

Hepatic NF- κ B-inducing kinase (NIK) suppresses mouse liver regeneration in acute and chronic liver diseases

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Abstract Reparative hepatocyte replication is impaired in chronic liver disease, contributing to disease progression; however, the underlying mechanism remains elusive. Here, we identify Map3k14 (also known as NIK) and its substrate Chuk (also called IKK α) as unrecognized suppressors of hepatocyte replication. Chronic liver disease is associated with aberrant activation of hepatic NIK pathways. We found that hepatocyte-specific deletion of *Map3k14* or *Chuk* substantially accelerated mouse hepatocyte proliferation and liver regeneration following partial-hepatectomy. Hepatotoxin treatment or high fat diet feeding inhibited the ability of partial-hepatectomy to stimulate hepatocyte replication; remarkably, inactivation of hepatic NIK markedly increased reparative hepatocyte proliferation under these liver disease conditions. Mechanistically, NIK and IKK α suppressed the mitogenic JAK2/STAT3 pathway, thereby inhibiting cell cycle progression. Our data suggest that hepatic NIK and IKK α act as rheostats for liver regeneration by restraining overgrowth. Pathological activation of hepatic NIK or IKK α likely blocks hepatocyte replication, contributing to liver disease progression.

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Introduction

The liver is an essential metabolic organ that experiences metabolic stress during fasting, refeeding, and overnutrition states (Rui, 2014). The liver is also responsible for detoxifications of endogenous and exogenous toxic substances, thus being frequently exposed to harmful insults. Dietary hepatotoxins and gut microbiota-derived toxic substances are transported to the liver through the entero-hepatic circulation, further increasing risk for liver injury. To compensate for hepatocyte loss, the liver evolves a powerful regenerative ability to maintain its homeostasis (Michalopoulos, 2017). After 70% of partial hepatectomy (PHx), rodents are able to regain normal liver mass within a week via reparative hepatocyte replications (Miyaoka et al., 2012). Nevertheless, hepatocyte proliferation is severely inhibited in chronic liver diseases, including nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease, and chronic exposures to hepatotoxins (Richardson et al., 2007; Inaba et al., 2015; Sancho-Bru et al., 2012; Michalopoulos, 2013). Impairment in hepatocyte replications considerably precipitates liver disease progression; however, the underlying mechanism responsible for defective hepatocyte replications remains poorly understood.

In response to liver injury induced by PHx, numerous growth factors and cytokines are secreted and delivered to hepatocytes where they stimulate hepatocyte proliferation by activating multiple mitogenic pathways, including the Janus kinase 2 (JAK2)/STAT3, MAPK, PI 3-kinase, and NF- κ B pathways (Michalopoulos, 2017). In contrast, TGF β 1 and interferon- γ inhibit hepatocyte proliferation, thereby preventing liver from overgrowth (Michalopoulos, 2013; Sato et al., 1993; Wu et al., 2015). Liver regeneration is fine-tuned by a balance between positive and negative regulators. We postulated that in chronic liver disease, the negative branch might be predominant and overcome the positive branch, leading to pathological suppression of hepatocyte proliferation and liver regeneration. However, intracellular pathways conferring hepatocyte proliferation inhibition remain elusive. In search for inhibitory pathways, we identified Map3k14, also called NF- κ B-inducing kinase (NIK), and its substrate Chuck, also referred to as I κ B kinase α (IKK α).

NIK is a Ser/Thr kinase known to activate the noncanonical NF- κ B2 pathway (Sun, 2012). It phosphorylates and activates IKK α (Xiao et al., 2001). IKK α in turn phosphorylates the precursor of NF- κ B2 p100, resulting in generation of the p52 form of NF- κ B2 (Sun, 2012; Xiao et al., 2001). Mature p52 is translocated into the nucleus to activate target genes. We previously reported that metabolic stress, oxidative stress, hepatotoxins, and cytokines stimulate hepatic NIK (Sheng et al., 2012; Jiang et al., 2015). Importantly, hepatic NIK is aberrantly activated in both mice and humans with NAFLD or alcoholic liver disease (Sheng et al., 2012; Shen et al., 2014). Hepatocellular stress and liver inflammation, which are associated with chronic liver disease, likely activate hepatic NIK. These observations prompted us to test the hypothesis that hepatic NIK/IKK α pathways cell-autonomously inhibit hepatocyte proliferation. In this work, we characterized hepatocyte-specific NIK (NIK^{Δhep}) and IKK α (IKK α ^{Δhep}) knockout mice, and examined reparative hepatocyte replications using PHx models. We found that the NIK/IKK α pathway suppresses reparative hepatocyte proliferation at least in part by inhibiting the JAK2/STAT3 pathway. This work unveils unrecognized crosstalk between the NIK/IKK α and the JAK2/STAT3 pathways involved in regulating liver regeneration.

Results

Hepatocyte-specific ablation of NIK accelerates liver regeneration

To assess the role of hepatic NIK in reparative hepatocyte proliferation, we performed 70% of PHx on mice at 8 weeks of age following the established protocols (Mitchell and Willenbring, 2008). NIK^{Δhep} mice were generated by crossing *Map3k14*^{flox/flox} (referred to as NIK^{f/f}) mice with *Albumin-Cre* drivers (Shen et al., 2017). Proliferating cells were detected by immunostaining liver sections with antibody against Ki67, a cell proliferation marker (Figure 1A). Baseline hepatocyte proliferation rates were low and comparable between NIK^{Δhep} and NIK^{f/f} mice (Figure 1B). Number of liver proliferating Ki67⁺ cells markedly increased within 48 hr following PHx, and Ki67⁺ cells were 85% higher in NIK^{Δhep} relative to NIK^{f/f} mice (Figure 1B). In line with these observations, the number of liver BrdU-labelled proliferating cells was also substantially higher in NIK^{Δhep} than in NIK^{f/f} mice (Figure 1C). Liver cell proliferation rates declined in both NIK^{Δhep} and NIK^{f/f} mice after 48 hr post-PHx, and became comparable between these two groups at 96 hr post-PHx (Figure 1B).

To verify hepatocytes proliferating, we costained liver sections with anti-Ki67 and anti-HNF4 α (a hepatocyte marker) antibodies, or with anti-Ki67 and anti-F4/80 (a Kupffer cell/macrophage marker) antibodies. HNF4 α ⁺ hepatocytes accounted for 96% of Ki67⁺ proliferating cells in NIK^{Δhep} mice at 48 hr post-PHx (Figure 1D,F) while F4/80⁺ Kupffer cells/macrophages for <4% of Ki67⁺ cells (Figure 1E,F). These data indicate that hepatic NIK is an intrinsic suppressor of hepatocyte proliferation.

We also examined the effect of NIK deficiency on hepatocyte death using TUNEL assays. The number of liver TUNEL⁺ apoptotic cells was slightly lower in NIK^{Δhep} relative to NIK^{f/f} mice, but the difference was not statistically significant (Figure 1G). Plasma alanine aminotransferase (ALT) activity, a liver injury index, was comparable between NIK^{Δhep} and NIK^{f/f} mice either under basal conditions or after PHx (Figure 1H). Thus, accelerated hepatocyte proliferation cannot be explained by changes in liver injury in NIK^{Δhep} mice.

