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ORIGINAL ARTICLE

Phosphoproteomics identifies pathways underlying the role of receptor-interaction protein kinase 3 in alcohol-associated liver disease and uncovers apoptosis signal-regulating kinase 1 as a target

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Abstract

Receptor-interaction protein kinase 3 (RIP3), a critical determinant of the necroptotic pathway of programmed cell death, contributes to injury in murine models of alcohol-associated liver disease (ALD); however, the underlying mechanisms are unknown. We investigated the effect of chronic ethanol feeding on the hepatic phosphoproteome in C57BL/6 and RIP3-deficient (*Rip3*$^{-/-}$) mice, focusing on death receptor (DR) signaling pathways. C57BL/6 and *Rip3*$^{-/-}$ mice were fed an ethanol-containing liquid diet or pair-fed control diet. A label-free mass spectrometry-based approach identified differentially phosphorylated proteins that were mapped to pathways affected by ethanol and *Rip3* genotype. Identified targets were validated in both the murine model of ALD and in liver tissue from patients with alcohol-associated hepatitis (AH) and healthy controls. Chronic ethanol dysregulated hepatic tumor necrosis factor-induced DR signaling pathways. Of particular importance, chronic ethanol feeding to C57BL/6 mice decreased the phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) at serine (S)1036/S1040 (S1029/S1033 human), sites linked with the inhibition of ASK1 death-promoting activity. This decrease in phosphorylation of inhibitory sites was muted in *Rip3*$^{-/-}$ mice. Decreased phosphorylation at S1033 was also lower in liver of patients with severe AH compared to healthy controls, and phosphorylation at the ASK1 activation site (threonine [Thr]-838) was increased in patients with AH. The net impact of these changes in phosphorylation of ASK1 was associated with

ABBREVIATIONS: AH, alcohol-associated hepatitis; ALD, alcohol-associated liver disease; ASK1, apoptosis signal-regulating kinase 1; B-H, Benjamini-Hochberg; C57BL/6, wild-type mice; DR, death receptor; FADD, FAS-associating death domain-containing protein; IPA, Ingenuity Pathway Analysis; NASH, nonalcoholic steatohepatitis; MAP3K5, mitogen-activated protein kinase kinase kinase 5; MAP3K7, mitogen-activated protein kinase kinase kinase 7/transforming growth factor-beta-activated kinase 1; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4/hepatocyte progenitor kinase-like; MS, mass spectrometry; MLKL, pseudokinase mixed lineage kinase-like protein; MS-MS, tandem mass spectrometry; nLC, nanoscale liquid chromatography; RIP1, receptor interacting protein kinase 1; RIP3, receptor-interacting protein kinase 3; *Rip3*$^{-/-}$, RIP3 deficient; Ser/S, Serine; Thr, threonine; TNFα, tumor necrosis factor α; TNFR1, tumor necrosis factor receptor 1; TRAD, tumor necrosis factor receptor-associated death domain; Tyr, tyrosine.

increased phosphorylation of p38, a downstream target of ASK1, in patients with AH and C57BL/6 but not *Rip3*$^{-/-}$ mice. Similarly, chronic ethanol feeding affected the c-Jun N-terminal kinase pathway in C57BL/6 but not *Rip3*$^{-/-}$ mice. Taken together, our data indicate that changes in inhibitory phosphorylation of ASK1 are an important target in ALD and suggest the involvement of non-canonical functions of *Rip3* in ALD.

INTRODUCTION

Although alcohol-associated liver disease (ALD) is a major type of chronic liver injury, it lacks effective treatments. It is a progressive disease marked by fatty liver, inflammation, hepatocellular death, and fibrosis, with an increased risk for cirrhosis and hepatocellular carcinoma.[1,2] While the pathophysiological mechanisms driving ALD are complex and not well understood, evidence indicates a clear association between inflammation and hepatocellular death.[1,2] The role of tumor necrosis factor alpha (TNFα) in driving the progression of ALD is of particular importance. While TNFα is hepatoprotective in healthy liver, exposure to chronic alcohol sensitizes hepatocytes to TNFα-induced cell death.[3] TNFα, acting through TNF receptor 1 (TNFR1), can trigger cell death by apoptosis or necroptosis, depending on the intricate balance of proteins and regulatory events stimulated in response to death signals. TNFR1 initially recruits receptor-interacting protein kinase (RIP) 1 and other cellular factors. TNFR1 promotes apoptosis by adaptor proteins TNFR1-associated death domain (TRADD)/Fas-associated death domain (FADD) and the activation of caspase-8. When caspase-8 is inhibited,[4,5] activation of RIP1 promotes its interaction with RIP3,[6–8] which in turn phosphorylates mixed-lineage kinase domain-like pseudokinase (MLKL), to mediate necroptosis by forming pores at the plasma membrane.

While multiple forms of cell death are associated with ALD,[9] the causal relationship between different cell death pathways and the progression of ALD is not well understood. Apoptosis does not appear to be causal in models of early steatosis and hepatocyte injury because mice deficient in *bid,* an effector common to both the extrinsic and intrinsic pathways of apoptosis, or treated with pan-caspase inhibitors are not protected from the early stages of ethanol-induced liver injury.[10] In contrast, mice deficient in *Rip3* (*Rip3*$^{-/-}$), a key effector of necroptotic cell death, are protected from liver injury following chronic ethanol feeding,[11,12] as well as acetaminophen-induced hepatotoxicity[13,14] and concanavalin-induced autoimmune hepatitis.[15] Indeed, RIP3 protein expression is higher in liver of patients with ALD compared with healthy controls as well as in liver from mice exposed to chronic ethanol.[11] If the association of RIP3 with ethanol-induced liver injury was a result of activation of necroptosis, we would expect that *Mlkl*-deficient mice (*Mlkl*$^{-/-}$ mice) would also be protected from injury because MLKL is the critical effector protein for necroptosis. However, *Mlkl*$^{-/-}$ mice are not protected from either chronic ethanol and chronic-plus-binge ethanol-induced liver injury,[16] suggesting that RIP3 acts independently of MLKL and that noncanonical functions of RIP3 are critical for ethanol-induced liver injury.[17]

