Predisposition to apoptosis in keratin 8-null liver is related to inactivation of NF-κB and SAPKs but not decreased c-Flip

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Summary
Keratin 8 and 18 (K8/K18) are major intermediate filament proteins of liver hepatocytes. They provide mechanical and nonmechanical stability, thereby protecting cells from stress. Hence, K8-null mice are highly sensitive to Fas-mediated liver cell apoptosis. However, the role of c-Flip protein in K8-null related susceptibility to liver injury is controversial. Here we analyzed c-Flip protein expression in various K8 or K18 null/mutant transgenic livers and show that they are similar in all analyzed transgenic livers and that previously reported c-Flip protein changes are due to antibody cross-reaction with mouse K18. Furthermore, analysis of various apoptosis- or cell survival-related proteins demonstrated that inhibition of phosphorylation of NF-κB and various stress activated protein kinases (SAPKs), such as p38 MAPK, p44/42 MAPK and JNK1/2, is related to the higher sensitivity of K8-null hepatocytes whose nuclear NF-κB is rapidly depleted through Fas-mediated apoptosis. Notably, we found that NF-κB and the studied protein kinases are associated with the K8/K18 complex and are released upon phosphorylation. Therefore, interaction of keratins with cell survival-related protein kinases and transcription factors is another important factor for hepatocyte survival.

Key words: Keratin, Intermediate filaments, Liver, c-Flip, Apoptosis, NF-κB, SAPK

Introduction
Keratins are important cytoskeletal proteins and their expression is restricted to epithelial cells. Keratin monomers – one from type I (K9–K28, K31–K40) and the other from type II (K1–K8, K71–K86) – assemble into heteropolymers forming intermediate filaments (IF) (Eriksson et al., 2009; Herrmann et al., 2009; Kim and Coulombe, 2007; Moll et al., 2008; Schweizer et al., 2006). Keratins are tissue-specific cytoskeletal proteins and their mutations cause organ-specific human diseases (Coulombe et al., 2009; Omary et al., 2004; Omary et al., 2009). For example, K8 and K18 proteins are the major cytoskeletal proteins of epithelial cells in digestive organs such as liver. The K8/K18 expression in hepatocytes relates to liver-specific diseases observed in mutant K8 or K18 transgenic mouse models (Ku et al., 2007; Omary et al., 2009). These experimental findings led to human liver-disease related studies that identified K8/K18 variants in patients with liver diseases of multiple etiologies (Ku et al., 2007; Omary et al., 2009; Strnad et al., 2012). In addition, transgenic mice models showed the significance of K8/K18 proteins for liver protection against both mechanical and nonmechanical stress caused by toxic chemicals and apoptosis-inducing agents (Ku et al., 2007; Magin et al., 2007; Marceau et al., 2007).

The death receptor CD95 (Fas)-mediated apoptosis can be induced by Fas ligand (FasL) binding (Taylor et al., 2008; Wilson et al., 2009). In mouse liver, it can be induced by the administration of Fas antibodies (Ogasawara et al., 1993). This extrinsic pathway requires the recruitment of the Fas-associated protein with death domain (FADD) that binds to the intracellular region of the death receptor and forms the death-inducing signaling complex (DISC) along with procaspase-8. Autoproteolytic conversion of procaspase-8 into activated caspase-8 initiates a caspase cascade leading to apoptosis. Fllice-like inhibitory protein (c-Flip) is a protein that takes part in the regulation of apoptosis by preventing caspase-8 activation at the DISC, thereby blocking the caspase cascade (Taylor et al., 2008; Wilson et al., 2009).

