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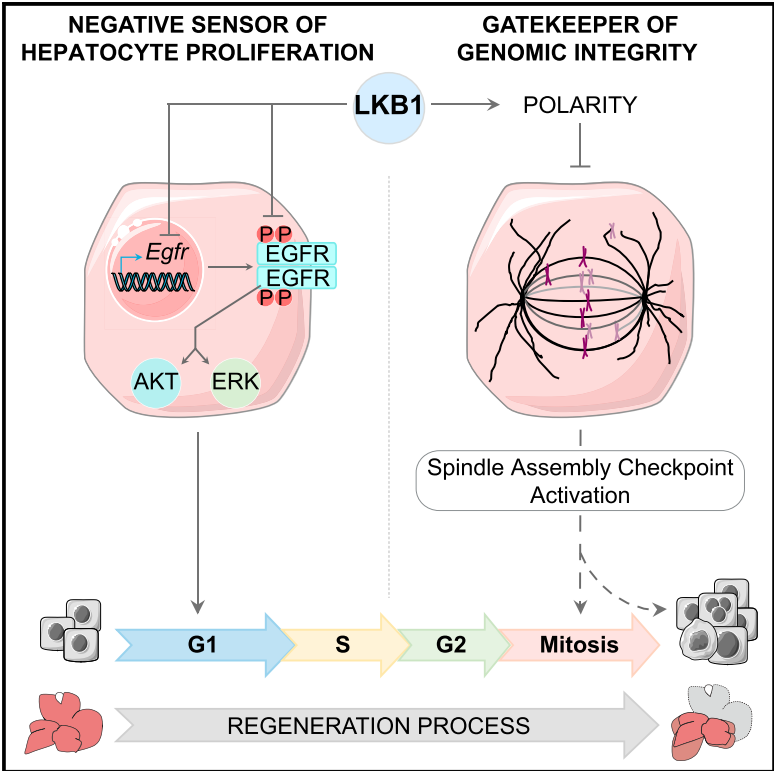
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LKB1 as a Gatekeeper of Hepatocyte Proliferation and Genomic Integrity during Liver Regeneration

Graphical Abstract



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In Brief

Maillet et al. demonstrate that the master kinase LKB1 plays a dual role in liver regeneration, independently of its major target, AMPK. LKB1 acts as a guardian of hepatocyte proliferation by controlling G₀/G₁ transition through EGFR activation. These findings also show that LKB1 is a gatekeeper of mitotic fidelity and hepatocyte ploidy integrity.

Highlights

- LKB1 inactivation promotes mass recovery during liver regeneration
- LKB1 regulates hepatocyte proliferation via EGFR signaling
- LKB1 regulates metaphase spindle orientation and organization
- LKB1 controls the genomic integrity of hepatocytes during liver regeneration

Data and Software Availability

GSE100605



LKB1 as a Gatekeeper of Hepatocyte Proliferation and Genomic Integrity during Liver Regeneration

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SUMMARY

Liver kinase B1 (LKB1) is involved in several biological processes and is a key regulator of hepatic metabolism and polarity. Here, we demonstrate that the master kinase LKB1 plays a dual role in liver regeneration, independently of its major target, AMP-activated protein kinase (AMPK). We found that the loss of hepatic *Lkb1* expression promoted hepatocyte proliferation acceleration independently of metabolic/energetic balance. LKB1 regulates G₀/G₁ progression, specifically by controlling epidermal growth factor receptor (EGFR) signaling. Furthermore, later in regeneration, LKB1 controls mitotic fidelity. The deletion of *Lkb1* results in major alterations to mitotic spindle formation along the polarity axis. Thus, LKB1 deficiency alters ploidy profile at late stages of regeneration. Our findings highlight the dual role of LKB1 in liver regeneration, as a guardian of hepatocyte proliferation and genomic integrity.

INTRODUCTION

Lkb1 (liver kinase B1) encodes an evolutionarily conserved serine/threonine protein kinase, originally identified as the causal gene of familial Peutz-Jeghers syndrome (PJS) (Hemminki et al., 1998). *Lkb1* is now considered to be a tumor suppressor gene frequently mutated in diverse human cancers (Momcilovic and Shackelford, 2015). LKB1 has been shown to act upstream to 14 kinases that belong to the ARK (AMP-activated protein kinase-related kinase) family including AMPK (AMP-activated protein kinase) (Shackelford and Shaw, 2009). LKB1 has pleiotropic activity in various biological processes including cellular metabolism, cell polarity, growth control, genomic integrity, and stem cell maintenance (Shackelford and Shaw, 2009; Williams and Brennan, 2008).

Tissue-specific deletion of *Lkb1* has revealed that this master kinase has organ-specific functions (Ollila and Mäkelä, 2011; Shorning and Clarke, 2011). During liver development, LKB1 is essential for the establishment of hepatocyte polarity, the main-

tenance of functional hepatocyte tight junctions, and the formation of the bile duct and canalicular networks (Just et al., 2015; Porat-Shliom et al., 2016; Woods et al., 2011). In adult mice, the hepatocyte-specific disruption of *Lkb1* expression has revealed the crucial role of this master kinase in the control of glucose and lipid homeostasis (Foretz et al., 2010; Patel et al., 2014; Shaw et al., 2005). Interestingly, the action of LKB1 as a key gluconeogenic suppressor is independent of AMPK and involves SIK (salt-inducible kinase, belonging to ARKs) signaling (Foretz et al., 2010; Patel et al., 2014).

Not only is the liver an essential organ for metabolic function, it also has a unique capacity to regulate its own growth and mass. In resting adult liver, less than 0.1% of hepatocytes are cycling at any given time (Fausto and Campbell, 2003). These cells have a tremendous potential for regeneration after liver resection (Fausto et al., 2006; Michalopoulos, 2017). Liver regeneration after partial hepatectomy has been studied extensively in rodents. One particularly fascinating feature of this process is that almost all quiescent and differentiated hepatocytes exit G₀ phase in a tightly synchronous manner, restoring initial hepatocyte mass after one or two rounds of replication (Michalopoulos, 2017). In addition to its regenerative capacity, the liver is also characterized by ploidy variations (Celton-Morizur and Desdouets, 2010; Duncan, 2013; Gentric and Desdouets, 2014). Unlike most tissues, liver tissues consist of a mixture of hepatocytes with one or two nuclei, each with two, four, eight, or more haploid chromosome sets. In rodent livers, hepatocyte polyploidization begins during weaning (Celton-Morizur et al., 2009). In adult livers, polyploidy increases with age but is also induced during regeneration after partial hepatectomy (Miyaoka et al., 2012; Toyoda et al., 2005). Polyploidization, which leads to increased cell size and genetic diversity, has been shown to promote adaptation to chronic injury or stress (Duncan et al., 2012).

In various organisms (e.g., *Drosophila*, *C. elegans*, mammals), LKB1 is a key actor in cell growth and genomic integrity. Given the unique and specific properties of liver parenchyma, we investigated the role of LKB1 in liver regeneration and ploidy. We found that LKB1 plays a dual role in liver regeneration independently of its major target, AMPK. It acts as both a regulator of hepatocyte proliferation through the control of EGFR signaling and as a gatekeeper of mitotic fidelity controlling hepatocyte ploidy integrity.