Posttranslational modifications (PTMs), in particular phosphorylation events, are critical to the regulation of programmed cell death.[18] Here, we applied nanoscale liquid chromatography-based mass spectrometry (nLC/MS) to characterize the interaction of chronic ethanol and RIP3 on hepatic phosphorylation events in order to better understand the contribution of RIP3 in the progression of ALD. We report that chronic ethanol feeding perturbed the hepatic phosphorylation landscape, including multiple proteins involved in death receptor (DR) signaling. Mitogen-activated protein kinase kinase kinase 5 (MAP3K5, commonly known as apoptosis signal-regulating kinase 1 [ASK1]) was prominent among the phosphoproteins affected by chronic ethanol. ASK1 expression is activated by different types of stress, such as oxidative stress and inflammatory cytokines, including TNFα. ASK1 is a member of the MAP3K family and an upstream activator of p38 MAPK and c-Jun N-terminal kinase (JNK) signaling cascades.[19] ASK1 mediates diverse biological signals regulating cell survival, proliferation, inflammation, and cell death.[20,21] ASK1 undergoes dynamic PTMs, particularly phosphorylation, which positively and/or negatively regulate its kinase activity. Phosphorylation of threonine (Thr)838 (corresponding to murine Thr845) enhances ASK1 activity,[22] while phosphorylation of serine (Ser or S)-1033/1034 (murine Ser-1040) negatively regulates ASK1 activity.[23]

ASK1 and its downstream target p38 are implicated in chronic-ethanol-induced injury in murine models,[24] but the role of ASK1 phosphorylation is not well understood. Here, we show that chronic ethanol feeding reduced the phosphorylation of key inhibitory phosphorylation sites on ASK1 in mice. In *Rip3*$^{-/-}$ mice, changes in ASK1 inhibitory site phosphorylation were

not as responsive to chronic-ethanol compared to wild-type (C57BL/6) mice. Because *Rip3*$^{-/-}$ mice are protected from chronic-ethanol-induced liver injury, these data suggest that loss of inhibitory site phosphorylation contributes to injury. Importantly, we also find that phosphorylation of one of the ASK1 inhibitory sites, Ser-1033 (murine Ser-1040), was lower in liver samples from patients with AH and was associated with an increase in phosphorylation of p38 compared to liver from healthy controls. Taken together, these data identify novel changes in inhibitory phosphorylation of ASK1 in response to ethanol that are associated with RIP3-dependent hepatocellular injury.

MATERIALS AND METHODS

Mouse models

Female C57BL/6J mice (8–10 weeks old) were from Jackson Labs (Bar Harbor, ME). *Rip3*$^{-/-}$ mice were obtained from Genentech (San Fransisco, CA).[25] Lieber-DeCarli high-fat ethanol and control diets were purchased from Dyets (Bethlehem, PA). All studies were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Mice were housed in pairs in standard cages with microisolator lids in a 12:12-hour light–dark cycle.

Chronic ethanol feeding

Age-matched mice were randomized into ethanol-fed and pair-fed groups and then adapted to a control liquid diet for 2 days. The ethanol-fed groups were given access to the ethanol-containing diet. Control mice were pair fed diets, which iso-calorically replaced maltose dextrins for ethanol.[11] The feeding model is described in the Supporting Materials and Methods. Liver samples used for phosphoproteomic analysis were archival samples from a published work; Roychowdhury et al.[11] provides specific data on the extent of chronic-ethanol-induced liver injury in C57BL/6 mice and the protective effects of *Rip3* deficiency on markers of liver injury.

Sample preparation and LC/MS-MS measurements

Liver tissue lysis, tryptic digestion, desalting of peptides on C18 Sep-Pak, and phosphopeptide enrichment

In brief, 100 mg of liver tissue was lysed in 2 mL lysis buffer (9 M urea, 200 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES] pH 8, containing Halt Protease and Phosphatase Inhibitor Cocktail [Thermo Scientific, Mannheim, Germany]); digested protein (1 mg) and phosphopeptides were enriched using the Thermo Scientific High-Select Titanium Dioxide (TiO2) kit.[26] This enrichment was followed by label-free analysis on the nLC system coupled to a tribrid linear ion trap/Quadrupole/Orbitrap. The LC/MS system was a Thermo Scientific Ultimate 3000 nano-flow ultra-high performance LC interfaced with a Fusion Lumos MS system. LC/MS data were analyzed using Proteome Discoverer software (version 2.3; Thermo Fisher Scientific, Bremen, Germany). Detailed information on Sample Preparation, measurements, and phosphoprotein identification and quantification is available in the Supporting Materials and Methods.

Quality controls

Details and evaluation of quality controls are included as Supporting Results (Figures S1–S3).

Statistical evaluation

Statistical analysis was performed on phosphorylation sites utilizing their corresponding abundances (precursor ion/MS1 peak area) to identify differentially phosphorylated sites in protein after chronic ethanol feeding in C57BL/6 and *Rip3*$^{-/-}$ *mice*. Group comparisons were made by the unpaired two-tailed *t* test or two-way analysis of variance (ANOVA). For the volcano plots, a two-tailed *t* test was used that compared control pair-fed C57BL/6 to ethanol-fed C57BL/6 or pair-fed *Rip3*$^{-/-}$ to ethanol-fed *Rip3*$^{-/-}$ liver tissues. ANOVA was performed using the general linear models procedure (SAS, Carey, IN) to carry out a comparison of the four groups (ethanol-fed C57BL/6, ethanol-fed *Rip3*$^{-/-}$, pair-fed C57BL/6, and pair-fed *Rip3*$^{-/-}$). Post hoc comparisons were made by least-square means testing. $p < 0.05$ was considered statistically significant. R statistical computing and GraphPad Prism 5.0 (GraphPad Software, Inc.) were used for data visualization. Values shown in all figures represent the mean ± SD. To visualize common and unique phosphorylation events between genotype and ethanol, Venn diagrams were generated using InteractiVenn, a web-based tool. Any redundancies in protein modifications were not counted in Venn diagram analysis.

Ingenuity pathway analysis and alignment of phosphoproteins from mouse in human

Detailed methods on ingenuity pathway analysis (IPA) and alignment of phosphoproteins from mouse in humans can be found in the Supporting Materials and Methods.

Western blot of human liver samples and immunohistochemistry of mouse liver

Detailed methods for western blotting (WB) and immunohistochemistry (IHC) can be found in the Supporting Materials and Methods; the lists of the antibodies for WB and IHC are also available in the Supporting Table.