Mitogen-activated protein kinases (MAPKs) are critical signaling molecules to transmit extrinsic stimuli into diverse cellular processes including apoptosis, mitosis, cell motility, differentiation and development (Cuevas et al., 2007; Gallo and Johnson, 2002; Wagner and Nebreda, 2009). MAPKs are divided into three subfamilies, namely JNK1/2, p38 MAPK and p44/42 MAPKs (ERK1/2). Their substrates are transcription factors, membrane receptors and other downstream protein kinases that become activated and are linked to the regulation of gene expression under various stimuli. For example, activation of transcription factors (e.g., p53, NF-κB, c-Jun, ATF2), and protein
kinases (e.g., RSKs, MKs, MSKs) is directly or indirectly associated with MAPK signaling pathways (Avruch, 2007; Wagner and Nebreda, 2009). The variety of their substrates confers broad impact on gene regulation, which is ultimately under the control of MAPKs. Notably, all three subfamilies of MAPKs bind K8/K18 and phosphorylate K8 (Gilbert et al., 2004; He et al., 2002; Ku et al., 2002). The abundance of cytoplasmic keratins provides plentiful binding capacity for MAPKs, whereas keratin ablation or mutations that are no more MAPK substrates may lead to increased MAPK availability to other molecules that modulate cell function.

Indeed, K8-null mice are highly susceptible to Fas-mediated liver injury (Gilbert et al., 2001). The increased susceptibility in K8-null liver was reported to be due to a drastic reduction of c-Flip protein and associated with inhibition of phosphorylation/activation of p44/42 MAPKs (Gilbert et al., 2004). On the contrary, our previous studies showed no change of mouse c-Flip protein in K8 or K18 ablation/mutation transgenic livers (Ku and Omary, 2006). This discrepancy may have resulted from the use of two different c-Flip antibodies, namely antibody (Ab) # 06-864 (Gilbert et al., 2004) and Ab # 06-697 (Ku and Omary, 2006). Here, we compare levels of mouse c-Flip protein expression in various mutant K8 or K18 transgenic mice liver. Our data show that the c-Flip protein levels remain unchanged in the keratin mutant transgenic livers including K8-null liver. The band detected by Ab # 06-864 and proposed to be mouse c-Flip protein (Gilbert et al., 2004), was found to be due to cross-reactivity of this antibody with mouse K18 protein, which is drastically reduced in K8-null liver and therefore excludes c-Flip as a pathogenic factor. Indeed, our results show that the higher sensitivity of K8-null hepatocytes to Fas-mediated apoptosis is due to inhibition of phosphorylation/activation of NF-κB and SAPKs, which results in depletion of nuclear NF-κB and leads to a rapid initiation of massive apoptosis.

**Results**

Ab # 06-864 against c-Flip cross-reacts with mouse K18

A previous study showed dramatic decrease of mouse c-Flip in K8-null hepatocytes (Gilbert et al., 2004) using Ab # 06-864. In contrast, we reported that the level of c-Flip protein was similar in mouse liver irrespective of the genotype, including K8-null liver, by using a different c-Flip antibody, namely Ab # 06-697 (Ku and Omary, 2006). Of note, different immunogens were used to raise these antibodies (Fig. 1A). The immunogen for Ab # 06-864 was a peptide corresponding to amino acid residues 447–464.
of human c-Flip. When we compared the 18 amino acids of the human c-Flip sequence with the corresponding mouse c-Flip, 10 matches were found which corresponded to 56% identity (Fig. 1A). The immunogen for Ab # 06-697 was a peptide corresponding to amino acid residues 2–17 of human c-Flip. Comparison between the amino acid sequence of this immunogen and mouse c-Flip showed 14 conserved residues out of the 16, which corresponded to 88% identity (Fig. 1A). In Fig. 1B, immunoblots using the two different antibodies show striking differences in the protein band pattern in transgenic as compared to nontransgenic mouse liver. Immunoblot with Ab # 06-697 showed a ~55 kd band regardless of the keratin mutation in the different transgenic mice (Fig. 1B). In agreement with this, we observed similar amounts of c-Flip mRNA in the analyzed mice livers by RT-PCR (Fig. 1C). However, in immunoblot using Ab # 06-864, the ~55 kd band was undetectable not only in K8-null, which confirms the results of the previous study of Gilbert et al. (Gilbert et al., 2004), but was also undetectable in K18-null mice, hK18 WT and hK18 R90C overexpressing mice (Fig. 1B). The obvious discrepancy between the expression of mouse c-Flip mRNA and c-Flip protein points to differences in the specificity of two used antibodies. We reasoned that the Ab # 06-864 might cross-react with mouse K18 for the following reasons. First, both mouse K18 and mouse c-Flip have a molecular mass of ~55 kd. Second, a common feature of the four different transgenic livers (K8 or K18-null and hK18 WT/mutant transgenic livers) in which no ~55 kd band was detected by Ab # 06-864 (Fig. 1B), was the absence or dramatic reduction of mouse K18 (Ku and Omary, 2006).