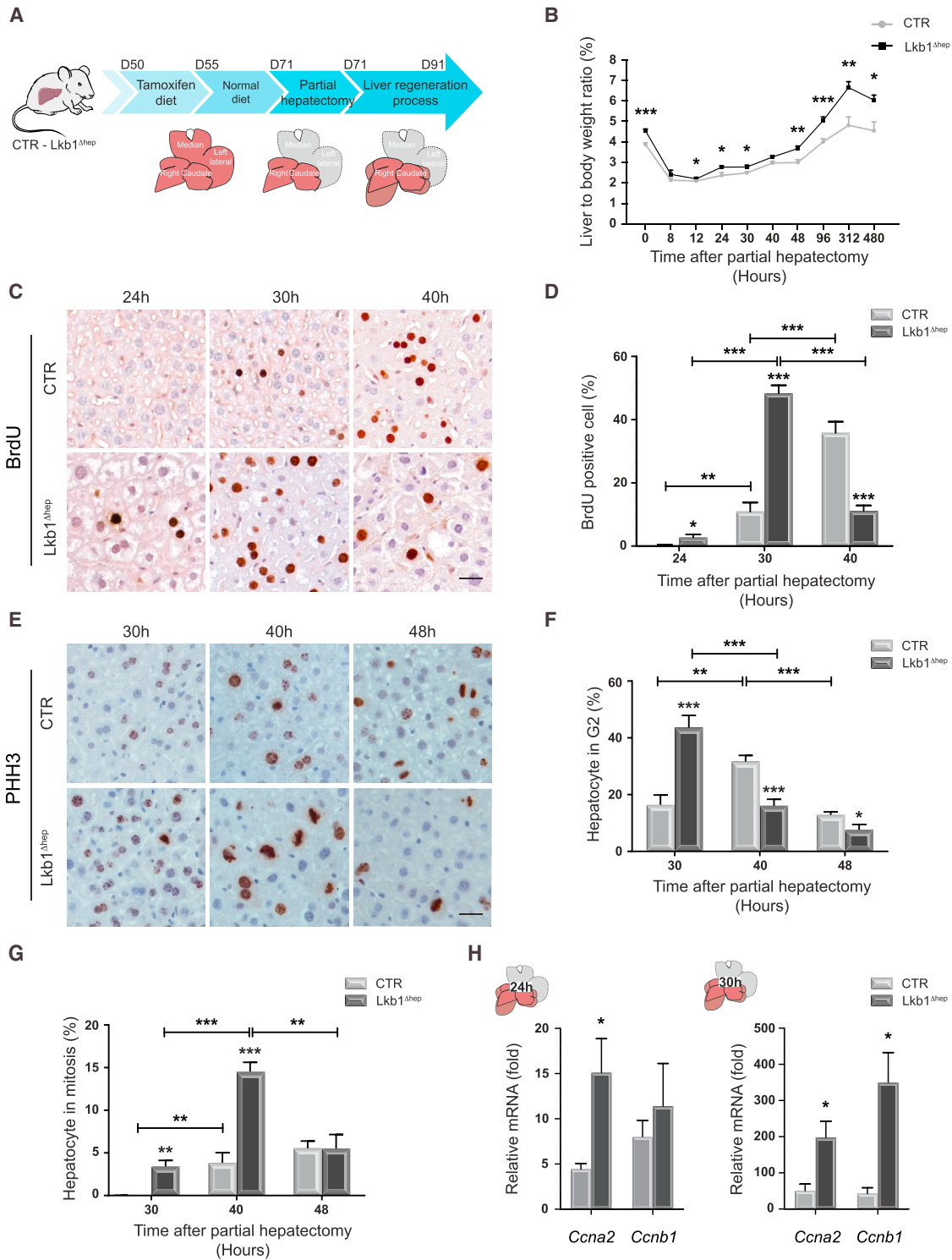


Figure 1. Hepatocyte-Specific Silencing of *Lkb1* Increases Cell Proliferation and Liver Mass during Liver Regeneration

(A) Representation of strategy for hepatocyte-specific deletion of *Lkb1* and experimental procedure for PHx.

(B) Hepatic regenerative index determined by measuring liver/body weight ratio in CTR and *Lkb1*^{Δhep} mice at indicated time points. Data represent the mean ± SEM (n ≥ 6 per group).

(C and E) Representative BrdU (C) and phospho-histone H3 (PHH3) (E) immunohistochemistry of CTR and *Lkb1*^{Δhep} liver tissues at different times after PHx (scale bar: 20 μm). PHH3 labeling discriminates between hepatocytes in G₂ (punctiform nuclei labeling) and hepatocytes in mitosis (condensed chromosome labeling).

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RESULTS

Lkb1 Deficiency Accelerates Hepatocyte Proliferation during Regeneration in Mouse Liver

We investigated the contribution of LKB1 to hepatocyte proliferation during liver growth, by performing a two-thirds partial hepatectomy (PHx) on age- and sex-matched *Lkb1* hepatocyte-specific knockout mice ($Lkb1^{\Delta hep}$) and control littermates (CTR) (Figure 1A). PHx in mice triggers a synchronized wave of cell cycling and growth in the remnant liver, restoring liver size to presurgical levels (Fausto, 2006). Almost all hepatocytes divide soon after PHx (between 0 and 48 hr after surgery). We found that LKB1 was phosphorylated in CTR livers at a quiescent stage (Figure S1A). The activation of LKB1 reached a peak when hepatocytes divide (24–48 hr) and decreased back to normal at the end of regeneration (Figure S1A). What is the impact of *Lkb1* deletion? First, *Lkb1* deletion in the resting liver resulted in a markedly higher liver-to-body weight ratio (Figure 1B). Relative liver weight was also monitored after PHx. CTR livers regenerated until 312 hr and re-established tissue homeostasis at 480 hr after PHx (Figure 1B). In $Lkb1^{\Delta hep}$ mice, liver-to-body weight ratio still increased at 312 hr (Figure 1B). However, $Lkb1^{\Delta hep}$ livers stopped growing at 480 hr, suggesting a response to homeostatic signals (Figure 1B). We then assessed hepatocyte proliferation by monitoring bromodeoxyuridine (BrdU) incorporation. In CTR livers, BrdU-positive hepatocytes were first detected 30 hr after PHx and their numbers peaked at 40 hr (Figures 1C and 1D). In $Lkb1^{\Delta hep}$ livers, BrdU-positive hepatocytes were detected as soon as 24 hr after PHx (Figures 1C and 1D) and the percentage of BrdU-positive hepatocytes peaked at 30 hr (Figure 1D). Consistent with these findings, we observed a faster entry into G₂ and M phases in $Lkb1^{\Delta hep}$ livers (Figures 1E–1G). Striking increases in the levels of cell cycle drivers were observed after *Lkb1* deletion. *Ccna2*, encoding a key actor of S-phase progression, was already upregulated 24 hr after PHx in $Lkb1^{\Delta hep}$ livers, with increases in both mRNA and protein levels (Figures 1H and S1B). Moreover, when CTR hepatocytes entered S phase (30 hr), *Ccnb1*, a crucial actor for G₂/M transition, was already expressed in $Lkb1^{\Delta hep}$ livers (Figures 1H and S1B). These results indicate that LKB1 is a key regulator of hepatocyte proliferation and mass recovery during liver regeneration.

