS-PAGE. Levels of RB/E2F targets (MCM7 and PCNA) were then analyzed in both RB/p130 and RB/p107 wild type and null conditions, compared to a CCl\textsubscript{4} positive control. Nuclear lysates of RB/p130 wild type and null conditions are on top and RB/p107 wild type and null conditions are below, in both cases Lamin B was used as a loading control.

Figure 4. RB loss deregulates the response to the genotoxic carcinogen DEN. (A) BrdU levels were detected at 24 hours post-treatment and counted then expressed as a percentage of BrdU positive hepatocytes. Ki-67 levels were also monitored. Positive hepatocytes for Ki67 at 24-hour post-treatment were expressed. (B) Levels of γH2AX was detected in all conditions and expressed as a percentage of total positive hepatocytes. Representative images were taken of γH2AX IF staining in DEN treated conditions. In addition, liver tissue samples of equal total protein were separated by SDS-PAGE and levels of γH2AX were analyzed. 2 samples were present per condition and Lamin B was utilized as a loading control. (C) Equal total protein was separated by SDS-PAGE. Levels of CDK1, MCM7, PCNA and Cyclin A were analyzed. 2 samples were present per condition and Lamin B was utilized as a loading control. (D) FFPE sections were stained for phospho-histone H3 serine10 and counted. Plot shows total positive hepatocytes for phospho-histone H3 serine10 as a percentage of total counted hepatocytes. Images at 24 hours post-treatment were taken to show typical mitotic figures. In addition, liver tissue samples of equal total protein were separated by SDS-PAGE and levels of Cyclin B1 were analyzed. 2 samples were present per condition and Lamin B was utilized as a loading control.

Figure 5. Distinct mechanisms define the critical role for RB in hepatic cell cycle control. (A) Equal total protein was separated by SDS-PAGE. Cyclin D1, pocket proteins p107 and p130 and E2F1 levels were analyzed and compared. In all conditions, 2 samples are present and all exposures were taken at the same time. Lamin B was used as a loading control (B) Equal total protein was separated by SDS-PAGE. RB/E2F targets (MCM7, PCNA and Cyclin A) were examined; in each condition 2 samples are represented.
Figure 2:

A.

|       | Rb\textsuperscript{fl} | Rb\textsuperscript{fl}; albcre\textsuperscript{*} |
|-------|-----------------------|-------------------|
| 0     | 48                    | 168               |
|       |                       |                   |
| MCM7  |                       |                   |
| PCNA  |                       |                   |
| β-actin |                     |                   |

B.

|       | Rb\textsuperscript{fl} | Rb\textsuperscript{fl}; albcre\textsuperscript{*} |
|-------|-----------------------|-------------------|
| 0     | 48                    | 168               |
|       |                       |                   |
| Cyclin D1 |                   |                   |
| Cyclin B1 |                   |                   |
| MCM7   |                       |                   |
| PCNA   |                       |                   |
| CDK2 kinase activity |            |                   |
| Lamin B |                       |                   |

C.

|       | Rb\textsuperscript{fl} | Rb\textsuperscript{fl}; albcre\textsuperscript{*} |
|-------|-----------------------|-------------------|
| 0     | 48                    | 168               |
|       |                       |                   |
| p107  |                       |                   |
| p130  |                       |                   |

D.

|       | Rb\textsuperscript{fl} | Rb\textsuperscript{fl}; albcre\textsuperscript{*} |
|-------|-----------------------|-------------------|
| 0     | 48                    | 168               |
| 1     | 2                     | 3                  |
| 4     | 5                     | 6                  |
| 7     | 8                     | 9                  |
| 10    | 11                    | 12                 |
| p130  |                       |                   |
| p107  |                       |                   |
| Lamin B |                      |                   |
Figure 4:

A. 

Brdu positive hepatocytes (%)

- Rb<sup>st</sup>
- Rb<sup>st</sup> + DEN
- Rb<sup>st</sup>; albcre<sup>+</sup>
- Rb<sup>st</sup>; albcre<sup>+</sup> + DEN

p = 0.003

B.

H2AX positive hepatocytes (%)

- Rb<sup>st</sup>
- Rb<sup>st</sup> + DEN
- Rb<sup>st</sup>; albcre<sup>+</sup>
- Rb<sup>st</sup>; albcre<sup>+</sup> + DEN

p = 0.03

24h post-DEN:

Rb<sup>st</sup> - +
Rb<sup>st</sup>; albcre<sup>+</sup> - +

γH2AX

Lamin B

C.

24h post-DEN:

- +
- +

CDK1
MCM7
PCNA
Cyclin A
Lamin B

D.

H3 positive hepatocytes/total hepatocytes (%)

- Rb<sup>st</sup> + DEN
- Rb<sup>st</sup>; albcre<sup>+</sup> + DEN

24h post-DEN:

Rb<sup>st</sup> - +
Rb<sup>st</sup>; albcre<sup>+</sup> - +

Phospho-histone H3 serine10

Cyclin B1
Lamin B
Figure 5:

A. 

|          | Rb^{ff}       | Rb^{ff}; albcre^{+} |
|----------|---------------|---------------------|
| CTRL.    | DEN 24h       | CCl_4 48h           |
| 1        |               |                     |
| 2        |               |                     |
| 3        |               |                     |
| 4        |               |                     |
| 5        |               |                     |
| 6        |               |                     |

B. 

|          | Rb^{ff}       | Rb^{ff}; albcre^{+} |
|----------|---------------|---------------------|
| CTRL.    | DEN 24h       | CCl_4 48h           |
| 1        |               |                     |
| 2        |               |                     |
| 3        |               |                     |
| 4        |               |                     |
| 5        |               |                     |
| 6        |               |                     |
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