To verify the cross-reactivity of Ab # 06-864 with mouse K18, high salt extracts (HSE) from livers of various transgenic mice were prepared to obtain the total liver keratin pool. Coomassie blue staining of the HSE showed no mouse K18 in K8-null/K18-null livers and dramatically reduced mouse K18 in hK18 WT/R90C overexpressing mice (Fig. 1D, upper panel). Overexpression of hK8 WT and hK8 G62C in transgenic liver did not affect the level of mouse K18 although it caused reduction in the amount of mouse K8 protein. Immunoblots with Ab # 06-697 showed no bands in the HSE samples (Fig. 1D, 2nd panel), whereas Ab # 06-864 produced ~55 kd bands in samples from nontransgenic mice, hK8 WT and hK18 G62C overexpressing mice (Fig. 1D, 3rd panel). The bands overlapped with the position of mouse K18 protein in those mice (Fig. 1D, 4th panel). These results demonstrate that Ab # 06-864 is cross-reactive with mouse K18.

Effect of K8 absence on apoptosis-associated cell signaling
Since we have shown that the Ab # 06-864 cross-reacts with mouse K18 and thus is not specific for mouse c-Flip, the proposed role of c-Flip in the pathogenesis of Fas-induced liver damage seems not to be justified. This is also supported by our lethality studies on hK18 WT mice. Like in K8-null liver, no band was detected by Ab # 06-864 in hK18 WT liver, in contrast to nontransgenic FVB/n liver (Fig. 1B). However, the lethality percentage of nontransgenic FVB/n mice and of hK18 WT mice caused by different forms of stress, i.e. administration of Fas antibody alone, streptozotin or Fas antibody combined with PUGNAc (an inhibitor of N-acetyl-D-glucosaminidase) (Ku et al., 2010), was similar (Table 1).

In terms of increased susceptibility of K8-null mice after Fas administration shown in previous study (Gilbert et al., 2001), we observed the same pattern of results that showed the increased mortality and liver damage in K8-null mice after Fas treatment (supplementary material Fig. S1). To gain further insight in the molecular mechanism of Fas-induced liver damage in K8-null mice, we analyzed the expression of several apoptosis-related proteins and the phosphorylation/activation of protein kinases and transcription factors, all of which are known to be associated with cell survival or apoptosis. Nontransgenic FVB/n mice and K8-null mice received Fas antibody intraperitoneally to induce liver apoptosis and liver lysates were analyzed by immunoblotting. Increased apoptosis in K8-null livers as compared to nontransgenic FVB/n mice was observed by increased formation of cleaved caspase 7 fragment (Fig. 2A). Furthermore, phosphorylation/activation of p38 MAPK, p44/42 MAPK, JNK1/2 and p90RSK, all regulators of cell-cycle progression and survival (Anjum and Blenis, 2008; Wagner and Nebreda, 2009), was dramatically inhibited in K8-null livers as compared to nontransgenic FVB/n mice under Fas-mediated apoptosis (Fig. 2A). Inhibition of phosphorylation of p44/42 MAPK in Fas-treated K8-null liver prevents subsequent phosphorylation of ribosomal S6 kinase (p90RSK) (Fig. 2A), known as a substrate of p44/42 MAPK (Anjum and Blenis, 2008). Notably, the protein amount of all studied kinases was similar in immunoblots of both nontransgenic FVB/n and K8-null livers (Fig. 2B). Consistent with these in vivo findings, a dramatic inhibition of p44/42 MAPK phosphorylation in ex vivo cultured K8-null hepatocytes was observed in a previous study (Gilbert et al., 2004). Next, we tested the effect of K8 ablation on phosphorylation/activation of transcription factors and the expression of several apoptosis-related proteins. Remarkably, phosphorylation