Effects of LKB1 on Hepatocyte Proliferation Are Independent of Metabolic Signals

Given the essential role of LKB1 in the control of liver metabolic balance, we investigated the metabolic response, a known source of proregenerative signals, in $Lkb1^{\Delta hep}$ livers following PHx (Huang and Rudnick, 2014). First, regarding the lipid metabolism, a significant accumulation of triglycerides (TGs) was observed in CTR livers at early time points after PHx, with a subsequent decrease as hepatocytes progressed through S phase

(40 hr, Figures 1D and 2A). Surprisingly, fat accumulation was not significantly affected in $Lkb1^{\Delta hep}$ livers (Figure 2A). Quantitative PCR confirmed that the expression of genes encoding key fatty acid synthesis enzymes (*Acc1*, *Srebp-1c*) was similar in both groups of animals during liver regeneration (Figure 2B). Given the key role of LKB1 in the control of glucose homeostasis, we also assessed glycogen content and gluconeogenic gene expression. High glucose concentrations in the liver parenchyma are generally considered to act as a brake on regeneration (Weymann et al., 2009). As expected (Huang and Rudnick, 2014), glycogen storage decreased strongly after PHx in CTR livers (Figure 2C). Consistent with previous observations (Shaw et al., 2005), hepatic glycogen content was higher in resting $Lkb1^{\Delta hep}$ livers (Figure 2C). Nevertheless, glycogen content clearly decreased in $Lkb1^{\Delta hep}$ livers as in CTR after PHx (Figure 2C). The expression of *Ppargc1 α* and *G6pc*, encoding two key gluconeogenesis enzymes, increased in resting $Lkb1^{\Delta hep}$ livers (Figure 2D) and was maintained at early time points after PHx (Figure 2D). Interestingly, the increased gluconeogenesis in regenerating $Lkb1^{\Delta hep}$ livers had no effect on pro-proliferative signaling. Finally, as LKB1 is a key regulator of cell energy levels through its control of AMPK, we assessed the AMPK activation. During liver regeneration, AMPK is activated before S-phase entry (Merlen et al., 2014). AMPK phosphorylation was lower in $Lkb1^{\Delta hep}$ than in CTR hepatocytes, but clear activation of this kinase was observed at 24 and 30 hr after PHx in both groups (Figure 2E). This result demonstrates that AMPK can be activated independently of LKB1 during liver regeneration. Collectively, our data indicate that LKB1 loss has a moderate impact on metabolic and energetic responses during liver regeneration.

LKB1 Loss in Quiescent Mouse Liver Enhances EGFR Activation

To further understand how LKB1 regulates the hepatocyte cell cycle, we focused on key pro-proliferative signals of liver regeneration. Cytokine signaling (IL6/TNF α /STAT3), which plays a key role in G₀ exit, was found activated early in regeneration in both $Lkb1^{\Delta hep}$ and CTR livers (Figures S2A–S2C). We then focused on the major growth factor signaling pathways in hepatocytes involving the MET receptor, activated by hepatocyte growth factor (HGF), and the epidermal growth factor receptor (EGFR), activated by EGF and related ligands. Resting $Lkb1^{\Delta hep}$ livers displayed a downregulation of MET mRNA levels and activity compared with CTR livers (Figures S2D and S2E). Conversely, *Egfr* mRNA levels were significantly higher in resting $Lkb1^{\Delta hep}$ than in CTR livers (Figure 3A). Furthermore, EGFR protein levels and activity (assessed by the level of phosphorylation) were also significantly increased in resting $Lkb1^{\Delta hep}$ livers (Figure 3B). This phenotype is independent of AMPK as resting AMPK α 1/ α 2 Δhep livers did not present an increase in EGFR expression or activity (Figures 3B and S2F). Consistent with an activation of EGFR signaling, downstream targets of this pathway (AKT, ERK, and

(D–G) Quantitative analysis of BrdU (hepatocytes in S phase) (D) and PHH3 labeling (hepatocytes in G₂ and mitosis) (G). Data represent the mean \pm SEM ($n \geq 6$ per group).

(H) Quantitative PCR analysis of genes associated with cell cycle progression (*Ccna2*, *Ccnb1*) in CTR and $Lkb1^{\Delta hep}$ liver tissues. Data represent the mean \pm SEM ($n = 8$ per group).

See also Figure S1.

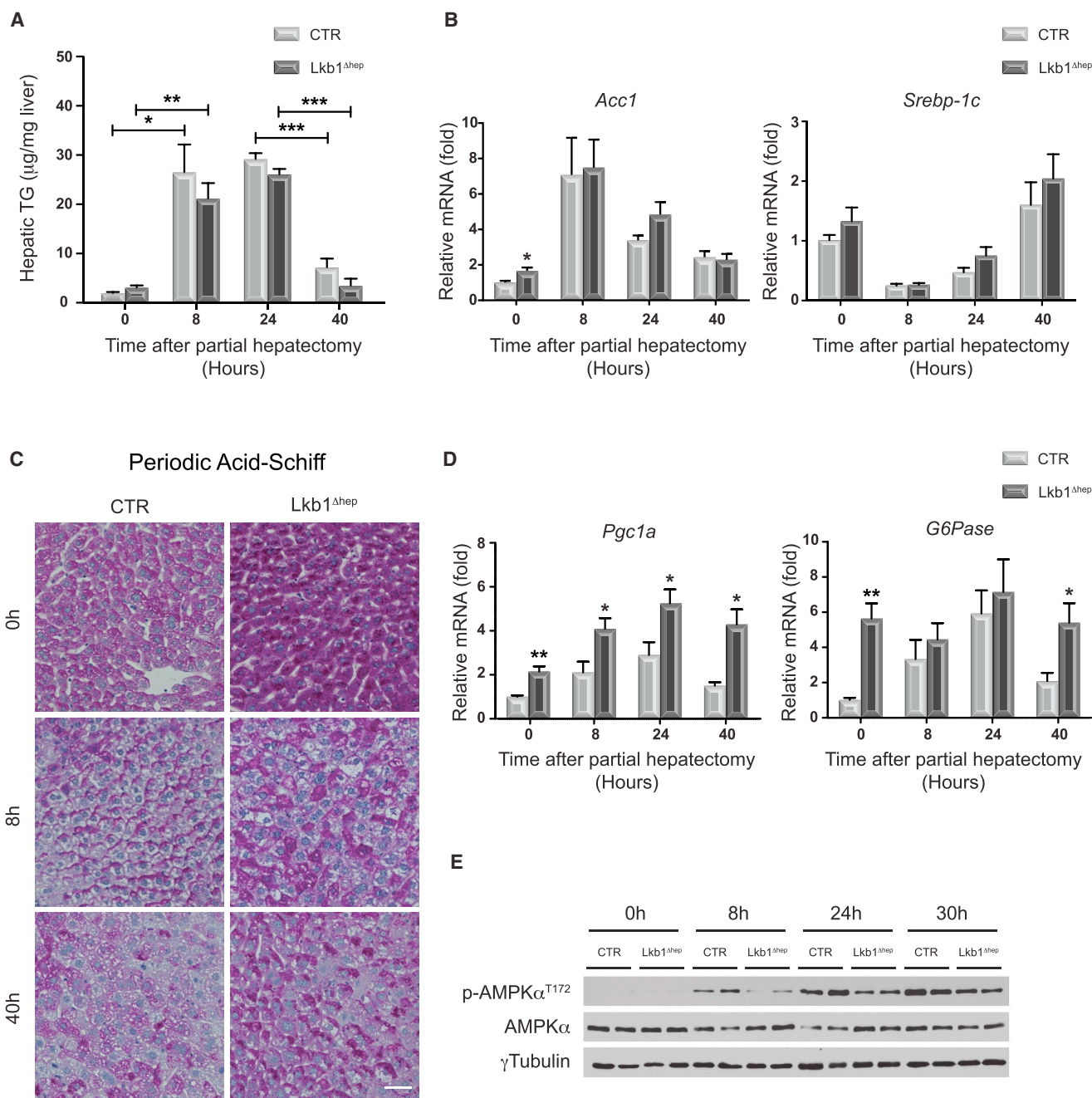
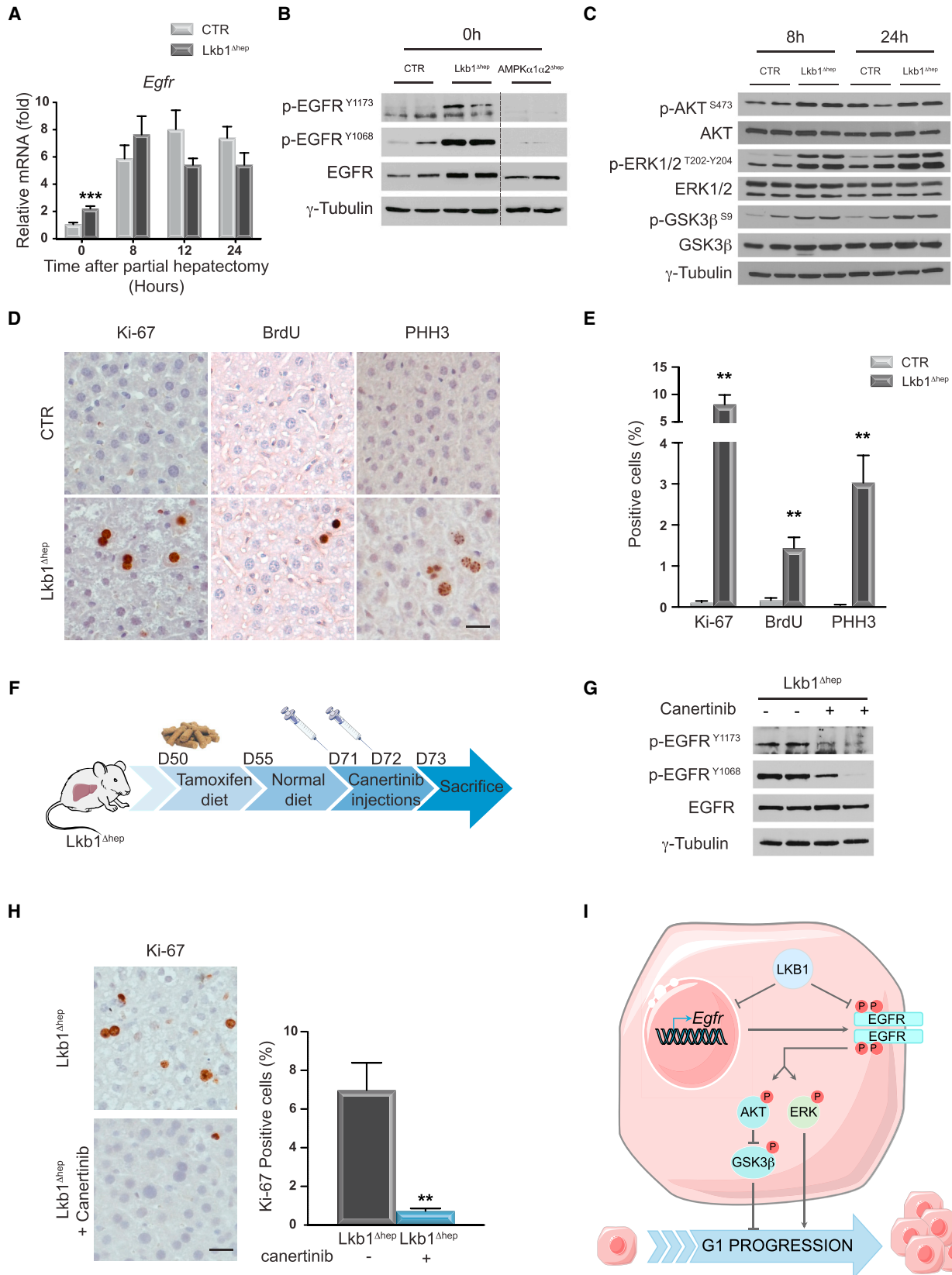