To further confirm the role of hepatic NIK in liver regeneration, we assessed liver to body weight ratios at 2 and 4 days post-PHx. Consistently, liver/body weight ratios were significantly higher in NIK^{Δhep} than in NIK^{f/f} mice at 4 days following PHx (Figure 1I). Of note, liver/body weight ratios

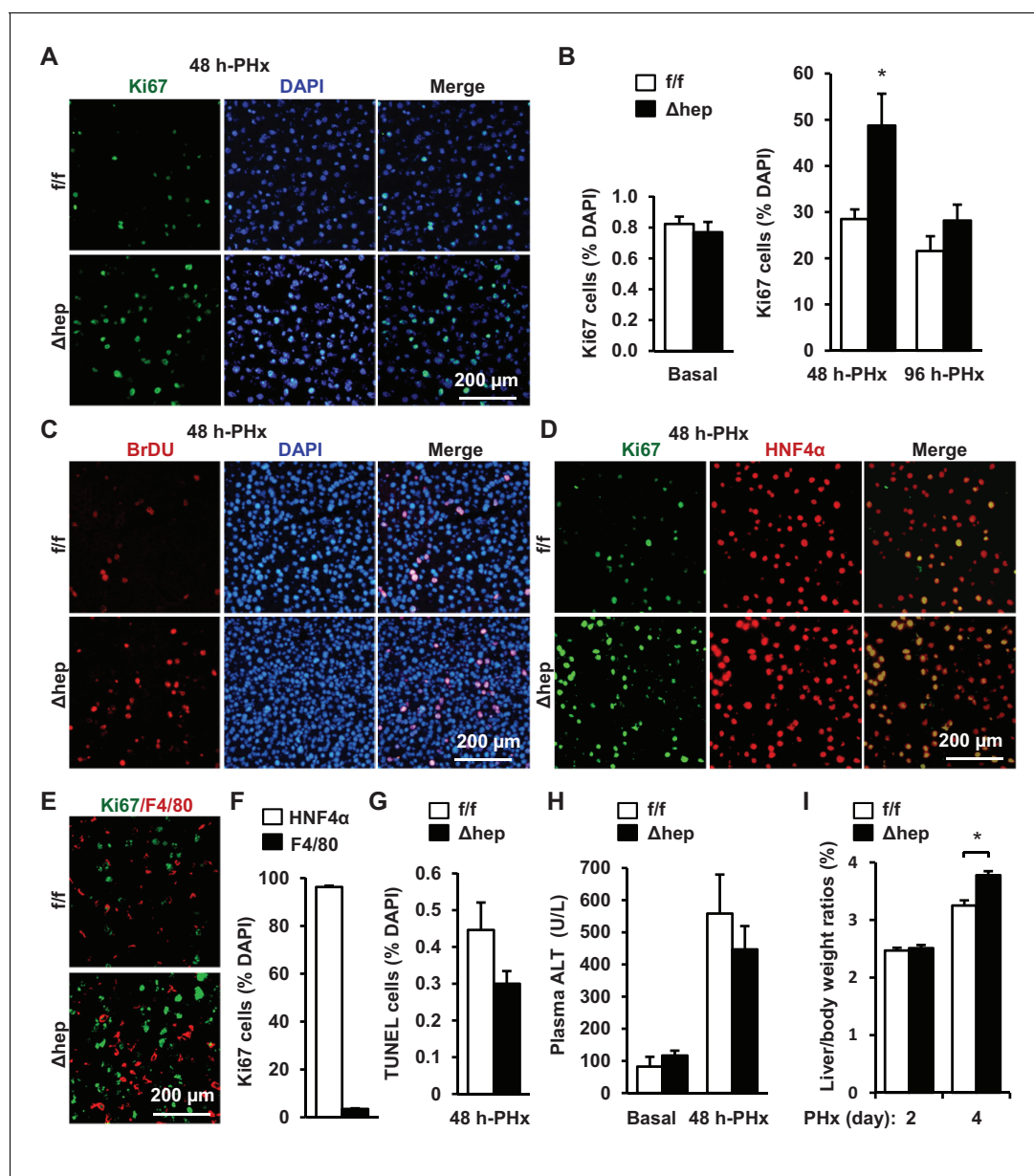


Figure 1. Hepatocyte-specific ablation of NIK accelerates reparative hepatocyte proliferation. $NIK^{f/f}$ ($n = 7$) and $NIK^{\Delta hep}$ ($n = 7$) male mice (8 weeks) were subjected to PHx, and livers were harvested 48 hr or 96 hr later. (A) Representative immunostaining of liver sections (48 hr after PHx) with anti-Ki67. (B) $Ki67^+$ cells were counted and normalized to total DAPI⁺ cells. (C) Representative immunostaining of liver sections (48 hr after PHx) with anti-BrdU antibodies. (D–E) Representative images of liver sections (48 hr after PHx) costained with anti-Ki67 and anti-HNF4 α antibodies (D) or anti-Ki67 and anti-F4/80 antibodies (E). (F) $Ki67^+HNF4\alpha^+$ and $Ki67^+F4/80^+$ cells were counted and normalized to total $Ki67^+$ cells. (G) Liver cell death were assessed 48 hr after PHx using TUNEL reagents. (H) Plasma ALT levels. (I) Liver to body weight ratios ($n = 8$ per group). Data were statistically analyzed with two-tailed Student's t test, and presented as mean \pm SEM. * $p < 0.05$.

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The following source data is available for figure 1:

Source data 1. Hepatic NIK deficiency accelerates liver regeneration.

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Source data 2. PHx increases hepatocyte replications.

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were similar between these two groups at 2 days post-PHx. One possible explanation is that a 2 day period may be too short for newly-generated hepatocytes to grow in size large enough to increase liver weight.

To determine whether NIK inhibits hepatocyte cell cycle progression, we measured the levels of cyclin D1, which is believed to drive hepatocyte proliferation following PHx (Michalopoulos, 2013). Hepatic cyclin D1 levels were undetectable in both NIK^{Δhep} and NIK^{f/f} mice under basal conditions, and were markedly increased by PHx (Figure 2A). Importantly, hepatic cyclin D1 levels were significantly higher in NIK^{Δhep} than in NIK^{f/f} mice (Figure 2A,B). Collectively, these results support the notion that hepatic NIK may act as an intrinsic rheostat for liver homeostasis by restraining liver overgrowth.

The role of NF-κB1, MAPK, and PI 3-kinase pathways in NIK-induced suppression of hepatocyte proliferation

We next sought to interrogate the molecular mechanism of the NIK action. Expression of liver NIK rapidly increased within 12 hr following PHx, but declined at 3 days post-PHx (Figure 2—figure supplement 1A). Consistently, PHx also increased phosphorylation of liver IKKα/β (Figure 2—figure supplement 1B,C). Interestingly, liver IKKα expression was also increased by PHx (Figure 2—figure supplement 1C). The NF-κB1, MAPK, and PI 3-kinase pathways are known to be involved in mediating PHx-stimulated liver regeneration (Michalopoulos, 2013; Wuestefeld et al., 2013; Pauta et al., 2016). Unexpectedly, phosphorylation of hepatic IκBα, p65 (the NF-κB1 pathway), Akt (pSer473) (the PI 3-kinase pathway), ERK1/2, and JNK (the MAPK pathway) was comparable between NIK^{Δhep} and NIK^{f/f} mice at 4 hr post-PHx (Figure 2C). We also did not detect difference in hepatic levels of reactive oxygen species (ROS) or hepatic expression of cytokines between NIK^{Δhep} and NIK^{f/f} mice (Figure 2D,E). Therefore, NIK suppression of liver regeneration cannot be explained by the above pathways.

NIK suppresses the JAK2/STAT3 pathway

JAK2 is known to phosphorylate and activate STAT3, which is believed to drive hepatocyte proliferation (Wang et al., 2011; Shi et al., 2017). We postulated that NIK might suppress hepatocyte proliferation by inhibiting the JAK2/STAT3 pathway. Liver extracts were prepared at 4 hr post-PHx and immunoblotted with anti-phospho-JAK2 (pTyr1007/1008) or anti-phospho-STAT3 (pTyr705) antibodies. Phosphorylation of both JAK2 and STAT3 was significantly higher in NIK^{Δhep} mice than in NIK^{f/f} littermates (Figure 3A). Baseline levels of JAK2 and STAT3 phosphorylation in the resected livers were similar between NIK^{Δhep} and NIK^{f/f} mice (Figure 2—figure supplement 1D).

To confirm that NIK directly inhibits the JAK2/STAT3 pathway, we transiently coexpressed JAK2 and STAT3 with NIK in HEK293 cells. In line with our previous reports (Rui and Carter-Su, 1999), overexpressed JAK2 robustly autophosphorylated as well as phosphorylated STAT3 (Figure 3B). Strikingly, overexpression of NIK dramatically decreased tyrosine phosphorylation of both JAK2 and STAT3 (Figure 3B). Consistently, NIK was coimmunoprecipitated with JAK2 (Figure 3C). These data indicate that NIK binds to JAK2 and inhibits JAK2 activity, thereby suppressing the JAK2/STAT3 pathway.

