Editorial

RIPK1 and allies in the battle against hepatocyte apoptosis and liver cancer

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Introduction

Liver cancer constitutes the third most frequent cause for cancer-related deaths worldwide. It typically appears in individuals with underlying liver diseases, which most commonly develop in the context of chronic infections with hepatitis viruses, alcoholic and non-alcoholic steatohepatitis or aflatoxin-mediated toxicity (1,2). Continuous death of hepatocytes is a central event in these liver pathologies and leads to a regenerative response characterized by hepatocyte proliferation, inflammation, steatosis and fibrosis that can progress to cirrhosis. This setting becomes a predisposing factor for the development of hepatocellular cancer (HCC), the most frequent form of primary liver cancer (3,4). Understanding the molecular mechanisms governing hepatocellular death is therefore crucial for designing preventive therapies.

In recent years, receptor interacting protein kinase 1 (RIPK1) has emerged as a key determinant of cell decisions to live or die by regulating pathways leading to cell survival or programmed cell death (5-7). Although RIPK1 plays a critical role downstream of many death receptors (DRs) and inflammatory signaling pathways, its functions have been best characterized in tumor necrosis factor (TNF) signaling cascade (*Figure 1*). Upon activation by TNF, TNF receptor 1 (TNFR1) undergoes trimerization and mediates the formation of a cytoplasmic complex (Complex I) by recruiting TNFR1 associated death domain protein (TRADD) and RIPK1. This leads to recruitment of TNF receptor-associated factor (TRAF2), the cellular inhibitors of apoptosis 1 and 2 (cIAP1/2) and the linear ubiquitin chain assembly complex (LUBAC). Several ubiquitination events are associated with RIPK1 and other Complex I components influencing the downstream functional outputs. First, cIAP1/2 mediate K11-, K48- and K63-linked ubiquitination of RIPK1 and cIAP1 itself allowing the recruitment of TAK1 (through its interacting proteins TAB2/3) and LUBAC subunits (HOIP, HOIL-1 and Sharpin), respectively. In turn, LUBAC adds M1-linked (linear) ubiquitin chains on RIPK1 and the NF-κB essential modulator (NEMO) (8). NEMO is the regulatory subunit of the IKK complex, which also includes IKK1/IKKα and IKK2/IKKβ kinases. The IKK complex is best known for mediating the activation of NF-κB signaling by phosphorylating the Inhibitor of NF-κB (IκB) family members, thus promoting their K48 ubiquitination and proteasomal degradation. This allows dimers of NF-κB transcription factors (RelA/p65, RelB, c-Rel, p50/NF-κB1 and p52/NF-κB2) to enter the nucleus and regulate the expression of proinflammatory but also pro-survival genes (9). More recently, NF-κB-independent, pro-survival functions of the IKK complex have also been described (10).

Destabilization of Complex I due to NF-κB inhibition or disruptions in its ubiquitin landscape results in the formation of distinct intracellular complexes that