**Table 1. Mouse lethality after injection of different agents**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mouse line</th>
<th>~55 kd band</th>
<th>Dead mice</th>
<th>Total mice</th>
<th>Lethality (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas antibody</td>
<td>Nontransgenic FVB/n</td>
<td>+</td>
<td>12</td>
<td>24</td>
<td>50</td>
<td>&gt;0.61</td>
</tr>
<tr>
<td></td>
<td>hK18 WT</td>
<td>-</td>
<td>26</td>
<td>44</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>Nontransgenic FVB/n</td>
<td>+</td>
<td>3</td>
<td>16</td>
<td>19</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td></td>
<td>hK18 WT</td>
<td>-</td>
<td>4</td>
<td>16</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>PUGNAc + Fas antibody</td>
<td>Nontransgenic FVB/n</td>
<td>+</td>
<td>3</td>
<td>9</td>
<td>33</td>
<td>&gt;0.99</td>
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<tr>
<td></td>
<td>hK18 WT</td>
<td>-</td>
<td>9</td>
<td>29</td>
<td>31</td>
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1Reanalyzed data that were originally published in Nature Cell Biology (Ku et al., 2010).

2A ~55 kd band detected with Ab # 06-864.
of NF-κB p65 was blocked in Fas treated K8-null livers and the expression of NF-κB target genes, such as Bax (Grimm et al., 2005) and c-Flip (Kreuz et al., 2001), was downregulated in the K8-null livers (Fig. 2C). Although the c-Flip band of K8-null (lane 4 in Fig. 2C) was weaker than that of FVB/n under basal conditions (lane 1 in Fig. 2C), it is likely due to the variation of c-Flip expression in individual mouse, which is independent of K8 expression. The densitometric quantification of c-Flip expression from 3 mice/strain showed the c-Flip expression in both mice strains was similar under basal conditions (supplementary material Fig. S2). On the other hand, p53 expression was similar in livers of both mice strains independent of Fas treatment (Fig. 2C). The phosphorylation of p53 could not be analyzed for technical reason. We observed no differences in other apoptosis-associated proteins and in stress-associated proteins such as Hsp70/Hsp60 in livers of both nontransgenic FVB/n and K8-null livers independent of Fas treatment (Fig. 2C).

Taken together, predisposition to apoptosis in K8-null liver is related to the lower level of phosphorylated kinases/NF-κB p65. The lower level is not likely due to rapid degradation of the proteins resulted from a faster apoptosis in K8-null livers since the amounts of each kinase (Fig. 2B) and NF-κB p65 (Fig. 2C) are similar in nontransgenic FVB/n liver and K8-null liver. In addition, the levels of cleaved caspase 7 in FVB/n and K8-null livers after 4 hr treatment of Fas antibody are similar, but the phosphorylation of the kinases/NF-κB p65 is dramatically inhibited in the K8-null liver (Fig. 2A) whereas the amount of the proteins are similar in both livers (Fig. 2B). Hence, it is likely that K8 is involved in phosphorylation/activation of the proteins by an unknown mechanism.

**Interaction between K8/K18 and protein kinases/transcription factors**

Given that the enhanced susceptibility to liver injury in K8-null liver is associated with a dramatic reduction in the level of phosphorylation/activation of protein kinases and NF-κB p65, we examined whether they interact with K8/K18. We used the HT29 colon carcinoma cell line, which expresses high level of endogenous K8/K18. The following conditions are tested: treatment with okadaic acid (OA, a phosphatase inhibitor), colcemid (Col, an antimitotic agent), and anisomycin (An, an apoptosis inducer). Strikingly, we observed an interaction between NF-κB p65 and K8/K18 under basal conditions, and the dissociation of the complexes under the various stress conditions including OA treatment (Fig. 3A). The phosphorylation of p53 could not be analyzed for technical reason. We observed no differences in other apoptosis-associated proteins and in stress-associated proteins such as Hsp70/Hsp60 in livers of both nontransgenic FVB/n and K8-null livers independent of Fas treatment (Fig. 2C).
be regulated by keratin complexes under apoptosis. Furthermore, interaction of p38, p44/42 and JNK1/2 to K8/K18 was also observed under basal conditions and remained almost unaffected by Col or An treatment (Fig. 3A). However, similar to the case of the transcription factors, OA treatment resulted in the dissociation of the complexes, implying the inhibition of their interactions by protein phosphorylation (Fig. 3A). Taken together, our findings indicate that the binding of keratins with cell survival-related protein kinases and transcription factors is important for cell survival and is pathogenic factor in liver injury.