Figure 2. Hepatocyte-Specific Silencing of *Lkb1* Has a Moderate Impact on Metabolic Regulation during Liver Regeneration

(A) Hepatic triglyceride (TG) content was measured in CTR and Lkb1^{Δhep} liver tissues before and after PHx. Data represent the mean ± SEM (n ≥ 6 per group). (B) Quantitative PCR analysis of genes associated with fatty acid synthesis: acetyl-CoA carboxylase 1 (*Acc1*), sterol regulatory element-binding protein 1c (*Srebp-1c*), in CTR and Lkb1^{Δhep} livers before and after PHx. Data represent the mean ± SEM (n = 9 per group). (C) Glycogen content analysis in CTR and Lkb1^{Δhep} liver tissues assessed by periodic acid-Schiff (PAS) staining before and after PHx (scale bar: 20 μm). Pink staining indicates the presence of hepatic glycogen. (D) Quantitative PCR analysis of genes associated with gluconeogenesis: PPARγ coactivator 1α (*Pgc1α*), glucose-6-phosphatase (*G6Pase*) in CTR and Lkb1^{Δhep} livers before and after PHx. Data represent the mean ± SEM (n = 9 per group). (E) Immunoblot analysis comparing the expression of phospho-AMPKα^{T172} and AMPKα in CTR and Lkb1^{Δhep} livers before and after PHx. Lanes show samples from independent biological replicates.



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GSK3 β) displayed higher levels of phosphorylation in early G₁ phase (8 and 24 hr) in Lkb1^{Δhep} livers than in CTR livers (Figure 3C). We then investigated whether this specific activation of EGFR was sufficient to induce hepatocyte proliferation. Ki-67, BrdU, and PHH3 labeling revealed that hepatocytes already proliferate in resting Lkb1^{Δhep} liver tissues (Figures 3D and 3E). This was confirmed by analyzing *Ccna2* and *Ccnb1* mRNA levels that were already expressed in Lkb1^{Δhep} contrary to CTR livers (Figures S3B and S3C). These results indicate that the resting Lkb1^{Δhep} liver is not quiescent due at least to the activation of EGFR. Consistent with our results on liver homeostasis, we observed a decrease in hepatocyte proliferation in Lkb1^{Δhep} livers compared to CTR livers, as well as a reduced expression/activity of EGFR, at the end of regeneration (Figure S3). We can suggest that factors controlling the termination of liver regeneration disable the cross talk between LKB1 and EGFR signaling.

We next tried to elucidate how LKB1 controls EGFR expression and/or activity. YAP was a good candidate as intrinsic signal due to its key role in liver regeneration and as regulator of EGFR expression (Tao et al., 2014). YAP has been also shown to be a downstream target of LKB1, particularly in the control of liver growth (Mohseni et al., 2014). However, in our model, we observed no activation of the YAP pathway. Indeed, in resting livers and early in regeneration, the levels of unphosphorylated YAP (activated form) were similar in Lkb1^{Δhep} and CTR tissues (Figure S4A). Furthermore, we observed no upregulation of known YAP target genes *Cyr61* and *Ctgf* (Figure S4B). Concerning extrinsic signals, several ligands like EGF, Amphiregulin (AR), heparin-binding EGF (HB-EGF), Neuregulin 1 (NRG1), and transforming growth factor α (TGF- α) also activate EGFR family in the liver (Berasain and Avila, 2014). We then measured these ligands in resting Lkb1^{Δhep} liver, and interestingly a specific upregulation of *Nrg1* mRNA was highlighted (Figure S4C). These results support the idea that LKB1 regulates EGFR signaling. To further support this assumption, we assessed whether the inhibition of EGFR activity in resting Lkb1^{Δhep} livers was sufficient to impair hepatocyte proliferation. We treated Lkb1^{Δhep} mice with injections of canertinib, an irreversible tyrosine-kinase inhibitor with specific activity against EGFR (Figure 3F). The effect of this treatment on the proliferative index was analyzed 48 hr after the first injection. As expected, the level of EGFR phosphorylation (both residues: Tyr^{1068/1173}) was strongly decreased by treatment in

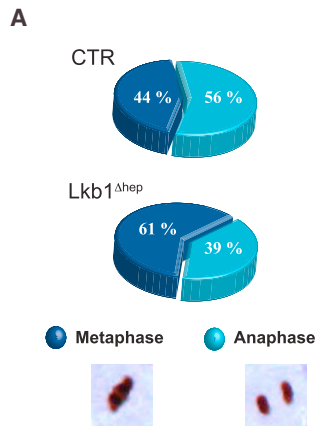
Lkb1^{Δhep} livers (Figure 3G). Importantly, the inactivation of EGFR in Lkb1^{Δhep} livers impaired hepatocyte proliferation (Figure 3H). Overall, these results demonstrate that LKB1 acts as a guardian of hepatocyte proliferation by controlling the G₀/G₁ transition through strong targeting of EGFR signaling (Figure 3I).