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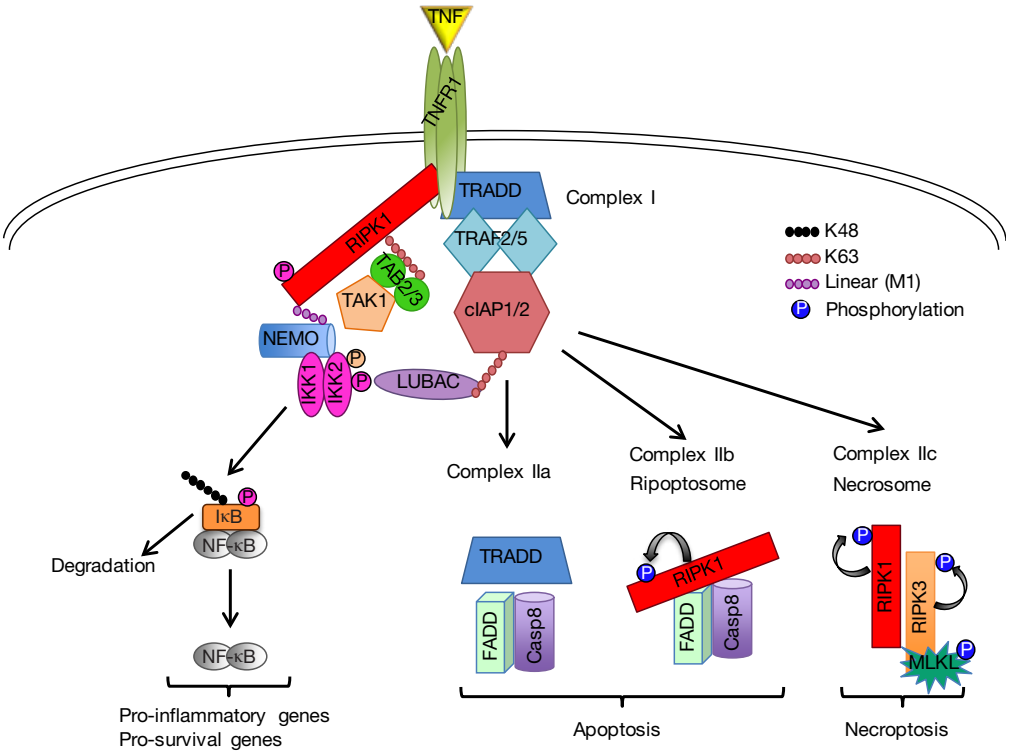

Figure 1 TNF signaling cascade. TNF binding to TNFR1 induces the formation of a receptor proximal complex (complex I). Ubiquitination and phosphorylation events taking place at complex I promote pro-survival mechanisms, including the activation of the IKK/NF-κB signaling pathway. In cells where pro-survival mechanisms are compromised, TNFR1 can induce apoptosis by TRADD-dependent (complex IIa) or RIPK1-dependent (complex IIb) activation of FADD/Caspase-8, or necroptosis through RIPK1/RIPK3/MKLK-containing complex IIc (necrosome). TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNF receptor-associated factor; NEMO, NF-κB essential modulator; IκB, inhibitor of NF-κB; TRADD, TNFR1 associated death domain protein; RIPK1, receptor interacting protein kinase 1; MLKL, mixed lineage kinase-like.

can induce cell death (*Figure 1*). Complex IIa, which predominantly forms upon NF-κB inhibition, involves the TRADD-dependent interaction between FADD and Caspase-8 and induces apoptosis. Complex IIb (or ripoptosome) comprises RIPK1, FADD and Caspase-8 and forms when RIPK1 ubiquitination (and possibly phosphorylation) is disrupted. Its formation is RIPK1 kinase activity-dependent and it also mediates apoptosis. When Caspase-8 is inhibited (upon FADD or Caspase-8 deficiency or treatment with the pan-caspase inhibitor zVAD-fmk), cells can form an alternative Complex IIb (or Complex IIc or necrosome) that is RIPK1 kinase activity-dependent and includes RIPK3 and its downstream target mixed lineage kinase-like (MLKL). Activation of this complex induces necroptosis and its formation appears to be cell type-specific depending on the expression levels of RIPK3 and MLKL (5-7,10,11).

RIPK1 is dispensable for normal liver development

RIPK1 is recognized as a protein with "two faces": one promoting cell survival through its scaffolding properties and one favoring cell death through its kinase activity (12). This was originally supported from the fact that RIPK1 constitutive knockout mice die perinatally (13-16), while mice expressing a kinase inactive form of RIPK1 (RIPK1^{D138N} or RIPK1^{K45A}) are viable and show resistance to TNF-induced inflammatory pathologies and necroptosis induction *in vitro* (17-19). Along this line, intestinal- and epidermis-specific deletion of RIPK1 leads to spontaneous death of epithelial cells and development of intestinal and skin inflammation (20,21). In sharp contrast, mice with liver parenchymal cell (LPC)-specific deletion of RIPK1 (RIPK1^{LPC-KO}) do not exhibit substantial spontaneous liver damage, suggesting that RIPK1

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is dispensable for liver homeostasis during development (22-27). This difference implies that hepatocytes may use redundant/multiple survival mechanisms that compensate for the lack of RIPK1. Alternatively, the stimuli that can trigger hepatocyte death in the absence of RIPK1 could be absent or below a critical threshold during organ development. Several genetic mouse models, such as NEMO^{LPC-KO}, IKK1/2^{LPC-KO} or TAK1^{LPC-KO} mice (22,28-30), were shown to develop spontaneous liver damage suggesting that death-inducing stimuli are present during liver development, and therefore, the presence of compensatory survival mechanisms operating in hepatocytes is likely the critical factor.