RESULTS

Characterization of the hepatic phosphoproteome in response to chronic ethanol feeding

Impaired regulation of DR signaling pathways and increased hepatocyte death are associated with the progression of ethanol-induced liver injury.[3,9] To gain insight into the mechanisms by which ethanol disrupts DR signaling, we conducted a quantitative, label-free, proteomic analysis of phosphorylation events in liver of ethanol-fed and pair-fed C57BL/6 and *Rip3*$^{-/-}$ mice. Phosphopeptide enrichment followed by nLC/MS-MS identified and quantified a total of 9785 phosphorylation sites corresponding to 2894 distinct phosphoproteins in the entire cohort (Figure 1A; Table S1). Phosphorylation sites were distributed across serines (pSer) at 77.06%, threonines (pThr) at 20.06%, and tyrosines (pTyr) at 2.88% (Figure 1B). Classification of the 2894 identified phosphoproteins by molecular function identified a broad range of molecular functions that were affected by chronic ethanol feeding (Figure 1C). Importantly, 217 kinases (7.62% of total phosphoproteins) and 53 phosphatases (1.86% of total phosphoproteins) were identified in the analysis of the entire cohort (Figure 1C).

Next, we analyzed the interactions between genotype and chronic ethanol feeding on the hepatic phosphoproteome; volcano plots illustrate the differences in fold expression of phosphoproteins (Figure 2). Principal component analysis (PCA) found differences in the hepatic phosphoproteome between pair-fed and ethanol-fed C57BL/6 and Rip3$^{-/-}$ mice and between genotypes for pair-fed and ethanol-fed mice (Figure S3). *Rip3* deficiency changed phosphorylation of 768 sites from 576 phosphoproteins; phosphorylation was increased on 402 sites (310 phosphoproteins) and decreased on 365 sites (317 phosphoproteins) compared to C57BL/6 mice under pair-fed conditions (Figure 2A). Among the phosphorylation sites up-regulated by *Rip3* deficiency in the pair-fed condition, 26 were kinases (6.5%) corresponding to 19 kinase phosphoproteins, 10 of which localized to the cytoplasm, seven to the nucleus, and two to the plasma membrane. Among the down-regulated phosphorylation sites by *Rip3* deficiency in the pair-fed condition, 30 (8.2%) were kinases corresponding to 22 phosphoproteins, three of which localized to the plasma membrane, 12 to the cytoplasm, six to the nucleus, and one to others. After chronic ethanol feeding,

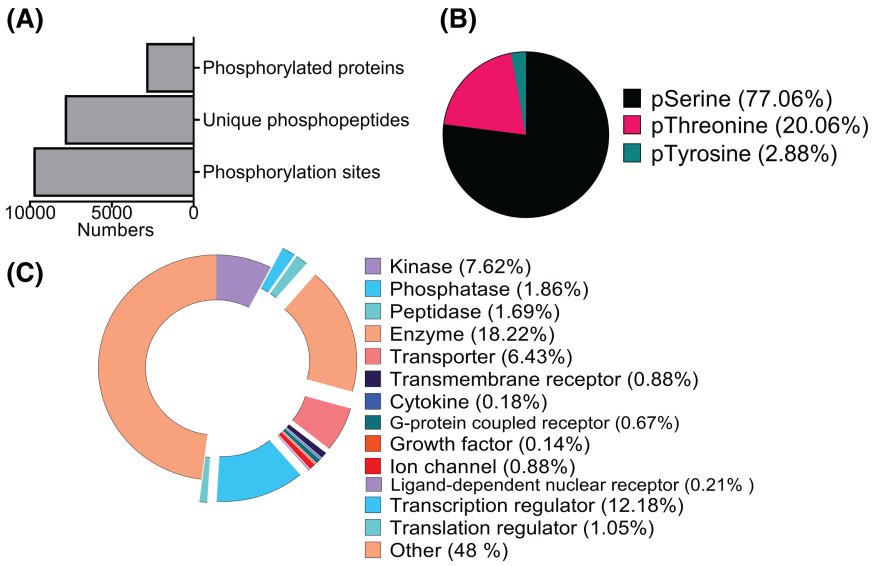


FIGURE 1 Overall characteristics of the hepatic phosphoproteome of C57BL/6 and *Rip3*-deficient (*Rip3*$^{-/-}$) mice. C57BL/6 and *Rip3*$^{-/-}$ mice were allowed free access to a liquid diet containing increasing concentrations of ethanol (1% volume [vol]/vol ethanol for 2 days, 2% ethanol for 2 days, 4% ethanol for 1 week, 5% ethanol for 1 week, and 6% ethanol for 1 week) or pair fed a control diet for 25 days. Proteins from liver lysates were digested and tryptic peptides processed to enrich for phosphopeptides, using the titanium dioxide procedure. Enriched peptides were analyzed by nanoscale liquid chromatography-based mass spectrometry by using a label-free approach. (A) Number of phosphoproteins, phosphopeptides, and phosphorylation sites; (B) distribution of phosphorylation on serine, threonine, and tyrosine residues for all phosphorylation sites; and (C) functional classifications/molecular functions of phosphoproteins identified in liver of ethanol-fed or pair-fed C57BL/6 and *Rip3*$^{-/-}$ mice. p, phosphorylated; Rip3, receptor-interacting protein kinase 1