Lkb1 Deletion Leads to a Higher Rate of Mitotic Errors and Changes in Ploidy

As LKB1 has proved critical for the maintenance of genomic integrity (Jansen et al., 2009), we next investigated whether this master kinase controls mitotic surveillance during liver regeneration. As described above, *Lkb1* deletion resulted in early entry into G₂ phase and mitosis (Figures 1F and 1G). However, at 48 hr, both groups had similar mitotic index (Figure 1G), and intriguingly the metaphase-to-anaphase ratio was higher in Lkb1^{Δhep} than in CTR liver (Figure 4A). These findings strongly suggest that *Lkb1* deletion affects spindle assembly checkpoint (SAC) activation. The SAC is responsible for ensuring that chromosomes segregate correctly by delaying the onset of anaphase in the presence of unattached kinetochores (Rieder and Maiato, 2004). We investigated the underlying mechanism by performing a transcriptomic analysis on liver samples 48 hr after PHx. We conducted pathway profiling for genes differentially expressed between Lkb1^{Δhep} and CTR livers (Table S1 and Figure 4B). Strikingly, the first enriched gene set in Lkb1^{Δhep} was involved in metaphase control (M phase of mitotic cell cycle, Figure 4B). Importantly, gene set enrichment analysis (GSEA) confirmed the association between a metaphase gene signature and genes upregulated in the Lkb1^{Δhep} model (Figure 4C). These genes encode mitotic kinases (*Aurka/b*, *Plk1*), kinetochore (*Mad2l1*, *Bub1*, *Cenpa/b/e*), and microtubule proteins (*Kif15/22*) (Table S2). Consistently, quantitative PCR analysis revealed that these metaphase actors were strongly upregulated in Lkb1^{Δhep} livers (Figure S4A). We investigated this specific signature by studying the formation and polarization of the microtubule spindle in mitotic hepatocytes of regenerating Lkb1^{Δhep} livers. Hepatocytes have a unique polarized architecture and predominantly orient their mitotic spindle axis toward the apicolateral subdomain (Slim et al., 2014) (Figure 4G). CTR hepatocytes mainly built normal bipolar spindles (Figure 4D) with a correct orientation toward the apicolateral domain (Figures 4D and 4E). As previously reported, 10% of mitotic spindles were multipolar during CTR regenerative proliferation (Duncan et al., 2010) (Figure 4F).

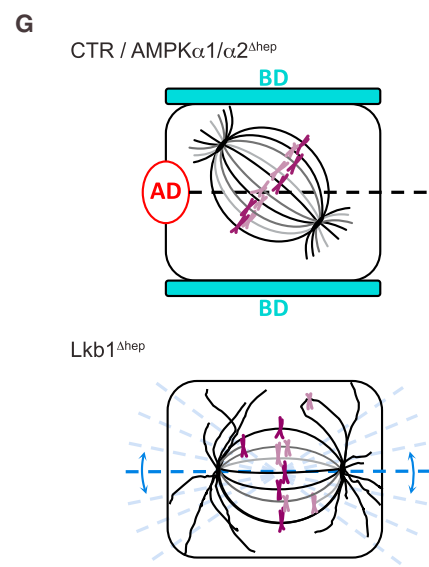
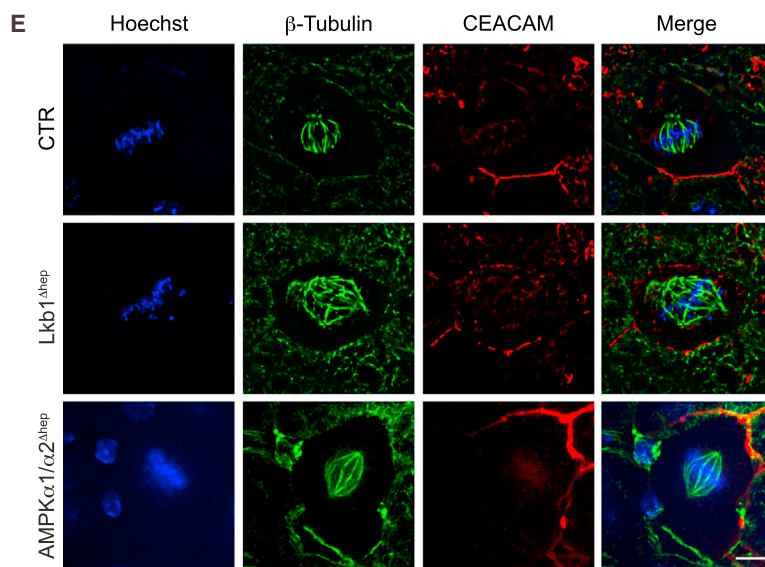
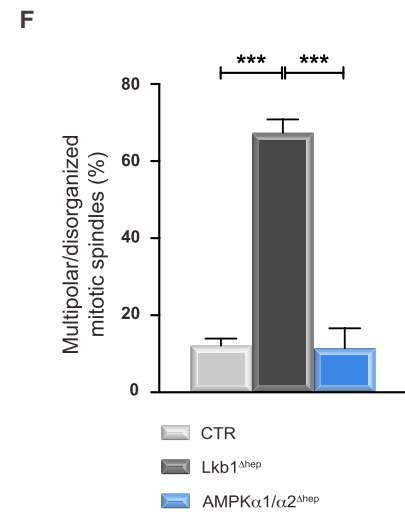
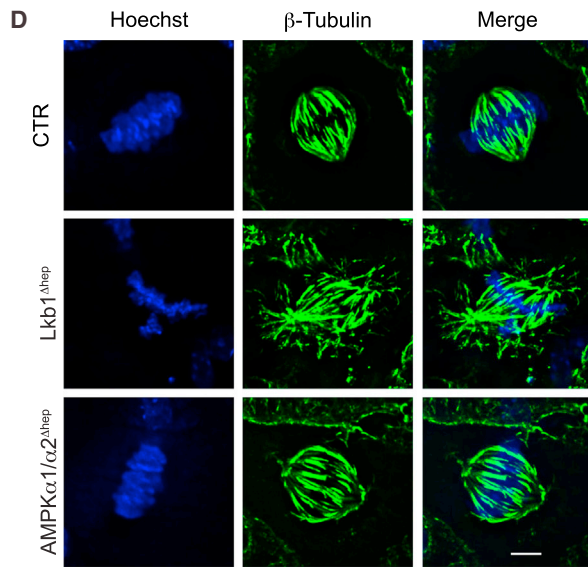
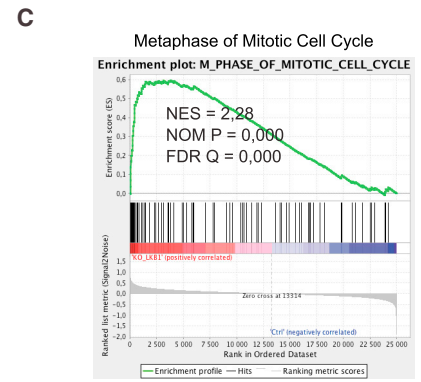
Figure 3. Hepatocyte-Specific Silencing of *Lkb1* Activates Specifically EGFR Signaling