The "two-faced" behavior of RIPK1 in liver disease pathogenesis has been genetically demonstrated in NEMO^{LPC-KO} mice, which show spontaneous hepatocyte apoptosis resulting in strong regenerative response, including hepatitis, and eventually in HCC formation (22,28). Expression of a kinase-inactive RIPK1^{D138N} mutant in NEMO-deficient hepatocytes significantly prevented spontaneous apoptosis, chronic liver pathology and HCC (22). The pro-apoptotic role of RIPK1 kinase activity has also been shown in cells depleted for cIAP1/2 using SMAC mimetics (SM) (31), or in TAK1- (32), IKK1/IKK2- (33) and LUBAC-deficient mouse embryonic fibroblasts (MEFs) (19). Conversely, complete absence of RIPK1 did not prevent apoptosis of NEMO-deficient or IKK1/2-deficient hepatocytes (22,23) underscoring the importance of RIPK1's scaffolding functions. In the absence of RIPK1, TRADD mediates an alternative pro-apoptotic pathway in hepatocytes, as NEMO/RIPK1/TRADD^{LPC-KO} mice showed no spontaneous liver damage (22). Both TRADD and RIPK1 interact with Caspase-8 upon TNF stimulation, but the presence of two independent complexes has not been demonstrated (34). While the presence of TRADD in Complex IIa has been inferred by studies showing that TRADD counteracts RIPK1-induced cell death (31,35), an interaction of TRADD with FADD or Caspase-8 was not observed in MEFs treated with TNF/Cycloheximide that induces TRADD-mediated apoptosis (33). Therefore, the precise molecular role of TRADD in the alternative apoptosis-inducing pathway remains elusive.

RIPK1 is indispensable for survival in mouse models of acute liver injury

In contrast to the lack of spontaneous liver phenotype in RIPK1^{LPC-KO} mice, mounting evidence show that the presence of RIPK1 in hepatocytes is essential for preventing acute hepatocellular damage in several *in vivo* models. Upon LPS- or CpG-DNA-induced liver damage, resident macrophages (Kupffer cells) become strongly activated and secrete high levels of TNF that initiates TNFR1 signaling cascade in hepatocytes (25,36). RIPK1^{LPC-KO} mice were shown to be highly sensitive to these stimuli and undergo apoptosis (25,27,37). Similarly, RIPK1^{LPC-KO} mice were greatly susceptible to apoptosis induced by Concanavalin-A (ConA) (24), a model of TNFR1-dependent, T cell-mediated autoimmune hepatitis (36). RIPK1 knock-down using anti-sense oligos had also a detrimental effect in mice injected with α-galactosyl ceramide (αGalCer), a liver injury model depending on the release of various cytokines, including TNF, by activated NKT cells (26). Finally, the impact of RIPK1 in acetaminophen (APAP)-induced hepatotoxicity, a TNF-independent acute liver injury model, has been more controversial. While RIPK1^{LPC-KO} mice were as sensitive to APAP toxicity as their wild-type controls (38), treatment of mice with RIPK1 anti-sense oligonucleotides significantly prevented liver damage and increased overall survival (39). Side-effects of using oligos, RIPK1 functions in non-parenchymal cells or adaptive responses of hepatocytes lacking RIPK1 already in utero have been proposed as arguments for explaining the opposite results (38,39). Additionally, RIPK1 depletion by the anti-sense oligos may not have been strong enough to trigger the TRADD-mediated apoptotic pathway that has been shown to operate in RIPK1-deficient hepatocytes (22,37).