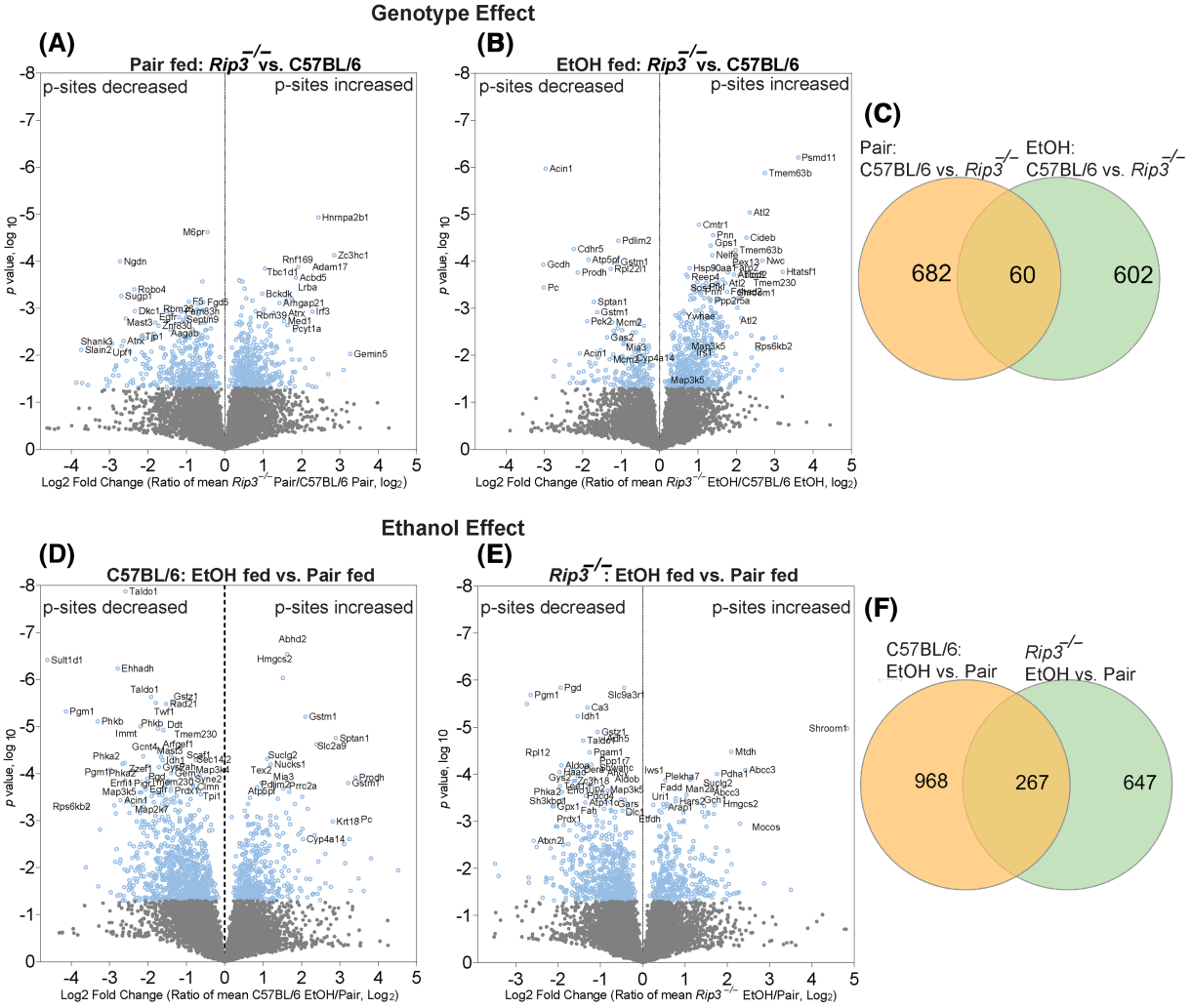


FIGURE 2 Effect of chronic ethanol on hepatic phoshoproteome. C57BL/6 and *Rip3*$^{-/-}$ mice were ethanol fed or pair fed for 25 days, as described in Figure 1. (A,B) Volcano plots of hepatic phosphoproteome comparing genotypes in (A) pair-fed controls (*Rip3*$^{-/-}$, n = 4) compared to C57BL/6 (n = 4) mice and (B) ethanol-fed (*Rip3*$^{-/-}$, n = 6) compared to C57BL/6 (n = 5) mice. (C) Venn diagram displays overlapping and unique phosphorylation sites on phosphoproteins identified between genotypes (*Rip3*$^{-/-}$ vs. C57BL/6) for ethanol-fed and pair-fed control mice. (D,E) Volcano plots of hepatic phosphoproteome in (D) ethanol-fed (n = 5) compared to pair-fed (n = 4) C57BL/6 mice and (E) ethanol-fed (n = 6) compared to pair-fed (n = 4) *Rip3*$^{-/-}$ mice. (F) Venn diagram of common and unique sets of changed phosphorylation sites after chronic ethanol feeding in C57BL/6 (ethanol-fed compared to pair-fed controls) and *Rip3*$^{-/-}$ (ethanol-fed compared to pair-fed controls) mice. In the volcano plots (A–E), phosphorylation sites display distribution of fold change and statistical significance. Each point represents one phosphorylation site and shows the ratio between liver samples of the ethanol-fed to pair-fed groups plotted against the level of statistical significance. Differentially abundant phosphorylation sites ($P \leq 0.05$) are highlighted in blue. Sites represented in gray were not affected by ethanol feeding. The *y* axis shows *P* values (plotted on log10) comparing the abundance of normalized phosphorylation sites. The *x* axis shows the ratio of the mean between the ethanol-fed and pair-fed groups (plotted on log2). EtOH, ethanol; p-site, phosphorylation site; Rip3, receptor-interacting protein kinase 1

Rip3 deficiency changed phosphorylation of 705 sites on 527 unique phosphoproteins; phosphorylation was increased on 528 sites (403 phosphoproteins) and decreased on 177 sites (144 phosphoproteins) compared to C57BL/6 mice (Figure 2B; Table S2). The Venn diagram summarizes the effects of genotype on the identified phosphosites in pair-fed and ethanol-fed conditions (Figure 2C). Among the phosphorylation sites up-regulated by *Rip3* deficiency in the ethanol-fed condition, 40 were kinases (7.6%) corresponding to 34 kinase phosphoproteins, 24 of which localized to the cytoplasm, six to the nucleus, and four to the plasma membrane. Among the down-regulated phosphorylation sites by *Rip3* deficiency, seven (4%) were kinases corresponding to seven phosphoproteins, of which five localized to the cytoplasm and two to the nucleus. Genotype-dependent changes in phosphorylation sites for cellular distribution and functions for the pair-fed (Figure 3A,B) and ethanol-fed (Figure 3C,D) condition are shown in Figure 3.

Next, we identified changes in phosphoproteome in response to chronic ethanol in C57BL/6 (Figure 2D)

and *Rip3*$^{-/-}$ mice (Figure 2E). In C57BL/6 mice, a total of 1317 (~13.45%) phosphorylation sites from 888 phosphoproteins were differentially phosphorylated in ethanol-fed mice compared to pair-fed controls. Phosphorylation was increased at 439 sites (on 320 phosphoproteins) and decreased at 878 sites (on 252 phosphoproteins) (Figure 2D; Table S3). In *Rip3*$^{-/-}$ mice, 948 phosphorylation sites (9.68%) from 682 phosphoproteins were differentially regulated in response to chronic ethanol. Phosphorylation increased at 330 sites (on 267 phosphoproteins) and decreased at 618 sites (on 457 phosphoproteins) in response to chronic ethanol feeding (Figure 2E; Table S4). The Venn diagram summarizes the effects of chronic ethanol feeding on the identified phosphosites in C57BL/6 and *Rip3*$^{-/-}$ mice (Figure 2F). In addition, three circle (Figure S4A) and four circle Venn diagrams (Figure S4B) further illustrating the interactions between genotype and chronic ethanol on the phosphoproteome are presented in Figure S4.