**Effect of K8 absence on localization of NF-κB p65**

Cytoskeletal proteins, such as F-actin, tubulin and vimentin, are involved in the transport and/or localization of p53 (O’Brate and Giannakakou, 2003). Since it is established that the function of NF-κB p65 is modulated by its subcellular localization (Meylan et al., 2009), we compared its distribution in nontransgenic FVB/n and K8-null liver. First, nuclear and cytosolic fractions were analyzed by immunoblotting. Localization of NF-κB p65 was similar in livers of both nontransgenic FVB/n and K8-null animals under basal conditions (Fig. 4A). However, NF-κB p65 nuclear retention was markedly diminished in K8-null liver under Fas treatment as compared with control liver (Fig. 4B). Next, we examined the cellular distribution of NF-κB p65 by immunofluorescence. Consistent with the immunoblot results, NF-κB p65 nuclear depletion rapidly occurred in Fas treated K8-null liver. Most nuclei of K8-null hepatocytes lacked nuclear NF-κB p65 immunofluorescence at 2 hr after Fas treatment, whereas ~15% nuclei of nontransgenic FVB/n hepatocytes lacked NF-κB p65 (Fig. 4C). Hence, in the case of increased susceptibility of K8-null mice to liver injury, a rapid depletion of nuclear NF-κB p65 (Fig. 4) together with dephosphorylation/inactivation of NF-κB p65 (Fig. 2C) occurred, and thereby blocked NF-κB p65 mediated pro-survival pathway, which leading to facilitate apoptosis under liver injury.

**Discussion**

The present study was prompted by discrepant findings of the importance of c-Flip for the enhanced susceptibility of K8-null mice towards liver-injury causing drugs (Gilbert et al., 2004; Ku and Omary, 2006). The main findings of our study are as follows. An antibody specific for c-Flip detects no decrease in c-Flip levels in liver of K8-null and other keratin transgenic mice. Thus, c-Flip seems not to be a pathogenic factor for the enhanced susceptibility of K8-null mice for Fas-mediated apoptosis. Rather, we find that inactivation of NF-κB and various SAPKs, together with a rapid depletion of nuclear NF-κB, is an important pathogenic factor (Fig. 5).

Stress activated protein kinases, such as p44/42 MAPKs, p38 MAPK and JNK1/2 convert a variety of environmental stress signals into diverse cellular responses (Avruch, 2007). p44/42 MAPKs are phosphorylated/activated by mitogens and are upregulated in human cancers, whereas p38 MAPK and JNK1/2 activation results either in apoptosis or in pro-survival depending on cell type and intensity of stress (Wagner and Nebra, 2009). Our data demonstrate inhibition of phosphorylation of all these protein kinases in K8-null liver under conditions of Fas-induced apoptosis. This implies that p38/JNK-mediated apoptosis may not be involved in increased lethality of challenged K8-null mice. Rather, SAPK-mediated pro-survival pathways may be blocked in K8-null liver under Fas-induced apoptosis. In agreement, inhibited phosphorylation/activation of NF-κB and deficiency in nuclear localization were observed in Fas-treated K8-null liver, despite constant p53 protein level. Interestingly, all these kinases were found to be associated with K8/K18 complexes under basal conditions, but were released in a phosphorylation-dependent manner. The biological significance of this interactions remains to be further investigated.

Another major finding in this work is the interaction of K8/K18 with the transcription factors p53 and NF-κB, which are apoptotic and pro-survival factors, respectively. Although the functional implications of this complex formation remain to be further determined, a biological significance of p53 binding to
other cytoskeletal proteins such as vimentin, F-actin and tubulin was demonstrated in previous studies (O’Brate and Giannakakou, 2003). It has been shown that p53 is transported along microtubules to the nucleus and that this is important for p53 accumulation in the nucleus after DNA damage (Giannakakou et al., 2000). On the other hand, p53 accumulation in the cytoplasm of glioma cells depends on presence of vimentin, while nuclear accumulation is observed in vimentin-negative glioma cells (Klotzsche et al., 1998). An aberrant cytoplasmic sequestration of p53, therefore unable to act as a transcription factor, was found in various cancers including undifferentiated neuroblastoma (Moll et al., 1995) and hepatocellular carcinoma (Ueda et al., 1995).