Interleukin 6 (IL6) stimulates the JAK2/STAT3 pathway, which is required for reparative hepatocyte proliferation (Riehle et al., 2008; Cressman et al., 1996). These observations prompted us to test if NIK negatively regulates the IL6/JAK2/STAT3 pathway. Mouse primary hepatocytes were transduced with NIK or β-galactosidase (β-gal) adenoviral vectors, followed by IL6 stimulation. IL6 robustly stimulated phosphorylation of STAT3 in β-gal-transduced, but not NIK-transduced, hepatocytes (Figure 3D). Collectively, these results unveil unrecognized crosstalk between NIK pathways and the JAK2/STAT3 pathway.

Hepatic IKKα suppresses liver regeneration following PHx

Given that IKKα acts downstream of NIK in the noncanonical NF-κB2 pathway, we reasoned that hepatic IKKα might also suppress liver regeneration. IKKα^{Δhep} mice were generated by crossing *Chuk*^{flox/flox} (referred to as IKKα^{f/f}) mice with *albumin-Cre* drivers (Liu et al., 2008). We confirmed that IKKα expression was disrupted specifically in the liver but not brain, heart, kidney, skeletal muscle, and spleen in IKKα^{Δhep} mice (Figure 4A). We performed PHx on IKKα^{f/f} and IKKα^{Δhep} male mice

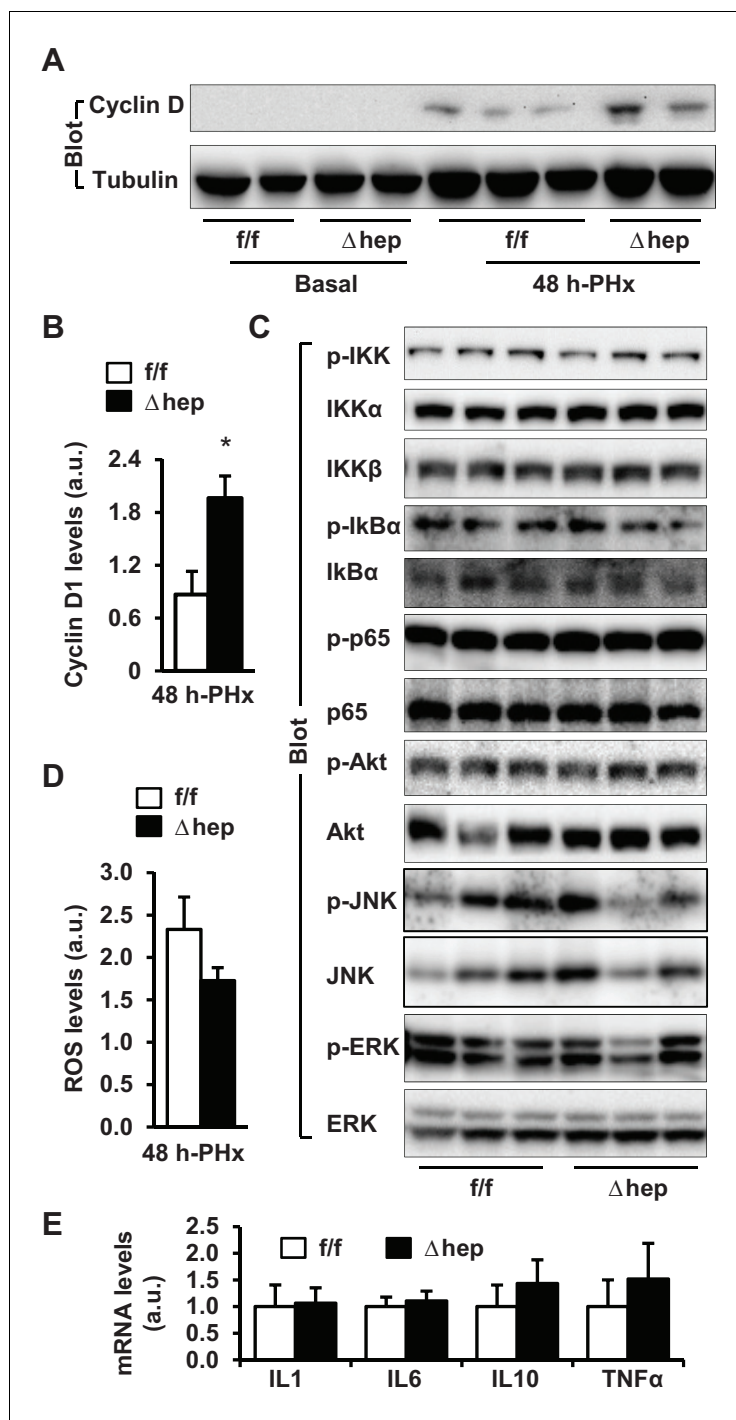


Figure 2. Hepatic NIK deficiency upregulates cyclin D1 without altering NF- κ B1, Akt, and MAPK pathways in the liver. NIK^{f/f} and NIK^{Δhep} male mice (8 weeks) were subjected to PHx. (A–B) Liver extracts were immunoblotted with anti-cyclin D1 antibody (48 hr after PHx). Cyclin D1 levels were quantified and normalized to α -tubulin levels (NIK^{f/f}: n = 4, NIK^{Δhep}: n = 4). (C) Liver extracts were immunoblotted with the indicated antibodies (4 hr after PHx). (D) Liver ROS levels 48 hr after PHx (normalized to liver weight). NIK^{f/f}: n = 5, NIK^{Δhep}: n = 6. (E) Liver cytokine expression was measured by qPCR and normalized to 36B4 expression (48 hr after PHx). NIK^{f/f}: n = 5, NIK^{Δhep}: n = 5. Data were statistically analyzed with two-tailed Student's t test, and presented as mean \pm SEM. *p < 0.05. DOI: <https://doi.org/10.7554/eLife.34152.005>

The following source data and figure supplements are available for figure 2:

Source data 1. Hepatic NIK regulates hepatocyte cell cycle progression.

Figure 2 continued on next page

Figure 2 continued

DOI: <https://doi.org/10.7554/eLife.34152.008>**Figure supplement 1.** Effect of PHx on liver NIK pathway activation.DOI: <https://doi.org/10.7554/eLife.34152.006>**Figure supplement 1—source data 1.** PHx stimulates hepatic NIK expression.DOI: <https://doi.org/10.7554/eLife.34152.007>

at 8–9 weeks of age. The number of liver proliferating Ki67⁺ cells was significantly higher in IKK $\alpha^{\Delta\text{hep}}$ than in IKK α^{ff} littermates at both 1 and 2 days post-PHx, and became similar between these two groups after 3 days following PHx (**Figure 4B**). HNF4 α^+ hepatocytes accounted for the majority of proliferating cells (**Figure 4C**). Consistently, liver cyclin D1 levels were significantly higher in IKK $\alpha^{\Delta\text{hep}}$ than in IKK α^{ff} mice (**Figure 4D**), while liver cell death was comparable between these two groups (**Figure 4E**). Consequently, liver to body weight ratios were significantly higher in IKK $\alpha^{\Delta\text{hep}}$ relative to IKK α^{ff} mice at both 5 and 7 days post-PHx (**Figure 4F**). Notably, liver/body weight ratios were comparable between these two groups within 3 days following PHx, likely due to lack of sufficient time for hepatocytes to grow their mass as discussed before. These results indicate that deficiency