Contrary to RIPK1 scaffolding function, the enzymatic activity of RIPK1 was also shown to play a pro-apoptotic role in acute liver injury models. Inhibition of RIPK1 kinase activity by Nec-1 or Nec-1s, a more specific inhibitor, and genetic expression of RIPK1^{D138N} or RIPK1^{K45A} significantly protected mice from APAP- (39,40), αGalCer- (26) and ConA-induced liver damage (41), though one study also reported exacerbated liver injury upon Nec-1 treatment in the later model (40). Another recent study also showed that RIPK1^{K45A} mice were completely protected from hepatocyte apoptosis induced by TNF and the IKK1/2 inhibitor, TPCA-1, whereas no protection was observed upon injection of TNF and D-galactosamine (inhibitor of hepatocyte gene transcription) (33), suggesting that RIPK1 kinase activity mainly mediates the NF-κB-independent pro-apoptotic pathway (see below).

Interplay between RIPK1, IKK complex and NF-κB

The resistance of RIPK1-deficient hepatocytes to

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spontaneous cell death during normal liver development implies RIPK1's close crosstalk with other key molecules/pathways regulating cell survival. The interplay of RIPK1 and NF-κB pathway is of particular interest. The importance of both NF-κB-dependent and independent mechanisms for spontaneous liver disease has been suggested in NEMO^{LPC-KO} mice. While NF-κB deficiency in RelA/RelB/cRel^{LPC-KO} (NF-κB^{LPC-KO}) mice did not recapitulate the pathology of NEMO^{LPC-KO} mice, the expression of a constitutively active form of IKK2 rescued the spontaneous apoptosis observed in NEMO^{LPC-KO} mice largely by activating canonical NF-κB signaling (22). This dual (NF-κB-dependent and independent) pro-survival function of NEMO is not only hepatocyte-specific, as similar results have been reported in intestinal epithelial cells (42) and Jurkat T cells (43). These findings are also consistent with the previously described existence of two distinct apoptotic pathways in cancer cells, an NF-κB/cFLIP-dependent and an NF-κB-independent but RIPK1/cIAP-dependent pathway (31). In a "two-hit model", simultaneous inhibition of RIPK1 scaffolding functions and NF-κB signaling would be required to induce spontaneous liver damage and chronic liver disease (*Figure 2*).

Schneider *et al.* recently provided additional support to this notion by showing that RIPK1/TRAF2^{LPC-KO} mice develop spontaneous liver damage, cholestasis, chronic hepatitis, fibrosis and HCC. Unlike the respective single knockout cells, RIPK1/TRAF2-deficient hepatocytes exhibited impaired canonical NF-κB signaling (27). RIPK1 was originally shown to be required for TNF-induced NF-κB activation (13), but this finding was subsequently challenged (44) and several studies have shown robust LPS/TNF-induced NF-κB activation in RIPK1-deficient hepatocytes (24,26,27). On the other hand, TRAF2-deficient hepatocytes can sustain NF-κB activation possibly due to the redundant function of TRAF5 (45). The presence of two independent pro-survival pathways is also supported by the fact that both NF-κB^{LPC-KO} and RIPK1^{LPC-KO} mice were susceptible to LPS-induced liver damage (22,27). Curiously, Schneider *et al*. show that although LPS/TNF stimulation induces rapid TRAF2 downregulation in RIPK1-deficient hepatocytes, this did not impair their ability to activate NF-κB, unlike what was observed in RIPK1/TRAF2-deficient hepatocytes (27). This implies that when the death-inducing stimulus is above a certain threshold, it can cause substantial liver damage even when NF-κB signaling is not significantly inhibited. Based on the proposed "two-hit model" (*Figure 2*), one would predict that combined ablation of RIPK1 and NF-κB signaling would culminate in stronger LPS-induced acute liver damage compared to the single knockouts.