NF-κB activation is involved in pro-survival pathways, leading to cancer development (Hayden and Ghosh, 2012; Perkins, 2012). However, little is known regarding the functional significance of cytoskeletal proteins in these signaling pathways. Although the molecular mechanism is not fully understood, the involvement of cytoskeletal proteins in the NF-κB signaling was previously reported. TNF-α mediated NF-κB activation is reduced by colchicine-mediated inhibition of microtubule formation, while taxol-mediated microtubule stabilization (even in the absence of

Fig. 4. Nuclear NF-κB p65 in K8-null hepatocytes is rapidly depleted following liver injury. (A) Hepatic nuclear and cytoplasmic fractions were prepared from nontransgenic FVB/n and K8-null mice under basal conditions and then were analyzed by immunoblotting. Each lane represents the analysis of one individual mouse liver. The subcellular distribution of NF-κB was similar in both control and K8-null hepatocytes under basal conditions. Nuclear lamin A/C and cytoplasmic tubulin were used to demonstrate the purity of cell fractions. (B) Immunoblot analysis as in panel A of nontransgenic FVB/n and K8-null mice livers after Fas administration. Note the absence of NF-κB p65 in the nuclear fraction of K8-null hepatocytes upon Fas treatment. Arrowhead indicates nonspecific bands. (C) Immunofluorescence for NF-κB p65 (red) in liver sections stained with DAPI (blue) to visualize nuclei. Livers from PBS- or Fas-treated mice for 2 hrs were isolated and analyzed by immunostaining. Note the loss of immunofluorescence for nuclear NF-κB p65 in most K8-null hepatocytes after Fas treatment. Arrowheads point to NF-κB p65 depleted nuclei. Scale bar: 10 μm.

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Fig. 5. Model depicting different patterns of SAPKs activation and NF-κB localization depending on the presence or absence of keratin filament under stress. Following Fas-mediated liver injury, SAPKs such as p44/42 MAPK and JNK1/2 are phosphorylated/activated in control FVB/n liver but not in K8-null liver, which indicates K8 involvement in the activation of SAPKs by an unknown mechanism. In the control liver, the activated SAPKs phosphorylate downstream kinases such as RSK1 that can finally phosphorylate NF-κB. This promotes NF-κB movement into the nucleus where it may initiate pro-survival signaling pathways, thereby delaying liver damage. On the other hand, the unphosphorylated SAPKs in K8-null livers cannot activate downstream kinases such as RSK1 and as a consequence NF-κB cannot be phosphorylated either. This hinders its nuclear localization, interrupts the initiation of the NF-κB-mediated pro-survival pathways and eventually leads to a rapid progression of apoptosis. Grey waved line and red dot denote keratin filaments and phosphorylation, respectively.
TNF-α enhances NF-κB activation (Jackman et al., 2009). Similarly, NF-κB phosphorylation/activation is interrupted in K8-null liver under stress as compared with control liver containing intact keratins (Fig. 2C). Together, this suggests that intact microtubules and keratins are important for the NF-κB signaling pathway.

NF-κB function is modulated by its subcellular localization. Under basal conditions, NF-κB p65/p50, which has a nuclear localization signal (NLS) is bound to IκB which has a nuclear export signal (NES). Thus, the complexes continuously shuttle between the nucleus and the cytoplasm although most of them are in the cytoplasm under steady state (Hayden and Ghosh, 2008). Previous studies on cell cultures showed that upon stimuli, IκB degradiation and p65 phosphorylation occur, which results in nuclear translocation of NF-κB (Chen and Greene, 2004). However, our in vivo studies in livers demonstrate that: (i) NF-κB p65 phosphorylation, at S534 in the mouse protein, can be detected under basal conditions in both nontransgenic FVB/n and K8-null livers (Fig. 2C), (ii) NF-κB p65 phosphorylation is diminished with the occurrence of Fas-mediated apoptosis (Fig. 2C), and (iii) a rapid depletion of nuclear NF-κB occurs in highly susceptible K8-null hepatocytes following liver injury (Fig. 4). The loss of nuclear NF-κB is probably due to failure of NF-κB translocation in the nucleus and may be related to the inactivation of stress-activated protein kinases (SAPKs), such as MAPKs. Various studies of the noncanonical pathway have shown that NF-κB p65 is phosphorylated at S276 by RSK1 (Bohuslav et al., 2004) and at S536 by MSK1 (Chen and Greene, 2004; Vermeulen et al., 2003). Both kinases are downstream kinases regulated by MAPKs, such as p44/42MAPK and p38MAPK (Cargnello and Roux, 2011). Hence, it is likely that inactivation of SAPKs in K8-null hepatocytes under conditions of liver injury results in a deficient phosphorylation state of NF-κB.