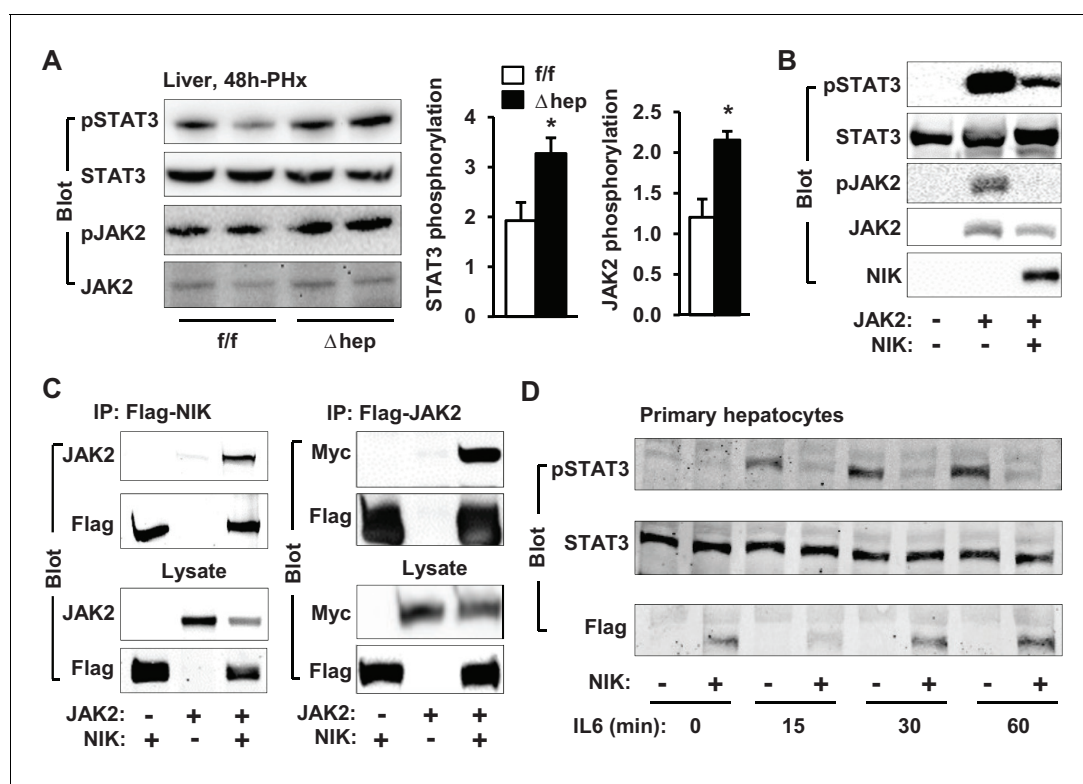


Figure 3. NIK inhibits the JAK2/STAT3 pathway. (A) Liver extracts were prepared from NIK^{f/f} and NIK ^{Δhep} males 4 hr after PHx and immunoblotted with anti-phospho-JAK2 and anti-phospho-STAT3 antibodies. Phosphorylation of JAK2 (pTyr1007/1008) and STAT3 (pTyr705) was normalized to total JAK2 and STAT3 levels, respectively. (B) STAT3 and JAK2 were coexpressed with or without NIK in HEK293 cells. Cell extracts were immunoblotted with the indicated antibodies. (C) NIK was coexpressed with JAK2 in HEK293 cells. Cell extracts were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. (D) Mouse primary hepatocytes were transduced with NIK or β -gal adenoviral vectors and stimulated with IL6 (10 ng/ml). Cell extracts were immunoblotted with the indicated antibodies. Data were statistically analyzed with two-tailed Student's t test, and presented as mean \pm SEM. * $p < 0.05$.

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The following source data is available for figure 3:

Source data 1. NIK inhibits the JAK2/STAT3 pathway.DOI: <https://doi.org/10.7554/eLife.34152.010>

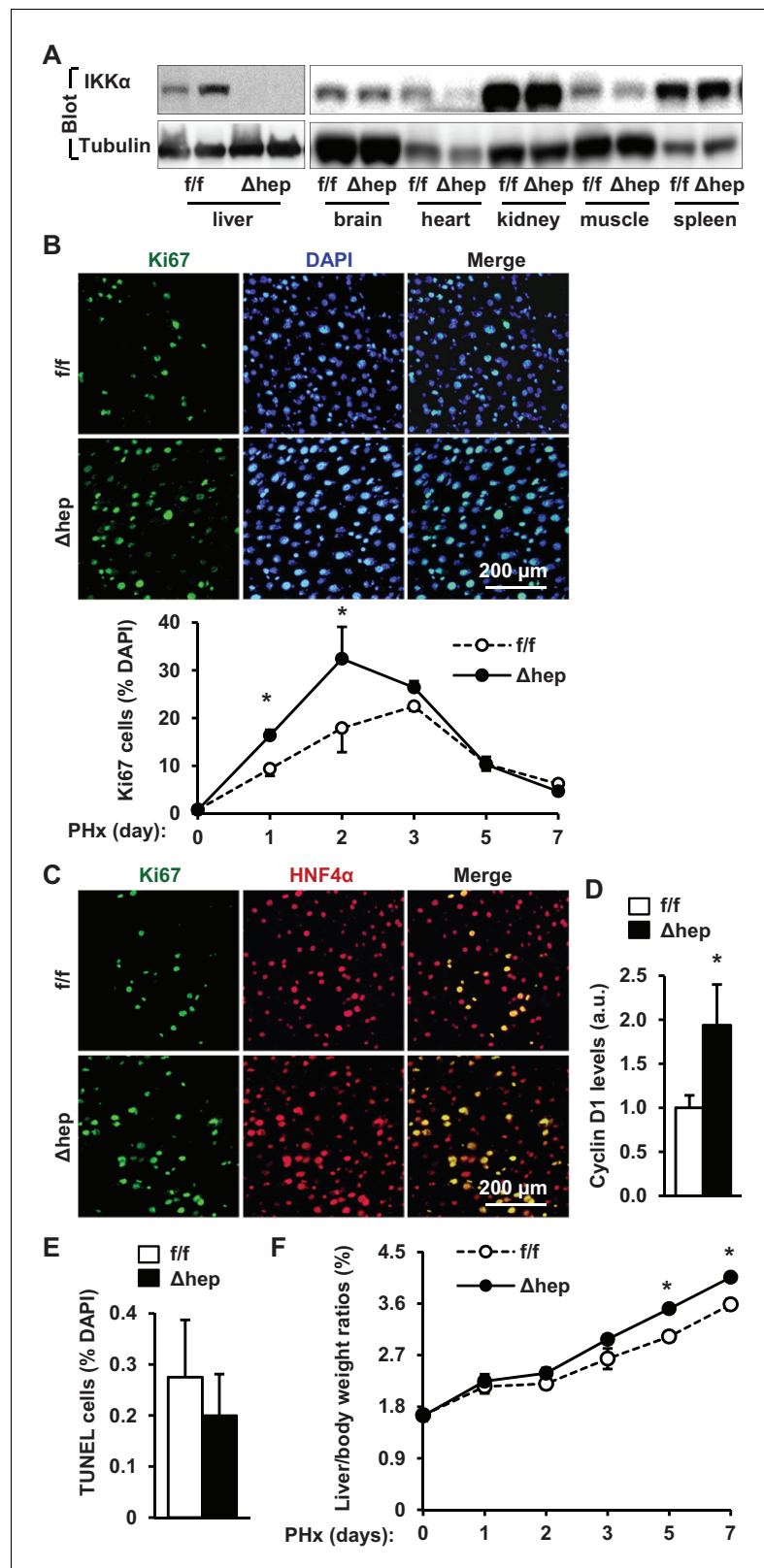


Figure 4. Ablation of hepatocyte IKK α accelerates hepatocyte reparative proliferation. (A) Tissue extracts were immunoblotted with anti- $\text{IKK}\alpha$ or anti- α -tubulin antibodies. (B–F) $\text{IKK}\alpha^{f/f}$ ($n = 6$) and $\text{IKK}\alpha^{\Delta\text{hep}}$ ($n = 6$) male littermates were subjected to PHx, and livers were harvested 48 hr later. (B) Liver sections were immunostained with anti-Ki67 antibody, and Ki67 $^{+}$ cells were counted and normalized to total DAPI $^{+}$ cells. Day 0 and 1: $n = 4$ per

Figure 4 continued on next page

Figure 4 continued

group; day 3: IKK $\alpha^{f/f}$: n = 6, IKK $\alpha^{\Delta hep}$: n = 8; day 5: IKK $\alpha^{f/f}$: n = 9, IKK $\alpha^{\Delta hep}$: n = 8; day 7: IKK $\alpha^{f/f}$: n = 6, IKK $\alpha^{\Delta hep}$: n = 5. (C) Representative images of liver sections costained with anti-Ki67 and anti-HNF4 α antibodies. (D) Liver cyclin D1 was measured by immunoblotting (normalized to α -tubulin levels). (E) TUNEL-positive cells in liver sections. (F) Liver to body weight ratios. Day 0 and 1: n = 4 per group; day 3: IKK $\alpha^{f/f}$: n = 6, IKK $\alpha^{\Delta hep}$: n = 8; day 5: IKK $\alpha^{f/f}$: n = 9, IKK $\alpha^{\Delta hep}$: n = 8; day 7: IKK $\alpha^{f/f}$: n = 6, IKK $\alpha^{\Delta hep}$: n = 5. Data were statistically analyzed with two-tailed Student's t test, and presented as mean \pm SEM. *p<0.05.