Schneider *et al.* also generated TRAF2/IKK2^{LPC-KO} mice as a way to genetically simulate their molecular findings in RIPK1/TRAF2^{LPC-KO} mice. These mice showed spontaneous liver damage, albeit less strong than in RIPK1/TRAF2^{LPC-KO} mice and without exhibiting cholestasis. One-year-old TRAF2/IKK2^{LPC-KO} mice developed HCC but of much smaller size than RIPK1/TRAF2 $^{LPC-KO}$ mice (27), likely reflecting the lower hepatocellular damage and lack of cholestasis in the former mice. Although the LPC-specific IKK2 deficiency was used to exemplify the contribution NF-κB blockade in the liver phenotype of RIPK1/TRAF2 $^{LPC-KO}$ mice, it is worth mentioning that Luedde *et al* have shown that IKK2 deficiency does not fully block NF-κB activation in hepatocytes due to a partial compensation by IKK1 (29). More importantly, RIPK1 has been identified as a phosphorylation substrate of IKK1/2 in MEFs and hepatocytes (23,33). This RIPK1 phosphorylation was shown to have an NF-κB-independent, prosurvival function. In MEFs, RIPK1 phosphorylation was abolished in the absence of IKK1/2, NEMO, TAK1 or cIAP1/2 suggesting that the event takes place at complex I (33). On the other side, the liver study showed that RIPK1 phosphorylation was inhibited in IKK1/2-deficient but not in NEMO-deficient hepatocytes (23). However, how IKK1/2 would be able to associate with and catalyze RIPK1 phosphorylation in the absence of NEMO remains unclear. The biological significance of this pro-survival, IKK-mediated RIPK1 phosphorylation will need to be validated using suitable RIPK1 mutants in *in vivo* mouse models.

Formal proof that the canonical NF-κB signaling and RIPK1 act synergistically to prevent spontaneous apoptosis in hepatocytes has come from a recent study reporting that RIPK1/RelA^{LPC-KO} mice develop spontaneous apoptosis, chronic liver disease and eventually HCC (37), as NEMO^{LPC-KO} and TRAF2/IKK2^{LPC-KO} mice do (22,27).

RIPK1 prevents hepatocyte apoptosis by stabilizing TRAF2 and cIAP1

Another important issue is how RIPK1 affects other components of Complex I to prevent apoptosis. The stability of cIAP1/2 and TRAF2 appear to be critical events for the sensitization to apoptosis, as they were downregulated in NEMO-deficient hepatocytes (22). The same was true for cIAP1 in liver lysates from RIPK1/TRAF2^{LPC-KO} mice (27). cIAP1 degradation correlated with

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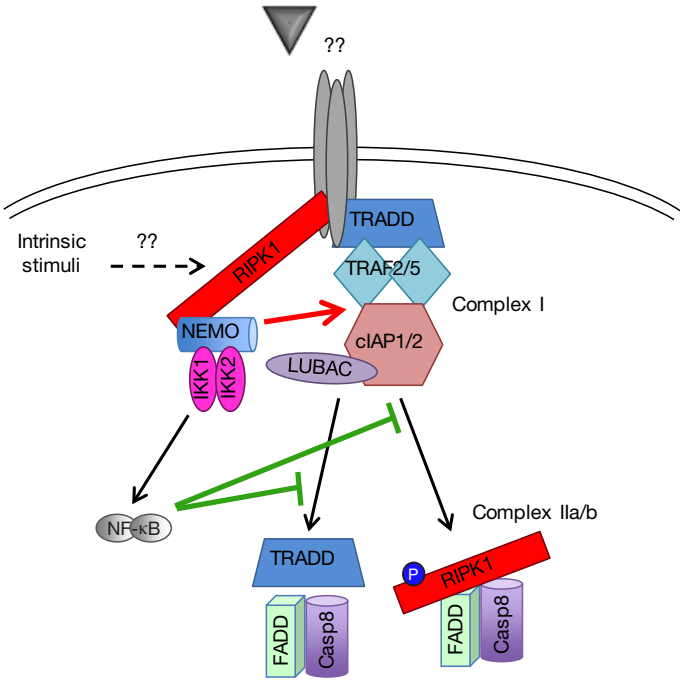