Genotype-dependent (Figure 3A,C) and chronic-ethanol-induced (Figure 3E,G) changes in phosphorylation sites were distributed between multiple cellular compartments, with plasma membrane, cytoplasm, and nucleus representing the dominant compartments. Among the phosphorylation sites up-regulated by chronic ethanol in C57BL/6 mice, 15 (3.4%) were kinases corresponding to 13 kinase phosphoproteins, nine of which localized to the cytoplasm, three to the nucleus, and one to the plasma membrane. Among the

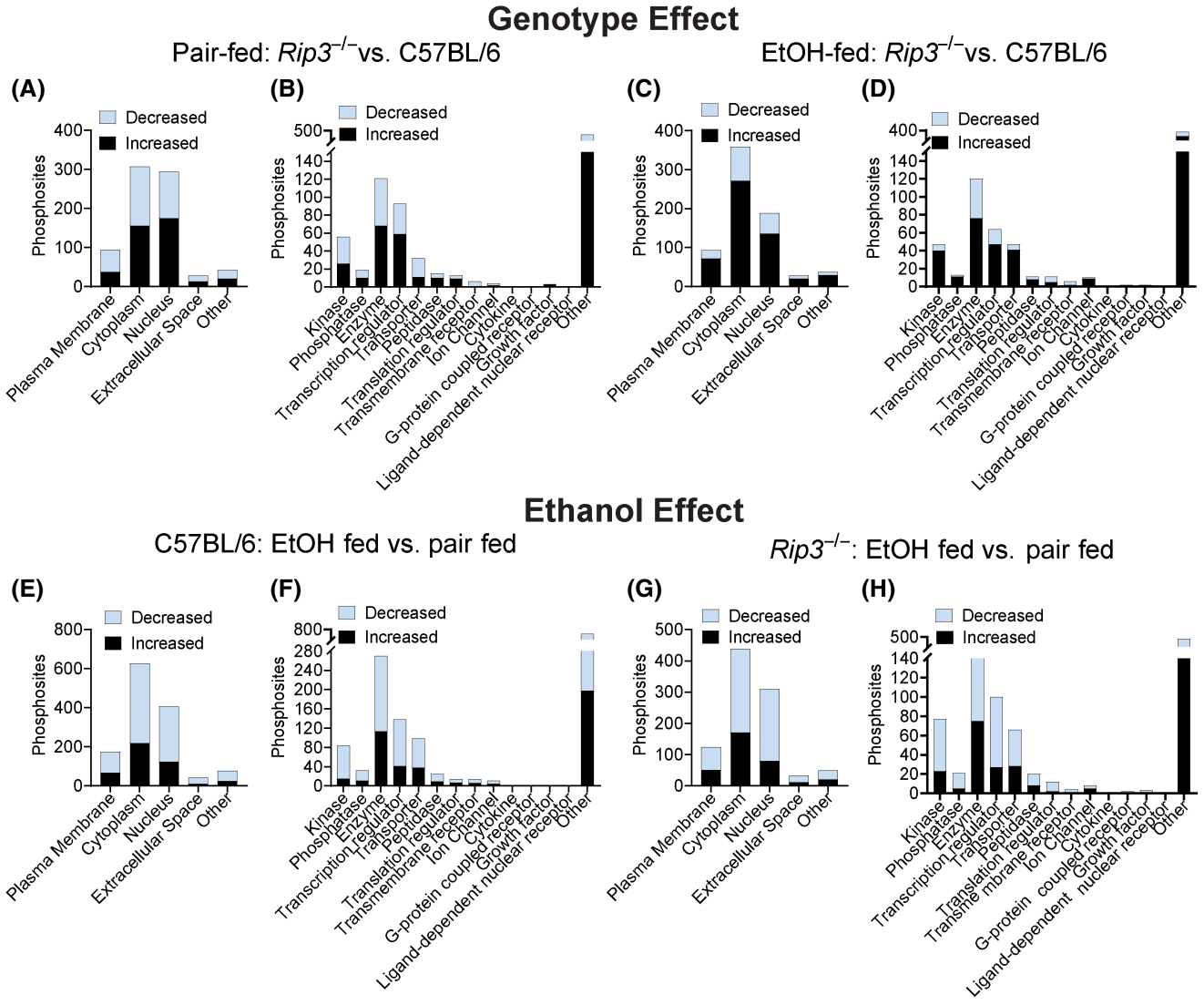


FIGURE 3 Subcellular distribution of phosphoproteins and type of phosphoprotein in response to chronic ethanol in C57BL/6 and *Rip3*-deficient mice. Differentially regulated phosphorylation sites in between genotypes for (A,B) pair-fed, (C,D) ethanol-fed, and in (E/F) C57BL/6 and (G/H) *Rip3*$^{-/-}$ mice were assigned a molecular classification with the use of the Ingenuity Knowledge Base (Qiagen) molecule option to infer the (A/C/E/G) subcellular compartment and (B/D/F/H) functions/types of genes. EtOH, ethanol; Rip3, receptor-interacting protein kinase 1

down-regulated phosphorylation sites in C57BL/6 mice, 68 (8%) were kinases corresponding to 47 phosphoproteins (Figure 3F), five of which localized to the plasma membrane, 31 to the cytoplasm, and 10 to the nucleus. The distribution of phosphorylation sites was similar in *Rip3*$^{-/-}$ mice, with 7% of up-regulated sites on kinases (23 sites) corresponding to 21 kinase proteins, 14 of which localized to the cytoplasm, two to the plasma membrane, and five to the nucleus. Among sites showing decreased phosphorylation, 8.7% were kinases (54 sites) corresponding to 34 kinase proteins, 23 of which localized to cytoplasm, four to plasma membrane, and seven to nuclei (Figure 3H).