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The following source data is available for figure 4:

Source data 1. Hepatic IKK α regulates liver regeneration.

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of hepatocyte IKK α , like NIK, also accelerates hepatocyte proliferation and liver regeneration in response to acute liver injury.

To gain insight into the molecular mechanism of the IKK α action, we examined the JAK2/STAT3 pathway. The levels of phosphorylation of JAK2 as well as STAT3 were significantly higher in IKK $\alpha^{\Delta hep}$ than in IKK $\alpha^{f/f}$ mice at 4 hr post-PHx (**Figure 5A,B**). We also compared phosphorylation time courses during days 0–7 following PHx. IKK α phosphorylation increased while JAK2 phosphorylation decreasing during days 1–5 (**Figure 5—figure supplement 1A,B**). This inverse relationship further supports the notion that the NIK/IKK α pathway inhibits the JAK2/STAT3 pathway. Ablation of hepatocyte IKK α increased phosphorylation of JAK2 and STAT3 during days 1–7 following PHx (**Figure 5—figure supplement 1B**). To confirm that IKK α cell-autonomously inhibits the JAK2/STAT3 pathway, IKK α was transiently coexpressed with JAK2 in HEK293 cells. IKK α was coimmunoprecipitated with JAK2 (**Figure 5C**), and markedly decreased JAK2 autophosphorylation and the ability of JAK2 to phosphorylate STAT3 (**Figure 5D**).

To determine whether NIK suppresses the JAK2/STAT3 pathway via IKK α , we transduced primary hepatocytes from IKK $\alpha^{\Delta hep}$ (IKK α -deficient) and IKK $\alpha^{f/f}$ (wild type) mice with NIK or green fluorescent protein (GFP) adenoviral vectors, followed by IL6 stimulation. The ability of NIK to inhibit IL6-stimulated phosphorylation of STAT3 was significantly reduced in IKK α -deficient hepatocytes compared to wild type hepatocytes (**Figure 5—figure supplement 1C,D**). Of note, NIK overexpression still considerably attenuated STAT3 phosphorylation in IL6-stimulated IKK α -deficient hepatocytes, compared with GFP overexpression (**Figure 5—figure supplement 1D**). These findings suggest that hepatic NIK suppresses the JAK2/STAT3 pathway, and possibly liver regeneration, by both IKK α -dependent and IKK α -independent mechanisms.

Deficiency of hepatic NIK accelerates liver regeneration in mice with hepatotoxin-induced liver injury

Hepatic NIK is highly activated in mice and humans with chronic liver disease (**Sheng et al., 2012; Shen et al., 2014**), raising the possibility that hepatic NIK might impair liver regeneration in these disease conditions. To model chronic liver disease, we treated NIK Δhep and NIK f/f male mice with 2-acetylaminofluorene (AAF), a hepatotoxin (**Laishes and Rolfe, 1981**), for 10 days prior to PHx. Liver cell proliferation was assessed at 48 hr post-PHx. AAF treatment considerably increased hepatic levels of NF- κ B2 p52 in wild type mice, indicative of NIK activation (**Figure 6A**). Baseline levels of proliferating Ki67 $^+$ hepatocytes in the resected liver (<2%) were comparable between NIK f/f and NIK Δhep mice (**Figure 6B**). PHx markedly increased hepatocyte proliferation rates in NIK f/f mice, which was substantially inhibited by AAF pretreatment (**Figure 6C,D**). Remarkably, the number of Ki67 $^+$ hepatocytes was significantly higher in NIK Δhep relative to NIK f/f littermates following AAF and PHx treatments (**Figure 6C,D**). Liver to body weigh ratios were slightly higher in NIK Δhep relative to NIK f/f mice at 2 days post-PHx, but not statistically different (**Figure 6—figure supplement 1A**). As discussed above, a 2 day period may be too short for newly-generated hepatocytes to grow in size to significantly increase liver weight. Plasma ALT levels were also similar between NIK f/f and NIK Δhep mice (**Figure 6E**).

We next examined cell signaling that drives cell cycle progression. We detected baseline levels of phosphorylation of hepatic STAT3 in NIK Δhep but not NIK f/f mice after AAF pretreatment (**Figure 6F**). PHx stimulated STAT3 phosphorylation in both NIK Δhep and NIK f/f mice, but to a substantially higher

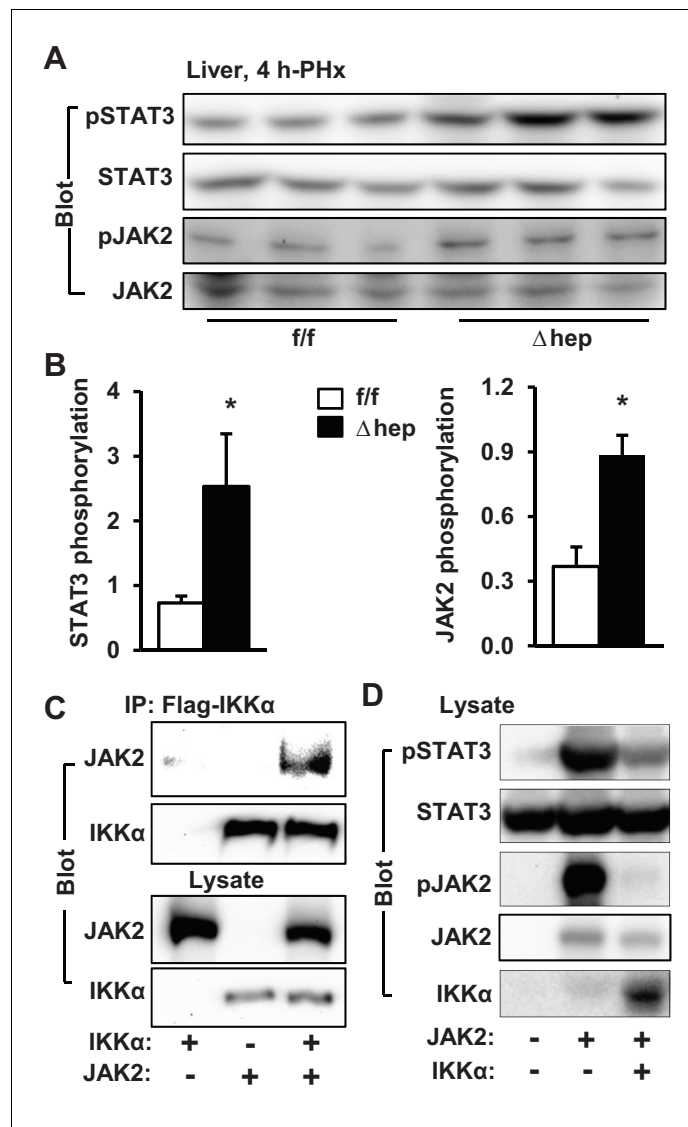


Figure 5. IKK α inhibits the JAK2/STAT3 pathway. (A–B) Liver extracts were prepared 4 hr after PHx and immunoblotted with anti-phospho-JAK2 and anti-phospho-STAT3 antibodies. Phosphorylation of JAK2 (pTyr1007/1008) and STAT3 (pTyr705) was normalized to total JAK2 and STAT3 levels, respectively. IKK $\alpha^{f/f}$: n = 6, IKK $\alpha^{\Delta hep}$: n = 6. (C) IKK α and JAK2 were coexpressed in HEK293 cells. Cell extracts were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. (D) STAT3 and JAK2 were coexpressed with IKK α in HEK293 cells. Cell extracts were immunoblotted with the indicated antibodies. Data were statistically analyzed with two-tailed Student's t test, and presented as mean \pm SEM. *p<0.05.

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The following source data and figure supplements are available for figure 5:

Source data 1. IKK α regulates the JAK2/STAT3 pathway.

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Figure supplement 1. The effect of PHx on activation of liver IKK α and JAK2/STAT3 pathways.