- (A) Relative *Egfr* mRNA level measured by quantitative PCR in CTR and Lkb1^{Δhep} livers before and after PHx. Data represent the mean \pm SEM (n = 9 per group).
 (B) Immunoblot analysis comparing the expression of phospho-EGFR^{Y1173}, phospho-EGFR^{Y1068}, EGFR in CTR, AMPK α 1/ α 2^{Δhep}, and Lkb1^{Δhep} resting livers. Lanes show samples from independent biological replicates. The blot was derived from parallel samples run on a separate gel, as indicated by the black line.
 (C) Immunoblot analysis comparing the expression of phospho-AKT^{S473}, AKT, phospho-ERK1/2^{T202-Y204}, ERK1/2, phospho-GSK3 β ^{S9}, and GSK3 β in regenerating CTR and Lkb1^{Δhep} livers. Lanes show samples from independent biological replicates.
 (D) Representative Ki-67, BrdU, and PHH3 immunohistochemistry on CTR and Lkb1^{Δhep} resting liver tissues (scale bar: 20 μ m).
 (E) Quantitative analysis of Ki-67, BrdU, and PHH3 labeling. Data represent the mean \pm SEM (n \geq 6 per group).
 (F) Representation of experimental procedure for canertinib treatment.
 (G) Immunoblot analysis comparing the expression of phospho-EGFR^{Y1173}, phospho-EGFR^{Y1068}, and EGFR in Lkb1^{Δhep} livers treated or not with canertinib. Lanes show samples from independent biological replicates.
 (H) Representative Ki-67 immunohistochemistry on Lkb1^{Δhep} liver tissues treated or not with canertinib (scale bar: 20 μ m) and quantitative analysis. Data represent the mean \pm SEM (n = 4 per group).
 (I) A proposed working model for LKB1 regulating hepatocyte proliferation during liver regeneration.
 See also Figures S2–S4.



B

Enriched Gene Set	NES	p-Value
M Phase of Mitotic Cell Cycle	2.28	0.000
Spindle	2.27	0.000
Mitosis	2.26	0.000
M Phase	2.21	0.000
Chromosomepericentric Region	2.21	0.000
Mitotic Cell Cycle	2.16	0.000
Regulation of Mitosis	2.07	0.000
Kinetochores	2.04	0.000
Spindle Microtubule	2.03	0.000
Condensed Chromosome	2.02	0.002
Cell Cycle Process	2.00	0.000
Microtubule	1.98	0.000
Regulation of Cyclin Dependent Protein Kinase Activity	1.94	0.000
Chromosome Segregation	1.94	0.000
Cell Cycle Phase	1.94	0.000
Spindle Pole	1.92	0.002
Chromosome	1.89	0.002
Chromosomal Part	1.87	0.000
Sister Chromatid Segregation	1.86	0.002
Establishment of Organelle Localization	1.84	0.002



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In $Lkb1^{\Delta hep}$ livers, metaphase hepatocytes had no mitotic spindle axis, due to a loss of division polarity (Figures 4D, 4E, and 4G). Consequently, the majority of spindle microtubules appeared to be multipolar/disorganized (Figure 4F). In other cellular systems, LKB1 is essential for mitotic spindle formation and orientation through the activation of AMPK (Nakano and Takashima, 2012; Shackelford and Shaw, 2009). Thus, we evaluated the role of AMPK in division polarity during liver regeneration. First, a strong activation of AMPK was detected at 48 hr after PHx in $Lkb1^{\Delta hep}$ livers (Figure S4B). Furthermore, $AMPK\alpha1/\alpha2^{\Delta hep}$ regenerating livers exhibited no alteration of hepatocytes mitotic spindle formation and orientation (Figures 4D–4F). We therefore concluded that LKB1 controls hepatocyte metaphase spindle orientation and organization independently of AMPK activation during liver regeneration.

Previous works demonstrated that cells that cannot satisfy the SAC are delayed in mitosis (Rieder and Maiato, 2004). However, this delay is seldom permanent. In fact, cells can escape metaphase and “slip” into the next G_1 phase as mononucleate polyploid cells (mitotic slippage, Figure 5A). Alternatively, cells can bypass the SAC and progress into anaphase with a high risk of abnormal chromosome migration (e.g., lagging chromosomes, tripolar anaphase) and the genesis of an aneuploid contingent (Figure 5A). What could be the fate of $Lkb1^{\Delta hep}$ hepatocyte stuck in metaphase? To answer this point, the hepatocyte ploidy profile was characterized. Polyploidy is defined on the basis of the number of nuclei per cell (cellular ploidy) or the DNA content of each nucleus (nuclear ploidy). As already published (Miyaoka et al., 2012), cellular ploidy of CTR and $Lkb1^{\Delta hep}$ livers decreased during regeneration and shift from predominantly binucleate before PHx to predominantly mononucleate upon completed liver regeneration (Figure S4E). We next assessed nuclear ploidy. In resting $Lkb1^{\Delta hep}$ livers, we observed a trend toward a reduction in the diploid fraction and an increase in the tetraploid and octoploid fractions (Figure S4G). By contrast, at the end of regeneration, $Lkb1^{\Delta hep}$ livers were enriched in mononucleate tetraploid and octoploid hepatocytes, associated with a decrease of the diploid fraction (Figure 5B). We reinforced these data by analyzing cellular hypertrophy. *Lkb1* loss in resting livers was associated with a significant increase in hepatocyte size (Figure S4D) reflecting that $Lkb1^{\Delta hep}$ hepatocytes are already engaged in the cell cycle without a modification of nuclear ploidy (Figures 3E and S4G). At the end of liver regeneration (low proliferative index), $Lkb1^{\Delta hep}$ hepatocytes were markedly larger,

reflecting the enrichment in polyploid nuclei (Figures 5B and 5C). These results strongly suggest that mitotic slippage events occur in $Lkb1^{\Delta hep}$ regenerating livers, leading to the genesis of mononucleate polyploid contingent (Figure 5A). Finally, we looked for evidence of the SAC bypass in $Lkb1^{\Delta hep}$ livers at the end of regeneration (Figure 5A). We found that regenerating $Lkb1^{\Delta hep}$ livers have abnormal anaphase figures with the presence of trinucleate hepatocytes (Figures 5D and S4C). Consistent with these observations, comparative genomic hybridization (CGH) analysis revealed many chromosomal aberrations in $Lkb1^{\Delta hep}$ livers, with both losses and gains of chromosomal regions (Figure 5E). Overall, these findings highlight the role of LKB1 in the control of hepatocyte genomic integrity during liver regeneration.

DISCUSSION

The liver differs from other organs in its unique capacity to recover from injury by regeneration rather than scar formation. Liver regeneration has been studied in detail over the years, in the hope of developing effective clinical treatment strategies. During this process, various intracellular and extracellular signals are induced, activating quiescent hepatocytes, which then proliferate to restore the liver mass (Fausto et al., 2006; Michalopoulos, 2017). Here, we show that the master kinase LKB1 plays a dual role in liver regeneration independently of its major target, AMPK. LKB1 is a guardian of hepatocyte proliferation and acts on the G_0/G_1 transition by controlling EGFR activation, but is also a gatekeeper of mitotic fidelity and hepatocyte ploidy integrity.