IPA analysis of proteins with dysregulated phosphorylation sites in liver of ethanol-fed and pair-fed C57BL/6 and *Rip3*$^{-/-}$ mice

To investigate the functional significance of chronic ethanol-induced changes in the hepatic phosphoproteome, over-represented pathways were identified by IPA analysis. We first analyzed the impact of *Rip3* deficiency within pair-fed controls and chronic-ethanol-fed groups for genotype-dependent effects. Multiple signaling and metabolic pathways were affected (Benjamini-Hochberg false discovery rate, $p < 0.05$) in pair-fed (Figure 4A) and ethanol-fed mice (Figure 4B). Interestingly, differential responses in phosphoproteins between genotypes (*Rip3*$^{-/-}$ vs. C57BL/6) in response to chronic ethanol (Figure 2B) showed enrichment for several pathways, including DR signaling, apoptosis, and 14-3-3-mediated and p-38 MAPK pathways (Figure 4B). In contrast, fewer pathways were enriched when comparing the impact of genotype (*Rip3*$^{-/-}$ vs. C57BL/6) in pair-fed controls and, in particular, cell death pathways were not affected (Figure 4A).

Multiple pathways were also affected after chronic ethanol feeding in C57BL/6 (Figure 4C; Table 1A) and *Rip3*$^{-/-}$ mice (Figure 4D; Table 1B). Sixty percent of the pathways were modulated by at least two or more associated kinases.

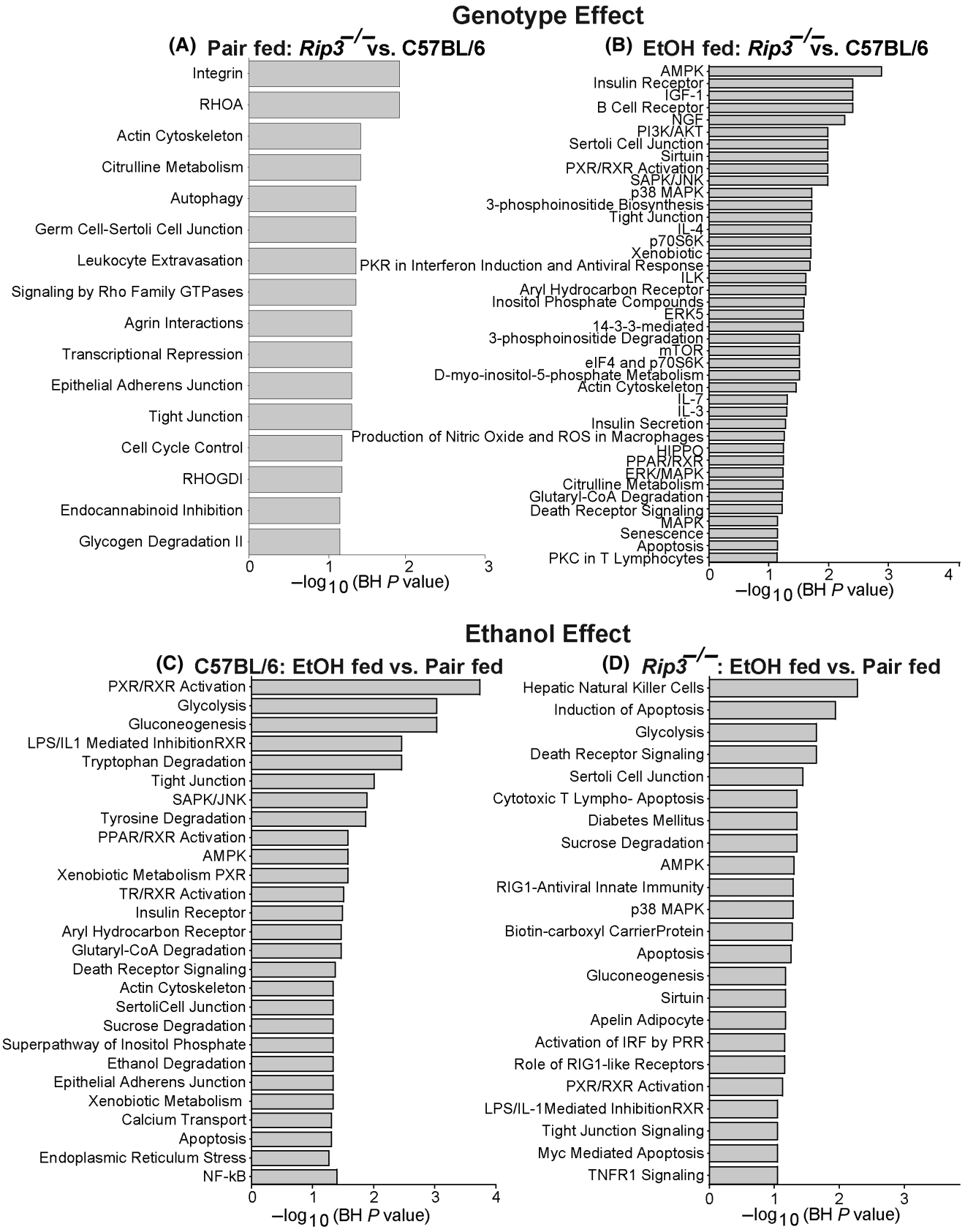
Multiple signaling pathways regulating cell death were dysregulated. While chronic ethanol affected DR signaling in both genotypes, the specific pathways affected were dependent on genotype. In particular, chronic ethanol feeding of C57BL/6 mice altered phosphorylation of proteins involved in DR pathways, apoptosis, and stress-activated protein kinase/JNK (SAPK/JNK) signaling pathways (Table 1A). The specific phosphorylation sites targeted by chronic ethanol in C57BL/6 mice are shown in Table 2. In contrast, pathways associated with apoptosis were more prominently regulated by chronic ethanol in *Rip3*$^{-/-}$ mice, including induction of apoptosis, cytotoxic T lymphocyte-mediated apoptosis of target cells, apoptosis and myc-mediated apoptosis, and DR signaling and TNFR1 pathways (Figure 4D; Table 1B). Specific phosphorylation sites affected by chronic ethanol in *Rip3*$^{-/-}$ mice are listed in Table 3.