Figure 2 "Two-hit model" of inducing liver damage during early liver development. Hepatocyte apoptosis can be induced during postnatal liver development by yet unidentified extrinsic or intrinsic stimuli. For this to happen, two pro-survival mechanisms [or checkpoints (10)] have to be simultaneously compromised: (I) the stability of TRAF2 and cIAP1/2 at complex I that is regulated by the interplay between the IKK complex subunits and RIPK1 (red arrow); and (II) the cell competence for canonical NF-κB activation, which can block either the formation of the pro-apoptotic complexes or the execution of apoptosis (green inhibitory symbols). RIPK1 deficiency alone neither induces strong TRAF2/cIAP degradation (likely because the currently unidentified stimulus/stimuli are below a critical threshold) nor significantly blocks NF-κB activation. TRAF2 deficiency does not impair both pro-survival mechanisms, likely due to the redundant function of TRAF5. NEMO-, IKK1/IKK2-, TAK1-, RIPK1/TRAF2-, RIPK1/IKK2-, RIPK1/RelA-, and LUBAC-deficient hepatocytes impair both pathways and could reduce the threshold of stimuli required to induce apoptosis. Additionally, destabilization of complex I could result in unprompted, stimulus-free formation of apoptosis-inducing complexes II. TRAF, TNF receptor-associated factor; cIAP1/2, cellular inhibitors of apoptosis 1 and 2; RIPK1, receptor interacting protein kinase 1; NEMO, NF-κB essential modulator; LUBAC, linear ubiquitin chain assembly complex; TRADD, TNFR1 associated death domain protein.

Caspase-8 activation in Complex IIb both in $NEMO^{LPC-KO}$ and $RIPK1/TRAF2^{LPC-KO}$ mice (22,27), as previously described (46). Accordingly, both TRAF2 and cIAP1 were shown to undergo TNF-induced proteasomal degradation in $RIPK1^{-/-}$ but not in WT MEFs (47).

An additional molecule that synergizes with RIPK1 to prevent cell death is cFLIP that negatively regulates apoptosis and necroptosis (12). cFLIP protein levels were strongly reduced in NEMO- and RIPK1/TRAF2-deficient hepatocytes (22,27). Interestingly, its levels remained low in $RIPK1/TRAF2/Caspase\text{-}8^{LPC-KO}$ mice, where apoptosis was prevented, and this was attributed to the observed NF-κB inhibition (27). However, this is unlikely because cFLIP levels were restored in $NEMO^{LPC-KO}/RIPK1^{D138N}$ mice, where apoptosis was strongly prevented but NF-κB was still inhibited due to the lack of NEMO (22). Unlike cancer cells where cFLIP is a typical NF-κB-responsive gene (48), its mRNA expression in NEMO- or NF-κB-deficient hepatocytes was not significantly affected. Thus, its protein levels at least in primary hepatocytes are likely regulated by additional NF-κB-independent transcription factors and post-translational mechanisms.

Different stimuli induce spontaneous and acute liver damage

Contrary to most of the acute liver injury models, where the cell death stimulus is defined, what triggers the spontaneous

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death of hepatocytes in all genetic mouse models of chronic liver disease leading to HCC is still unresolved. Considering that deletion of FADD or Caspase-8 prevents hepatocytes apoptosis in most of these mice (27,28,49,50), one would expect that death ligands that initiate extrinsic apoptotic pathways are important. Surprisingly, combined LPC-specific deletion of TNFR1, TRAILR and Fas did not protect NEMO-deficient hepatocytes from spontaneous death *in vivo* (49). Likewise, TNFR1 deletion in LPCs did not ameliorate the liver pathology in RIPK1/RelA^{LPC-KO} mice (37). The same was recently shown in HOIP^{LPC-KO} mice that develop a spontaneous liver pathology as NEMO^{LPC-KO} mice (51). This raises the question whether any of the remaining DRs (DR3 or DR6) may be involved (52). The most likely, however, is that low levels of multiple stimuli, including ligands activating DRs or Toll-like receptors, are the inducers of spontaneous liver damage. Moreover, intrinsic pathways inducing apoptosis could also contribute. For instance, unresolved ER stress was shown to induce DR-independent, RIPK1- and Caspase-8-dependent apoptosis in MEFs (53). Additionally, TAK1/NEMO/RIPK1-mediated activation of NF-κB signaling is shown to be part of the DNA damage response (54,55), while a DR-independent formation of Complex IIb/Ripoptosome has been described upon degradation of cIAPs induced by genotoxic stress (56,57). The multiple cell divisions that hepatocytes undergo during liver organogenesis in the first weeks after birth (when spontaneous apoptosis first occurs) could induce replicative stress leading to activation of analogous signaling pathways (58).