The role of LKB1 in liver growth has been little investigated. It seems to depend on the type of lesion and, thus, on the proliferative signal received by hepatocytes. In the context of metabolic disorders, the activation of HGF-LKB1/AMPK signaling has been shown to promote hepatocyte proliferation and viability (Varela-Rey et al., 2009; Vázquez-Chantada et al., 2009). Conversely, Nakau et al. (2002) observed the emergence of hepatic lesions associated with high rates of proliferation in a mouse model of *Lkb1* haploinsufficiency. This model displayed a particularly high incidence of hepatocellular carcinoma (HCC) development, suggesting a tumor suppressor role for LKB1 in the mouse liver. Here, we report a strong increase of hepatocyte proliferation caused by *Lkb1* deletion during liver regeneration. In this context, $Lkb1^{\Delta hep}$ hepatocytes initiate DNA

Figure 4. LKB1 Is a Mitotic Regulator during Liver Regeneration

(A) Quantification of metaphase and anaphase events on CTR and $Lkb1^{\Delta hep}$ liver tissues sections immunostained with PHH3 48 hr after PHx (see Figure 1E). Data represent the mean \pm SEM (n = 6 per group).

(B) GSEA was performed with transcriptomic data from CTR and $Lkb1^{\Delta hep}$ liver tissues 48 hr after PHx (n = 4 per group) (GEO:GSE100605) and with BioCarta and Kyoto Encyclopedia of Genes and Genomes gene sets. All results are shown in Table S1, and only the best enrichments are shown (false-discovery rate q value < 0.001).

(C) GSEA plot for the top enriched pathway showing a significant enrichment in genes upregulated in liver from $Lkb1^{\Delta hep}$ mice, from the Kyoto Encyclopedia of Genes and Genomes list of metaphase genes (noted in the figure Metaphase-Phase of Mitotic Cell Cycle).

(D and E) Images of liver sections from $Lkb1^{\Delta hep}$, $AMPK\alpha1/\alpha2^{\Delta hep}$, and CTR mice immunostained with β -tubulin (microtubule labeling, green) (D and E), CEACAM (baso-lateral labeling, red) (E), and Hoechst (nucleus, blue) (D and E) 48 hr after PHx (scale bar: 3 μ m).

(F) Percentage of multipolar/disorganized spindles 48 hr after PHx. Results represent the mean \pm SEM (n \geq 4 per group).

(G) A proposed working model for mitotic spindle orientation related to hepatocyte polarity in $Lkb1^{\Delta hep}$, $AMPK\alpha1/\alpha2^{\Delta hep}$, and CTR mice (AD, apical domain; BD, basal domain; blue dotted line, misorientation of the mitotic spindle axis) (adapted from Lázaro-Diéguez et al. [2013]).

See also Tables S1 and S2 and Figure S4.

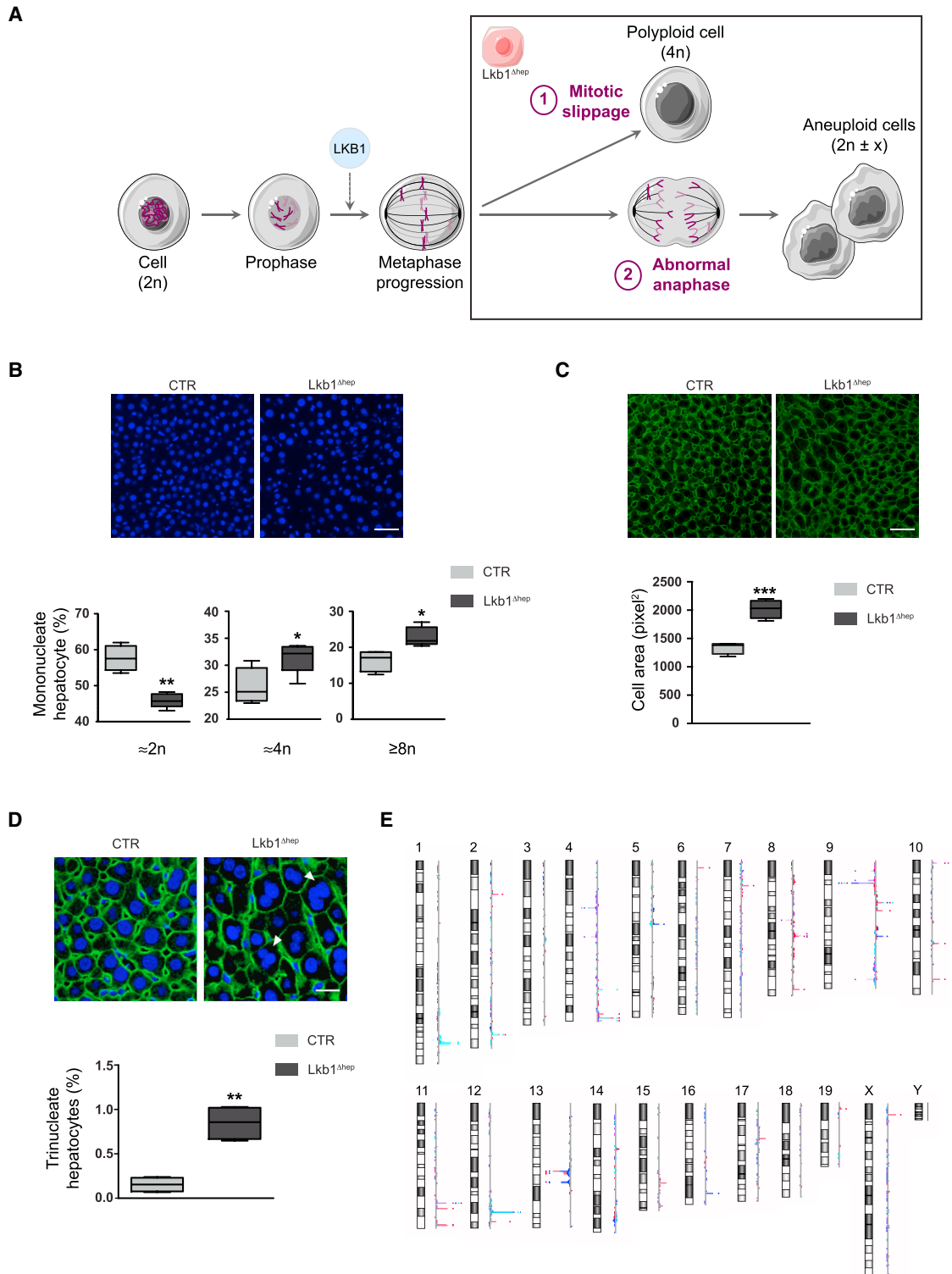


Figure 5. LKB1 Controls Hepatocyte Genomic Integrity during Liver Regeneration

(A) A working model explaining the fate of $Lkb1^{\Delta hep}$ hepatocytes arrested in metaphase, these cells could either perform (1) mitotic slippage leading to mononucleated polyploid cells genesis or (2) bypass metaphase block and progress to anaphase with lagging chromosomes, leading to aneuploid cells genesis. (B) Hoechst staining (nucleus, blue) of liver sections from $Lkb1^{\Delta hep}$ and CTR mice (scale bar: 50 μm). Boxplots of the percentage of mononucleate 2n, 4n, and $\geq 8n$ hepatocytes relative to total mononucleate hepatocytes in $Lkb1^{\Delta hep}$ and CTR mice, 13 days after PHX. The bottom, central, and top lines of each box represent the first quartile, median, and third quartile of the distribution ($n = 6$ per group).