Effect of chronic ethanol feeding on the DR signaling phosphorylation network in C57BL/6 and *Rip3*$^{-/-}$ mice

Because several pathways were involved in cell death, we next filtered all cell death-related phosphoproteins for more in-depth analysis. Phosphorylation sites regulated in response to chronic ethanol were differentially distributed across cell death networks in C57BL/6 (Figure 5A) and *Rip3*$^{-/-}$ mice (Figure 5B). Volcano plots illustrate the impact of chronic ethanol feeding on this subset of phosphorylation sites from 70 phosphoproteins in cell death networks in C57BL/6 (Figure 6A) and *Rip*$^{-/-}$ mice (Figure 6B) as well as the direct comparison for these subsets of phosphoproteins between genotypes in response to chronic ethanol feeding (Figure 6C). Twenty-three of these cell death-related phosphoproteins were kinases (32.8%), including MAPK (MAP3K5, MAP2K7, MAP4K4, and MAPK 1/3), members of the RIP family (RIP1 and RIP2), and the IkappaB kinase (IKK) complex, a core element of the nuclear factor kappa B (NF-κB) signaling cascade.

Among phosphoproteins annotated to the "kinase" protein class in the DR signaling pathway, the peptide most differentially phosphorylated (Figure 6A–C) during ethanol feeding was MAP3K5/ASK1 (TLFLGIPDENFEDHS (pS1036) APPS(pS1040)PEEK) (Figure 7C). The ASK1 phosphorylation sites S1036 and S1040 identified in this analysis are not in the activation loop of the kinase domain but are located close to the C-lobe coiled-coil domain[27] (Figure 7B). Phosphorylation of S1040 (corresponding to human S1033/1034) negatively regulates ASK1 activity, suggesting that it is an inhibitory phosphorylation site.[23] Chronic ethanol lowered the frequency of this inhibitory phosphorylation event in both C57BL/6 and *Rip3*$^{-/-}$ mice compared to pair-fed controls. However, chronic ethanol had less effect in *Rip3*$^{-/-}$ compared to C57BL/6 mice (Figure 7C,D).

Chronic ethanol also affected MAPKs downstream of ASK1; phosphorylation at S77/T82 on Map2k7, a specific activator of JNK, was decreased while phosphorylation at S794 on MAP4K4, a kinase that activates JNK, was increased in C57BL/6 mice compared to pair-fed controls. However, chronic ethanol had no effect on these phosphorylation sites in *Rip3*$^{-/-}$mice (Tables 2 and 3). Because ASK1 is regulated both by phosphorylation and protein–protein interactions (as a multiprotein complex called the signalosome), we next filtered for known ASK1 interacting proteins that are regulated by phosphorylation. Proteins suppressing ASK1 activity (e.g., 14-3-3 family proteins, RAF proto-oncogene serine/threonine-protein kinase, and thioredoxin reductase 3) and interacting proteins known to enhance ASK1



activity are listed in Table S1. Of these multiple ASK1-interacting proteins, chronic ethanol only affected the phosphorylation of the 14-3-3 epsilon isoform (Table S1; Figure S5), suggesting that chronic ethanol may also regulate ASK1 activity through changes to the phosphorylation state of ASK1-interacting proteins.

FIGURE 4 Top canonical pathways identified by IPA of the quantitative hepatic phosphoproteome in response to chronic ethanol in C57BL/6 and *Rip3*-deficient mice. IPA core analysis of phosphorylation sites in liver of (A) pair-fed controls between genotypes, (B) ethanol-fed mice between genotypes, (C) in response to chronic ethanol feeding in C57BL/6 and (D) *Rip3*-deficient mice with increased or decreased phosphorylation ($p < 0.05$). *P* values show the significance of enrichment for the most highly expressed genes. Fisher's exact test *P* values (hypergeometric test, $p < 0.05$) were corrected for multiple testing, using the Benjamini-Hochberg false discovery rate. AKT, protein kinase B; AMPK, adenosine monophosphate–activated protein kinase; B-H, Benjamini-Hochberg; CoA, coenzyme A; eIF4, eukaryotic initiation factor 4; ERK, extracellular signal-regulated kinase; EtOH, ethanol; GTPase, guanosine triphosphatase; IGF, insulin-like growth factor; IL, interleukin; ILK, integrin-linked kinase; IPA, Ingenuity Pathway Analysis; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor kappa B; NGF, nerve growth factor; p70S7K, protein 70 serine 6 kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKR, protein kinase R; PPAR, peroxisome proliferator-activated receptor; PRR, pattern recognition receptor; PXR, pregnane X receptor; RHOA, Ras homolog family member A; RHOGDI, Rho guanosine diphosphate dissociation inhibitor; RIG1, retinoic acid-inducible gene; Rip3, receptor-interacting protein kinase 1; ROS, reactive oxygen species; RXR, retinoid X receptor; SAPK, stress-activated protein kinase; TNFR, tumor necrosis factor receptor; TR, thyroid hormone receptor

TABLE 1 Cell death-relevant signaling pathways identified by IPA analysis and their relevant protein components from liver of C57BL/6 and *Rip3*$^{-/-}$ mice induced by chronic ethanol

Pathways	–log(B-H *P* value)	Identified components (molecules involved in the pathway)	Phosphorylation sites up-regulated	Phosphorylation sites down-regulated
C57BL/6:ethanol fed versus pair fed				
SAPK/JNK signaling	1.9	CRK,IRS1,MAP2K7,MAP3K3,MAP3K4,MAP3K5,MINK1,NFATC1,PIK3C2A,RIP1,SOS1 (102)	9	20
Death receptor signaling	1.38	ACIN1,CASP3,CASP7,IKBKG,LMNA,MAP2K7,MAP3K5,RIP1,SPTAN1 (91)	9	14
Apoptosis signaling	1.32	ACIN1,AIFM1,CASP3,CASP7,IKBKG,LMNA,MAP2K7,MAP3K5,SPTAN1(99)	13	14
Rip3$^{-/-}$:ethanol fed versus pair fed				
Induction of apoptosis by HIV1	1.95	CASP3,DFFA,FADD,FAS,IKBKE,MAP3K5,RIP1,SLC25A13 (61)	6	10
Death receptor signaling	1.66	ACIN1,CASP3,DFFA,FADD,FAS,GAS2,IKBKE,MAP3K5,RIP1 (91)	10	13
Cytotoxic T lymphocyte-mediated apoptosis of target cells	1.36	CASP3,DFFA,FADD,FAS,HLA-A (34)	2	5
Apoptosis signaling	1.27	ACIN1,AIFM1,CASP3,DFFA,FAS,GAS2,IKBKE,MAP3K5 (99)	10	17
Myc-mediated apoptosis signaling	1.06	CASP3,FADD,FAS,IKBKE,PRKAG2 (50)	7	7
TNFR1 signaling	1.06	CASP3,FADD,IKBKE,PAK4,RIPK1 (50)	6	8
p38 MAPK signaling	1.30	EEF2K,FADD,FAS,HMGN1,MAP2K3,MAP3K5,MAPT,MEF2A,RPS6KC1 (118)	11	15

Abbreviations: HIV, human immunodeficiency virus; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Rip3, receptor-interacting protein kinase 1; SAPK, stress-activated protein kinase; TNFR, tumor necrosis factor receptor.