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Figure supplement 1—source data 1. PHx regulates the hepatic JAK2/STAT3 pathway.

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Figure supplement 1—source data 2. Regulation of the JAK2/STAT3 pathway by NIK/IKK α pathways.

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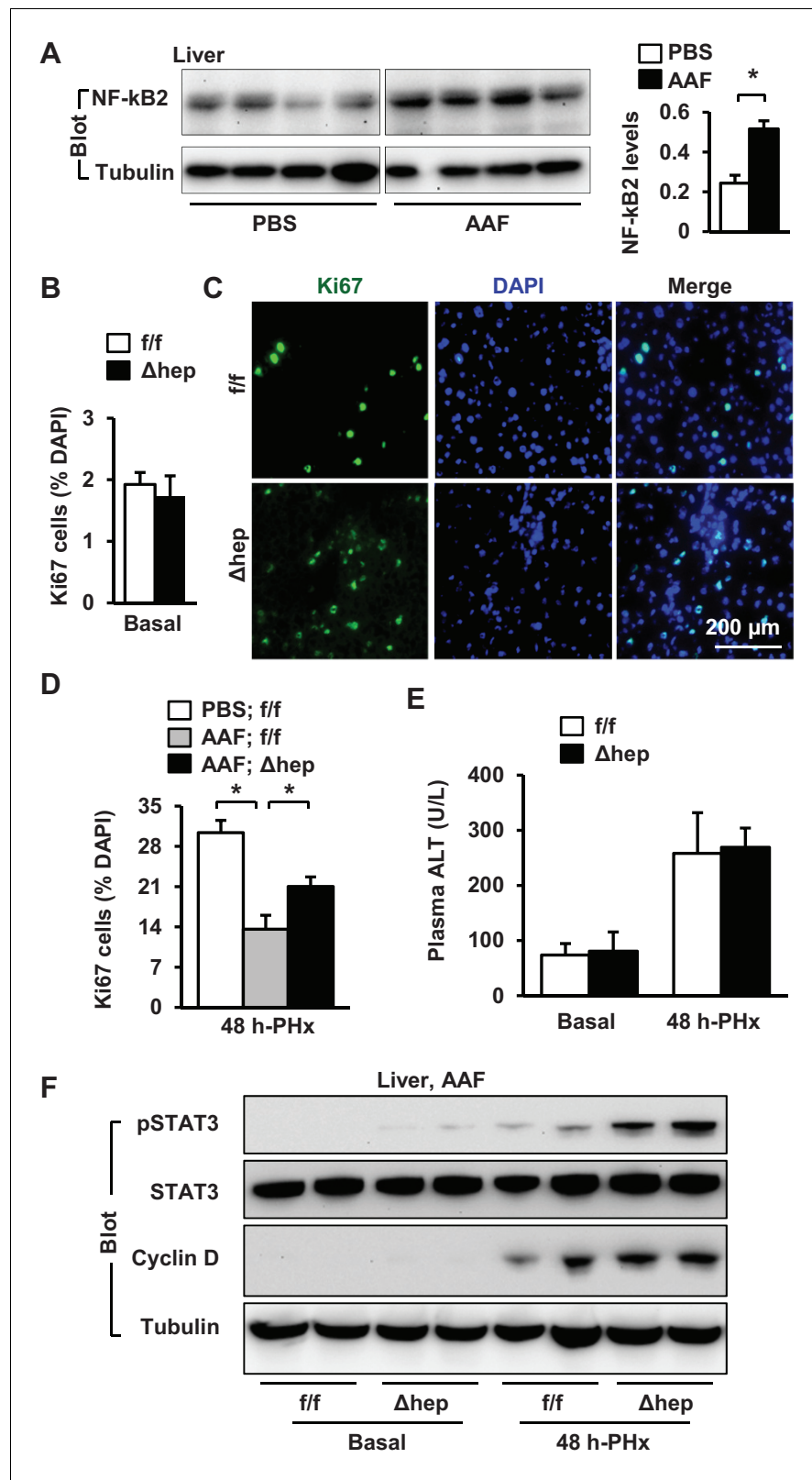


Figure 6. Ablation of hepatocyte NIK reverses AAF-induced impairment in hepatocyte reparative proliferation. (A) C57BL/6 males (8 weeks) were treated with PBS or AAF (10 mg/kg body weight, gavage) daily for 10 days. NF-kB2 p52 in liver extracts was immunoblotted with anti-NF-kB2 antibody (normalized to α -tubulin levels). PBS: n = 4, AAF: n = 4. (B–G) NIK^{f/f} and NIK ^{Δ hep} males were treated with PBS or AAF (10 mg/kg body weight) for 10 days and

Figure 6 continued on next page

Figure 6 continued

then subjected to PHx. Livers were harvested 48 hr later. (B) Baseline Ki67⁺ cell number in resected liver sections obtained from PHx. NIK^{f/f}: n = 5, NIK^{Δhep}: n = 4. (C) Representative immunostaining of liver sections (AAF-treated) with anti-Ki67 antibody. (D) Ki67⁺ cell number in liver sections (normalized to DAPI⁺ cells). PBS;NIK^{f/f}: n = 3, AAF; NIK^{f/f}: n = 5, AAF;NIK^{Δhep}: n = 5. (E) Plasma ALT levels. NIK^{f/f}: n = 3, NIK^{Δhep}: n = 4. (F) Liver extracts were immunoblotted with the indicated antibodies. Data were statistically analyzed with two-tailed Student's t test, and presented as mean ± SEM. *p<0.05.

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The following source data and figure supplements are available for figure 6:

Source data 1. Hepatic NIK regulates hepatocyte proliferation in AAF-treated mice.

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Figure supplement 1. Hepatic NIK inhibits reparative hepatocyte proliferation.

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Figure supplement 1—source data 1. Baseline hepatocyte proliferation in AAF-treated mice.

DOI: <https://doi.org/10.7554/eLife.34152.020>

Figure supplement 1—source data 2. Baseline hepatocyte proliferation in HFD-fed mice.

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level in NIK^{Δhep} mice (**Figure 6F**). Baseline hepatic cyclin D1 levels were undetectable in both NIK^{Δhep} and NIK^{f/f} mice pretreated with AAF, and PHx increased cyclin D1 levels to a higher extent in NIK^{Δhep} than in NIK^{f/f} mice (**Figure 6F**). Together, these data support the notion that abnormal activation of hepatic NIK contributes to hepatotoxin-induced impairment in liver regeneration.

Inactivation of hepatic NIK increases reparative hepatocyte proliferation in mice with NAFLD

NAFLD is associated with both arrest of hepatocyte proliferation and upregulation of hepatic NIK (*Richardson et al., 2007; Inaba et al., 2015; Sheng et al., 2012; Shen et al., 2014; Collin de l'Horset et al., 2014*), prompting us to test if elevated hepatic NIK is responsible for impairment in liver regeneration under the disease conditions. To model NAFLD, we placed NIK^{Δhep} and NIK^{f/f} mice on a high fat diet (HFD) for 10 weeks. Both NIK^{Δhep} and NIK^{f/f} mice similarly developed liver steatosis, as assessed by liver triacylglycerol (TAG) levels (**Figure 7A**). HFD feeding increased hepatic NF-κB2 p52 levels, indicative of NIK activation (**Figure 7B**). To assess liver regeneration, we performed PHx after HFD feeding for 10 weeks. Hepatocyte proliferation was assessed at 48 hr post-PHx by staining liver sections with anti-Ki67 antibody (**Figure 7C**). Baseline levels of hepatocyte proliferation in the resected liver were comparable between NIK^{Δhep} and NIK^{f/f} mice (**Figure 7D**). PHx markedly increased hepatocyte proliferation in chow-fed NIK^{f/f} mice, which was substantially inhibited by HFD feeding (**Figure 7E**). Importantly, number of proliferating Ki67⁺ hepatocytes was significantly higher in NIK^{Δhep} than in NIK^{f/f} littermates after HFD feeding (**Figure 7E**). Liver/body weight ratios were slightly higher in NIK^{Δhep} relative to NIK^{f/f} mice at 2 days post-PHx, but not statistically different (**Figure 6—figure supplement 1B**). This modest difference can be explained by the short duration that limits the capacity of newly-generated hepatocytes to significantly grow in size and increase liver weight. Plasma ALT levels were comparable between NIK^{Δhep} and NIK^{f/f} littermates under both basal and PHx conditions (**Figure 7F**).