As a consequence, this hinders its nuclear localization, interrupts the initiation of the NF-κB-mediated pro-survival pathway and eventually leads to a rapid progression of apoptosis. A major challenge ahead is to address how keratins modulate NF-κB or p53 signaling. Future studies should aim to identify the interaction sites between keratins and the protein kinases/ transcription factors and to reveal the relation between the intracellular distribution of the transcription factors and specific keratins by analyzing transgenic tissues. This may result in information about a possible survival advantage for hepatocytes provided by intact keratins.

Materials and Methods
Reagents and keratin-mutant mice
The following antibodies were used: c-Flip antibodies (Ab # 06-697 and Ab # 06-864, Upstate Biotechnology/Millipore); Fas antibody (for mouse injection; BD Biosciences); phospho- or nonphospho-p38, p90RSK, phospho-p90RSK (human T359/S363, mouse T348/S352), phospho-NF-κB p65 (human S536, mouse S534), phospho-p42, c-Jun N-terminal protein kinase (JNK) and c-Jun (Cell Signaling); Fas antibody (for immunoblotting), FADD and Bax (Upstate Biotechnology/Millipore); Fas-ligand (Santa Cruz Biotechnology). K8-null and hK18 WT mice were provided by Robert Oshima (The Burnham Institute, La Jolla, CA), K18-null livers (Fig. 2C), (ii) NF-κB occurs

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As a consequence, this hinders its nuclear localization, interrupts the initiation of the NF-κB-mediated pro-survival pathway and eventually leads to a rapid progression of apoptosis. A major challenge ahead is to address how keratins modulate NF-κB or p53 signaling. Future studies should aim to identify the interaction sites between keratins and the protein kinases/ transcription factors and to reveal the relation between the intracellular distribution of the transcription factors and specific keratins by analyzing transgenic tissues. This may result in information about a possible survival advantage for hepatocytes provided by intact keratins.

Materials and Methods
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Apoptosis in K8-null liver


Fig. S1. Increased susceptibility of K8-null mice after Fas treatment.
Nontransgenic FVB/n or K8-null mice were treated with Fas Ab (0.15 mg/kg body weight) to induce liver injury. (A) Mouse mortality was shown. (B) Histologic analysis. The livers were isolated after 3 hrs, fixed with 10% formalin and stained with hematoxylin/eosin. Note more severe liver damage in K8-null liver as compared with nontransgenic FVB/n liver. Scale bars: 50 μm.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Dead mice</th>
<th>Total mice</th>
<th>Lethality %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/n</td>
<td>4</td>
<td>9</td>
<td>44</td>
</tr>
<tr>
<td>K8-null</td>
<td>7</td>
<td>9</td>
<td>78</td>
</tr>
</tbody>
</table>

*Nontransgenic mice

Fig. S2. c-Flip expression is similar in nontransgenic FVB/n and K8-null livers under basal conditions. Although the c-Flip band of K8-null (lane 4 in Fig. 2C) was a little bit weaker than that of FVB/n under basal conditions (lane 1 in Fig. 2C), it is likely due to the variation of c-Flip expression in the individual mouse, which is independent of K8 expression. The densitometric quantification of c-Flip expression from 3 mice/strain showed the c-Flip expression in both mice strains was similar under basal conditions. (A) Densitometric quantification of c-Flip expression. (B) Relative amount of c-Flip.