Translational aspects for human chronic liver disease and HCC patients

The type of programmed cell death has been associated with the elicited immunological responses thereby potentially affecting cancer development (5). While multiple mouse studies have demonstrated the relevance of apoptosis for liver disease development (22,25-28,37,49,50), the significance of hepatocyte necroptosis remains controversial (3,59). The absence or minimal expression of RIPK3, a bona-fide regulator of necroptosis, could explain why hepatocytes are resistant to necroptosis, while the positive or negative effect that RIPK3 KO mice showed in some liver injury models could reflect a role of RIPK3 in modulating necroptosis or inflammation in a non-LPC compartment [see (59) for a critical review].

Undoubtedly, the aforementioned mouse studies demonstrate a causal link between hepatocyte death and chronic steatohepatitis, which in turn promotes HCC development. These data highlight the potential benefit of targeting apoptosis (as the most relevant cell death type) in chronic liver disease patients as a preventive approach against HCC development. Accordingly, the pan-caspase inhibitor IDN-6556 is currently in clinical trials in such patients. Additionally, RIPK1 kinase inhibitors could also be efficient in preventing apoptosis in at least a subset of patients, and are likely to be well tolerated considering the lack of phenotype in mice expressing kinase-inactive RIPK1.

Despite the association between cell death and liver disease pathogenesis, no clear role for RIPK1 and other Complex I-related molecules in HCC initiation or progression has been so far demonstrated. Two opposing studies using the DEN-induced hepatocarcinogenesis model in mice have shown a detrimental or beneficial effect on liver tumor burden in IKK2^{LPC-KO} (60) and RIPK1^{LPC-KO} mice (37), respectively. However, these effects were attributed to a differential impact of the cell death observed early after DEN injection rather than a late effect of the deleted genes in tumor progression.

Complex I members were also not found significantly enriched in large exome sequencing projects on human HCC samples (61,62). However, there are studies showing a correlation between the expression of certain molecules and HCC patient prognosis. Loss of NEMO (63) or combined loss of RIPK1/TRAF2 immunoreactivity (27) in human HCC samples was associated with poor overall patient survival. Moreover, downregulation of RIPK1 and IKK1/2 was reported in a significant percentage of human HCC biopsies, albeit no correlation was observed to the underlying disease etiologies (23). Considering the pro-survival role of these molecules, the benefit of their downregulation for cancer cells is counterintuitive. Most probably, the reduction in protein levels has taken place at an early pre-cancerous stage leading to increased hepatocyte apoptosis and promoting the underlying hepatitis. However, during HCC development, tumor-initiating hepatocytes must have upregulated anti-apoptotic mechanisms to overcome this deficit and avoid cell death in a microenvironment with increased death-inducing stimuli. Indeed, hepatobiliary cancer cells are often resistant to TRAIL-induced death because they overexpress pro-survival proteins and targeting cIAP1 was shown to restore sensitivity to TRAIL (46).

Taken together, reduced immunoreactivity for RIPK1, TRAF2 and IKK complex subunits could have a prognostic

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value for chronic liver disease patients and HCC patient survival, although more extensive studies will need to be performed in larger patient cohorts. Based on our understanding of the RIPK1-mediated signaling pathways, it might be beneficial for HCC patients with low RIPK1 immunoreactivity to use NF-κB or cIAP inhibitors in their therapeutic scheme, as these HCCs are likely to have upregulated such anti-apoptotic pathways. In this case, the challenge will certainly be to develop ways for delivering these drugs preferentially in liver cancer cells.

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