We further explored liver mitogenic pathways in these mice. Baseline STAT3 phosphorylation levels in the resected liver were similar between NIK^{Δhep} and NIK^{f/f} mice fed HFD; however, liver STAT3 phosphorylation increased to a considerably higher level in NIK^{Δhep} relative to NIK^{f/f} mice at 48 hr post-PHx (**Figure 7G**). Hepatic cyclin D1 levels were also higher in NIK^{Δhep} than in NIK^{f/f} mice post-PHx (**Figure 7G**). These data suggest that aberrant activation of hepatic NIK suppresses hepatocyte proliferation and liver regeneration in NAFLD at least in part by inhibiting the JAK2/STAT3 pathway.

Discussion

Reparative hepatocyte proliferation plays a pivotal role in the maintenance of liver homeostasis and integrity by supplying new hepatocytes to replace lost ones. Liver regeneration impairment is likely involved in chronic liver disease. In this work, we identified hepatic NIK and IKKα as unrecognized

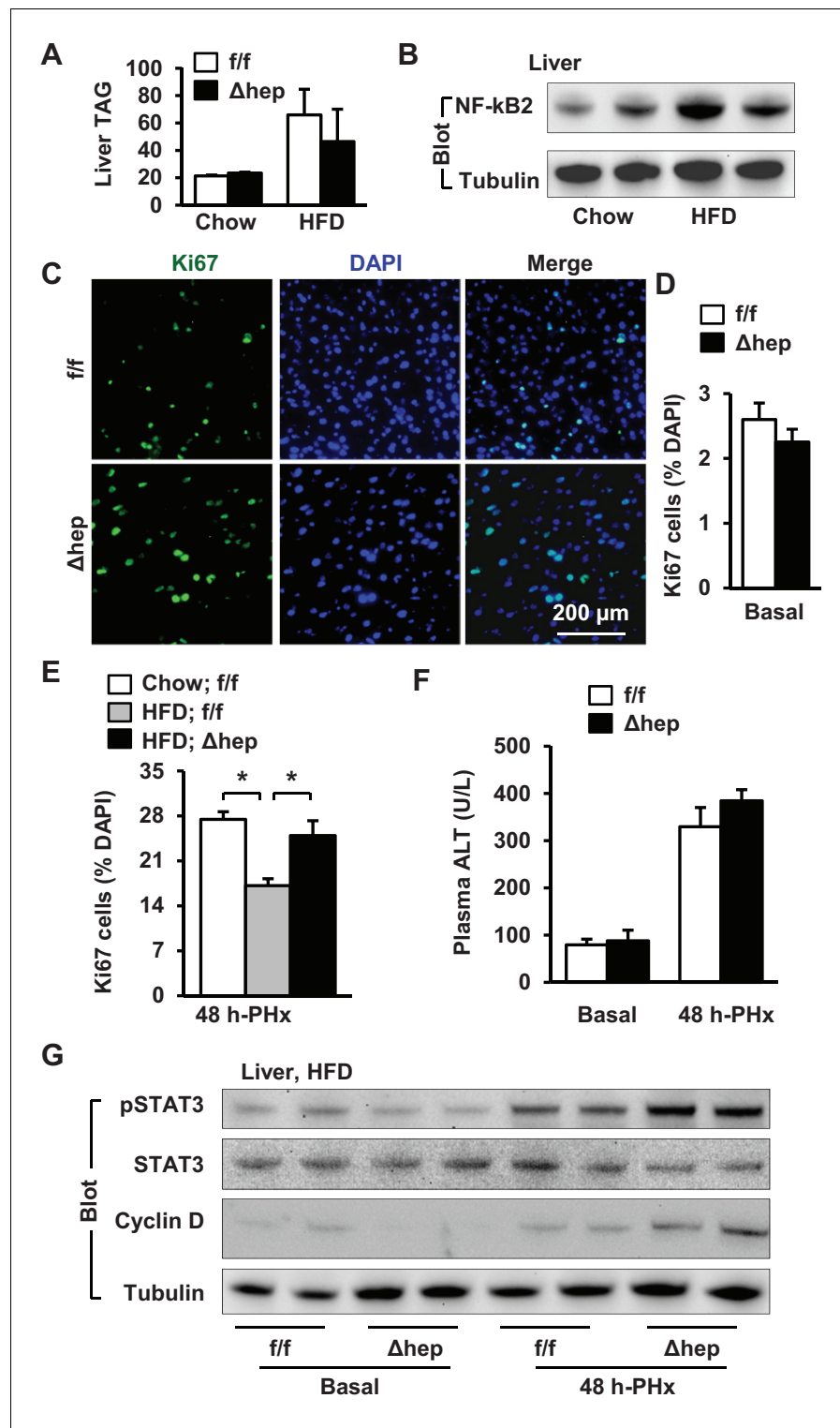


Figure 7. Hepatic NIK deficiency corrects impaired hepatocyte reparative proliferation in mice with NAFLD. (A–B) C57BL/6 males (8 weeks) were fed a normal chow diet (n = 5) or a HFD (n = 5) for 10 weeks. (A) Liver TAG levels (normalized to liver weight). (B) NF-kB2 p52 in liver extracts was immunoblotted with anti-NF-kB2 antibody (normalized to α -tubulin levels). (C–H) NIK^{f/f} and NIK ^{Δ hep} males were fed a HFD for 10 weeks followed by PHx, and livers were harvested 48 hr after PHx. (C) Representative immunostaining of liver sections with anti-Ki67 antibody. (D) Baseline Ki67⁺ cell number in resected liver sections obtained from PHx. NIK^{f/f}: n = 4, NIK ^{Δ hep}: n = 4. (E) Liver Ki67⁺ cell number (normalized to DAPI⁺ cells). Chow; NIK^{f/f}: n = 3, HFD; NIK^{f/f}: n = 5, HFD; NIK ^{Δ hep}: n = 4. (F) Figure 7 continued on next page

Figure 7 continued

Plasma ALT levels. NIK^{ff}: n = 3, NIK^{Δhep}: n = 4. (G) Liver extracts were immunoblotted with the indicated antibodies. Data were statistically analyzed with two-tailed Student's t test, and presented as mean ± SEM. *p < 0.05.

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The following source data is available for figure 7:

Source data 1. Hepatic NIK regulates hepatocyte proliferation in HFD-fed mice.

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suppressors of liver regeneration; moreover, NIK inhibits hepatocyte proliferation at least in part by activating IKK α . We previously demonstrated that hepatic NIK is aberrantly activated in mice and humans with chronic liver disease (Sheng et al., 2012; Shen et al., 2014). Our current results show that elevated activation of hepatic NIK pathways impairs liver regeneration, likely contributing to liver disease progression.

We found that hepatocyte-specific ablation of NIK or IKK α substantially increases hepatocyte proliferation in NIK^{Δhep} or IKK α ^{Δhep} mice following PHx. Accordingly, liver regeneration rates were higher both in NIK^{Δhep} relative to NIK^{ff} littermates and in IKK α ^{Δhep} relative to IKK α ^{ff} mice. We observed that both NIK and IKK α bound to JAK2 and substantially inhibited the ability of JAK2 to phosphorylate STAT3. Consistently, hepatocyte-specific ablation of either NIK or IKK α substantially increased phosphorylation of hepatic JAK2 and STAT3 in mice post-PHx. IKK α deficiency decreased the ability of NIK to suppress the JAK2/STAT3 pathway in hepatocytes, confirming that IKK α acts downstream of NIK. However, NIK overexpression still inhibited the JAK2/STAT3 pathway in IKK α -deficient hepatocytes, suggesting that hepatic NIK is able to suppress the JAK2/STAT3 pathway by an additional IKK α -independent mechanism. The JAK2/STAT3 pathway is known to drive hepatocyte proliferation, which is indispensable for liver regeneration (Wang et al., 2011; Shi et al., 2017; Riehle et al., 2008; Cressman et al., 1996). Therefore, hepatic NIK and IKK α inhibit liver regeneration at least in part by suppressing the JAK2/STAT3 pathway.