(legend continued on next page)

replication 10 hr before CTR hepatocytes. Furthermore, the resting $Lkb1^{\Delta hep}$ liver was not quiescent, as it displayed EGFR activation. Several studies have shown EGFR to be essential for liver regeneration (Berasain and Avila, 2014; Komposch and Sibilia, 2016). Due to this “pre-primed” state, $Lkb1^{\Delta hep}$ livers respond more rapidly to stimuli induced by PHx, compared to CTR livers. We found that preventing EGFR activation in the resting $Lkb1^{\Delta hep}$ liver was sufficient to restore quiescence. Further studies are required to elucidate the cross talk between LKB1 and EGFR. Our results suggest that at least NRG1 can activate EGFR signaling. NRG1 is not a canonical EGFR ligand for liver regeneration (Berasain and Avila, 2014). However, NRG1 binds ErbB3 receptor (EGFR family), which is expressed in the adult liver and relies on EGFR activity (Michalopoulos and Khan, 2005).

Our findings also show that LKB1 preserves hepatocyte genomic integrity during liver regeneration. *Lkb1* loss from proliferating hepatocytes activates the SAC during mitosis. Unattached or untensioned kinetochores trigger the SAC, leading to cell cycle arrest at metaphase, thereby preventing the missegregation of chromosomes. We found that $Lkb1^{\Delta hep}$ hepatocytes had abnormal mitotic spindle orientation and organization during metaphase. These results suggest that microtubule disorganization induces untensioned kinetochores in $Lkb1^{\Delta hep}$ hepatocytes, leading to SAC activation. In various systems, LKB1/AMPK signaling is detrimental to cell polarity and cell division, notably through effects on the control of spindle orientation (Brennan, 2007; Jansen et al., 2009; Nakano and Takashima, 2012). In kidney epithelial cells, Wei et al. (2012) showed that *Lkb1* mutations cause a marked mislocalization of activated AMPK on mitotic spindles. We found that AMPK depletion in liver parenchyma had no impact on mitotic spindle formation and orientation. Thus, LKB1 controls hepatocyte division polarity independently of AMPK. Which key targets of LKB1 ensure correct hepatocyte cell division? Hepatocytes adopt a specific polarity during mitosis and orient their mitotic spindle poles toward an area near the apical plasma membrane domain (Slim et al., 2014). Spindle orientation depends on the local positioning of two cortical cues that capture the two sets of astral microtubules at opposite lateral cell membranes equidistant from the basal domain (Slim et al., 2014). MARK (microtubule affinity-regulating kinase, belonging to ARKs) family members are known to regulate cellular polarity and this function is conserved (Jansen et al., 2009; Nakano and Takashima, 2012). These kinases include Par1b, also called MARK2, which could be seen as a potential candidate here. In kidney and hepatocyte cell lines, Par1b controls mitotic spindle orientation by acting on the attachment of astral microtu-

bules to the cortex (Lázaro-Diéguez et al., 2013; Slim et al., 2013). Further studies will be required to determine how LKB1 controls the activities of MARK2 and/or other MARK isoforms during liver regeneration.

Finally, our work shows that LKB1 controls hepatocyte ploidy during liver regeneration. In the adult liver, normal hepatocyte ploidy ranges from 2n to 8n (Duncan, 2013; Gentric and Desdouets, 2014). Following acute liver injury, polyploid hepatocytes re-enter the cell cycle and contribute to recovery (Melchiorri et al., 1993; Miyaoka et al., 2012). Under pathological conditions, hepatocyte polyploidization is strongly correlated with damage severity: the more severe injury, the higher the rate of polyploidization (Gentric and Desdouets, 2014; Wang et al., 2017). Genome integrity is protected in normal tissue by various means, including cell cycle checkpoints and DNA repairs mechanisms. Our study highlights that LKB1 acts as a key actor of genomic stability in liver parenchyma. In light of these findings, future studies should aim to assess the role of this master kinase in liver tumorigenesis related to chromosomal instability.

EXPERIMENTAL PROCEDURES

Animals

Mice carrying two floxed alleles of the *Lkb1* gene ($Lkb1^{lox/lox}$) (Bardeesy et al., 2002) were crossed with TTR-Cre-ER^{T2} mice expressing a tamoxifen-inducible Cre recombinase under control of the hepatocyte-specific transthyretin promoter (Tannour-Louet et al., 2002) to generate $Lkb1^{\Delta hep}$ ($Lkb1^{lox/lox}$, TTR-Cre-ER^{T2}) and control mice (CTR: $Lkb1^{lox/lox}$). All mice were of a C57Bl6J/FVB mixed background. To induce specific hepatocytes *Lkb1* deletion, 8-week-old male ($Lkb1^{\Delta hep}$, CTR) mice were fed with tamoxifen diet (1,000 mg/kg TAM A115-T7100, Ssniff) during 5 days (Figure 1A). Liver-specific AMPK knockout mice (AMPK $\alpha1/\alpha2^{\Delta hep}$) were generated by crossing Alfp-Cre mice with mice containing floxed AMPK $\alpha1/\alpha2$ subunits in a C57BL/6 background (Guigas et al., 2006). $Lkb1^{\Delta hep}$, AMPK $\alpha1/\alpha2^{\Delta hep}$, and CTR mice received care in compliance with institutional guidelines regulated by “Direction Départementale des Services Vétérinaires de Paris,” France (authorization number 75956). Mice were kept under a controlled humidity and lighting schedule with a 12-hr dark period with free access to food and water.

Statistical Analysis

Statistical significance was determined with a two-tailed Student's t test with Welch correction using GraphPad Software. All data are representative of 4–10 animals of each genotype and expressed as mean \pm SEM. A p value of less than 0.05 was considered statistically significant and represented in figures as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

DATA AND SOFTWARE AVAILABILITY

The accession number for the transcriptomic data reported in this paper is GEO: GSE1100605.

(C) β -Catenin staining (plasma membrane labeling, green) of liver sections from $Lkb1^{\Delta hep}$ and CTR mice (scale bar: 50 μ m). Boxplots of cell area in $Lkb1^{\Delta hep}$ and CTR mice, 13 days after PHx. The bottom, central, and top lines of each box represent the first quartile, median, and third quartile of the distribution (n = 6 per group).

(D) Immunostaining of β -catenin and Hoechst on liver sections from $Lkb1^{\Delta hep}$ and CTR mice (scale bar: 20 μ m). Arrows point trinucleate hepatocytes. Boxplots of trinucleate hepatocytes percentage in $Lkb1^{\Delta hep}$ and CTR mice, 13 days after PHx. The bottom, central, and top lines of each box represent the first quartile, median, and third quartile of the distribution (n = 6 per group).

(E) Alterations in chromosome copy number were assessed on $Lkb1^{\Delta hep}$ and CTR livers by CGH analysis. Each colored line represents genomic profile of one distinct $Lkb1^{\Delta hep}$ mouse. The log ratio decreases for chromosome loss (left shift); increases for chromosome gain (right shift).

See also Figure S4.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.01.086>.

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AUTHOR CONTRIBUTIONS

V.M. designed and performed experiments and interpreted data. N.B. and J.L. performed experiments. V.F. performed partial liver hepatectomy experiments. J.-P.C. and S.C.-M. performed microarray analysis and interpreted data. M.F. and B.V. generated AMPK α 1/2^{Δhep} mice and interpreted data. C.P. provided Lkb1^{Δhep} mice and interpreted data. C.D. conceived and planned the experiments and interpreted data. V.M. and C.D. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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