Mounting evidence shows that hepatic NIK is aberrantly activated in chronic liver disease, likely due to liver inflammation and hepatocellular stress (Sheng et al., 2012; Shen et al., 2014). We modeled chronic liver disease by chronically treating mice with hepatotoxin AAF or placing them on HFD. We found that hepatocyte-specific inactivation of NIK substantially increases the ability of PHx to stimulate hepatocyte proliferation in both AAF-treated mice and HFD-fed NIK^{Δhep} mice. Consistently, in mice pretreated with AAF or HFD, ablation of hepatic NIK increased phosphorylation of both hepatic JAK2 and STAT3 post-PHx. It is worth mentioning that NIK in nonparenchymal cells (e.g. immune cells) also contributes to obesity-associated liver steatosis (Liu et al., 2017). These observations raise the possibility that in chronic liver disease, NIK in Kupffer cells/macrophages, and possibly other nonparenchymal cells, may indirectly inhibit reparative hepatocyte replication by a paracrine mechanism. Collectively, our results provide proof of concept evidence supporting the notion that aberrant hepatic NIK impairs reparative hepatocyte replication, thereby contributing to liver disease progression.

In conclusion, we have identified hepatic NIK and IKK α as unrecognized suppressors of reparative hepatocyte replication. NIK and IKK α suppress liver regeneration at least in part by inhibiting the hepatic JAK2/STAT3 pathway. Our findings suggest that pharmacological inhibition of hepatic NIK or IKK α may provide a new therapeutic strategy for liver disease treatment.

Materials and methods

Key resources table

Reagent type	Designation	Source or reference	Identifiers	Additional information
Antibody	Ki67	Vector lab	VP-RM04	1:100
Antibody	NIK	Abcam	ab191591	1:2000
Antibody	IKK beta	Cell Signaling Technology	8943	1:5000

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Continued

Reagent type	Designation	Source or reference	Identifiers	Additional information
Antibody	IKK alpha	Cell Signaling Technology	2682	1:5000
Antibody	p-IKKa/b	Cell Signaling Technology	2697	1:5000
Antibody	STAT3	Santa Cruz	sc-8019	1:1000
Antibody	p-STAT3	Cell Signaling Technology	9145	1:5000
Antibody	JAK2	Cell Signaling Technology	3230	1:5000
Antibody	p-JAK2 1007/1008	Cell Signaling Technology	3776	1:5000
Antibody	Myc	Santa Cruz	sc-40	1:1000
Antibody	Flag	Sigma	F1804	1:5000
Antibody	p85	Home-raised	N/A	1:5000
Antibody	α -tubulin	Santa Cruz	sc-5286	1:1000
Antibody	JNK	Cell Signaling Technology	9258	1:5000
Antibody	p-JNK	Cell Signaling Technology	4668	1:5000
Antibody	ERK1/2	Cell Signaling Technology	9102	1:5000
Antibody	p-ERK1/2	Cell Signaling Technology	4370	1:5000
Antibody	NF-kB2	Cell Signaling Technology	4882	1:5000
Antibody	p65	Cell Signaling Technology	8242	1:5000
Antibody	p-p65	Cell Signaling Technology	3033	1:5000
Antibody	IkB alpha	Cell Signaling Technology	4812	1:5000
Antibody	p-IkB alpha	Cell Signaling Technology	9246	1:5000
Antibody	AKT	Cell Signaling Technology	4091	1:5000
Antibody	p-Akt	Cell Signaling Technology	4060	1:5000
Antibody	Cyclin D1	Cell Signaling Technology	2978	1:5000
Antibody	F4/80	eBioscience	14-4801	1:100
Antibody	HNF4 alpha	Santa Cruz	sc-8987	1:100
Antibody	CK8	Developmental Studies Hybridoma Bank	Troma 1	1:100
Antibody	BrDU	Cell Signaling Technology	5292	1:100

Antibodies and animals

Antibodies were described in the key resources table. Animal experiments were conducted following the protocols approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). We generated NIK^{ff}, NIK^{Δhep}, and IKK α ^{Δhep} mice (C57BL/6 background). IKK α ^{ff} mice (C57BL/6 background) were provided by Dr. Yinling Hu (the Inflammation and Tumorigenesis Section, National Cancer Institute). *Albumin-Cre* mice (C57BL/6 background) were from the Jackson laboratory (Bar Harbor, ME). Mice were housed on a 12 hr light-dark cycle and fed a normal chow diet (9% fat; Lab Diet, St. Louis, MO) or a HFD (60% fat in calories; D12492, Research Diets, New Brunswick, NJ) *ad libitum* with free access to water.

PHx models

We followed published 2/3 PHx protocols (Mitchell and Willenbring, 2008). Briefly, NIK^{ff}, NIK^{Δhep}, IKK α ^{ff}, and IKK α ^{Δhep} male mice (8–10 wks.) were anesthetized with isoflurane, followed by a ventral midline incision. The median and left lateral lobes (70% of the liver) were resected by pedicle ligations. Mice were euthanized after PHx, and tissues were harvested for histological and biochemical analyses. Mice were intraperitoneally injected, 12 hr before euthanization, with BrdU (40 mg/kg body weight, ip) to label proliferating cells. A separate cohort was fed a HFD for 10 weeks prior to PHx. An additional cohort was treated with hepatotoxin 2-acetylaminofluorene (AAF) (10 mg/kg body weight, gavage) daily for 10 days prior to PHx.

Immunostaining

Liver frozen sections were prepared using a Leica cryostat (Leica Biosystems Nussloch GmbH, Nussloch, Germany), fixed in 4% paraformaldehyde for 30 min, blocked for 3 hr with 5% normal goat serum (Life Technologies) supplemented with 1% BSA, and incubated with the indicated antibodies at 4°C overnight. The sections were incubated with Cy2 or Cy3-conjugated secondary antibodies.

Cell cultures, transient transfection, and adenoviral transductions

Primary hepatocytes were prepared from mouse liver using type II collagenase (Worthington Biochem, Lakewood, NJ) and grown on William's medium E (Sigma) supplemented with 2% FBS, 100 units ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin, and infected with adenoviruses as described previously (Zhou *et al.*, 2009). HEK293 cells were grown at 37°C in 5% CO₂ in DMEM supplemented with 25 mM glucose, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 8% calf serum. For transient transfection, cells were split 16–20 hr before transfection. Expression plasmids were mixed with polyethylenimine (Sigma, St. Louis, MO) and introduced into cells. The total amount of plasmids was maintained constant by adding empty vectors. Cells were harvested 48 hr after transfection for biochemical analyses.

Immunoprecipitation and immunoblotting

Cells or tissues were homogenized in a L-RIPA lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na₃VO₄, 100 mM NaF, 10 mM Na₄P₂O₇, 1 mM benzamidine, 10 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin, 1 mM phenylmethylsulfonyl fluoride). Tissue samples were homogenized in lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na₃VO₄, 100 mM NaF, 10 mM Na₄P₂O₇, 1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin; 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Real-time quantitative PCR (qPCR) and ROS assays

Total RNAs were extracted using TRIzol reagents (Life technologies). Relative mRNA abundance of different genes was measured using SYBR Green PCR Master Mix (Life Technologies, 4367659). Liver lysates were mixed with a dichlorofluorescein diacetate fluorescent (DCF, Sigma, D6883) probe (5 µM) for 1 hr at 37°C. DCF fluorescence was measured using a BioTek Synergy 2 Multi-Mode Microplate Reader (485 nm excitation and 527 nm emission).

Statistical analysis

Data were presented as means ± sem. Differences between two groups were analyzed using two-tailed Student's *t* tests. *p* < 0.05 was considered statistically significant.

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Author contributions

Yi Xiong, Adriana Souza Torsoni, Data curation, Formal analysis, Validation, Investigation, Methodology, Writing—review and editing; Feihua Wu, Hong Shen, Yan Liu, Mark J Canet, Data curation, Investigation, Writing—review and editing; Xiao Zhong, Data curation; Yatrik M Shah, M Bishr Omary, Resources, Investigation, Writing—review and editing; Yong Liu, Investigation, Writing—review and editing; Liangyou Rui, Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Ethics

Animal experimentation: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols (PRO00006638) of the University of Michigan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Michigan.

Decision letter and Author response

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Additional files

Supplementary files

- Transparent reporting form

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Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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