Effect of chronic ethanol on phosphorylation/activation of p38 MAPK in C57BL/6 and *Rip3*$^{-/-}$ mice

To assess the impact of decreased inhibitory phosphorylation on ASK1 in response to chronic ethanol, we evaluated the phosphorylation of p38 (a downstream target of ASK1) at Thr180/Tyr182, a phosphorylation site leading to activation of p38. Phosphorylated (phospho)-Thr180/Tyr182-p38 immunoreactivity was increased after chronic ethanol feeding in liver of C57BL/6 mice (Figure 8A), consistent with the loss of ASK1 inhibitory phosphorylation. Chronic ethanol also increased immunoreactive phospho-Thr180/Tyr182-p38 in liver of *Rip3*$^{-/-}$ mice; however, the response was lower in *Rip3*$^{-/-}$ compared

TABLE 2 Differentially regulated phosphorylation sites in C57BL/6 mice associated with death receptor signaling

Phosphorylations in proteins	Protein	Gene	Sequence	Number of PSMs	*P* value	Relative abundance log_2 FC (C57BL/6:EtOH fed/pair fed)	Regulated (in EtOH fed relative to pair fed)
Kinases							
O35099 S1036;S1040	Mitogen-activated protein kinase kinase kinase 5	Map3k5	TLFLGIPDENFEDHSAPPSPEEK	23	0.0004	–1.56	Down
O35099 S1040			TLFLGIPDENFEDHSAPPSPEEK	56	0.016	–0.53	Down
Q8CE90 S77;T82	Dual specificity mitogen-activated protein kinase kinase 7	Map2k7	SPSSESSPQHPTPPTRPR	2	0.0005	–2.11	Down
Q60855 S313	Receptor-interacting serine/threonine-protein kinase 1	Rip1	EYPDQSPVLQR	43	0.019	–0.32	Down
P97820 S794	Mitogen-activated protein kinase kinase kinase kinase 4	Map4k4	AASSPNLSNGETESVK	1	0.040	1.64	Up
O88522 S380	Nuclear factor kappa B essential modulator	Ikbkg	SPPEEPPDFCCPK	19	0.024	0.81	Up
Peptidases							
P70677 S26	Caspase-3	Casp3	SVDSGIYLDSSYK	13	0.0140	–1.98	Down
P97864 ST	Caspase-7	Casp7	MTDDQDCAAELEKVDSSSEDGVDAKPDR	7	0.004	1.06	Up
Caspase 3 substrates							
Q8CE90 S	Spectrin alpha chain, nonerythrocytic 1	Sptan1	SPSSESSPQHPTPPTRPR	28	0.021	–0.80	Down
P16546 S1217			SLQQLAEER	95	0.012	0.43	Up
Q9JIX8 S561	Apoptotic chromatin condensation inducer in the nucleus	Acin1	RASHALFPEHSGK	6	0.0004	–2.72	Down
Q9JIX8 S825			KISVVSATK	9	0.015	–1.77	Down
P48678 S390;S392	S390;S392 Prelamin-A/C	Lmna	LRLSPSPTSQR	90	0.009	0.60	Up

Note: Data are n = 6 ethanol-fed C57BL/6 mice versus four pair-fed C57BL/6 mice; $p < 0.05$.

Abbreviations: EtOH, ethanol; FC, fold change; PSM, peptide spectra matched.

TABLE 3 Differentially regulated phosphorylation sites in *Rip3*$^{-/-}$ mice associated with death receptor signaling

Phosphorylation in proteins	Protein	Gene		# PSMs	*P* value	Relative abundances $\log_2$ FC (*Rip3*$^{-/-}$:EtOH fed/pair fed)	Regulated (in *Rip3*$^{-/-}$ EtOH fed relative to *Rip3*$^{-/-}$ pair fed)
Kinases							
O35099 S1036;S1040	Mitogen-activated protein kinase kinase kinase 5	Map3k5	TLFLGIPDENFEDHSAPPSPEEK	23	0.0004	–0.77	Down
Q60855 S336	Receptor-interacting serine/threonine-protein kinase 1	Rip1	SNSEQPGSLHSSQGLQMGPVEESWFSSSPEYPQDENDR	6	0.024	–0.39	Down
Q9R0T8 S713	Inhibitor of nuclear factor kappa B kinase subunit epsilon	Ikbke	LHRVPSAPDV	3	0.020	1.67	Up
Q61160 S191	FAS-associated death domain protein	Fadd	SENMSPVLR	43	0.0004	0.57	Up
Peptidases							
P70677 S26	Caspase-3	Casp3	SVDSGIYLDSSYK	13	0.02	–1.03	Down
O89110 S188	Caspase-8	Casp8	MSLEGR	39	0.04	–0.74	Down
Caspase 3 substrates							
O54786 S314	DNA fragmentation factor subunit alpha	Dffa	SPLPGEPQRPK	37	0.0008	–0.66	Down
Q9JIX8 S655;S657	Apoptotic chromatin condensation inducer in the nucleus	Acin1	SLSPGVSR	45	0.001	–0.64	Down
Q9JIX8 S479;S482			SLSPLSGTTDTK	6	0.043	–0.54	Down
Q9JIX8 S710			HLSHPEPEQQHVIQR	87	0.003	–0.82	Down
Q9JIX8 S413;T417			APVVLQPEQIVSEEETPPPLLTK	63	0.04	–0.29	Down
P11862 S186;S	Growth arrest-specific protein 2	Gas2	EIEQEETLSAPSPSPSPSSK	4	0.002	–2.34	Down
P25446 S 220;S	Tumor necrosis factor receptor superfamily member 6	Fas	ETIPMNASNLSLSK	31	0.02	–1.17	Down

Note: Data are n = 5 ethanol-fed Rip3−/− versus four Rip3−/− pair-fed mice; $p < 0.05$.

Abbreviations: EtOH, ethanol; FC, fold change; PSM, peptide spectra matched; Rip3, receptor-interacting protein kinase 1.

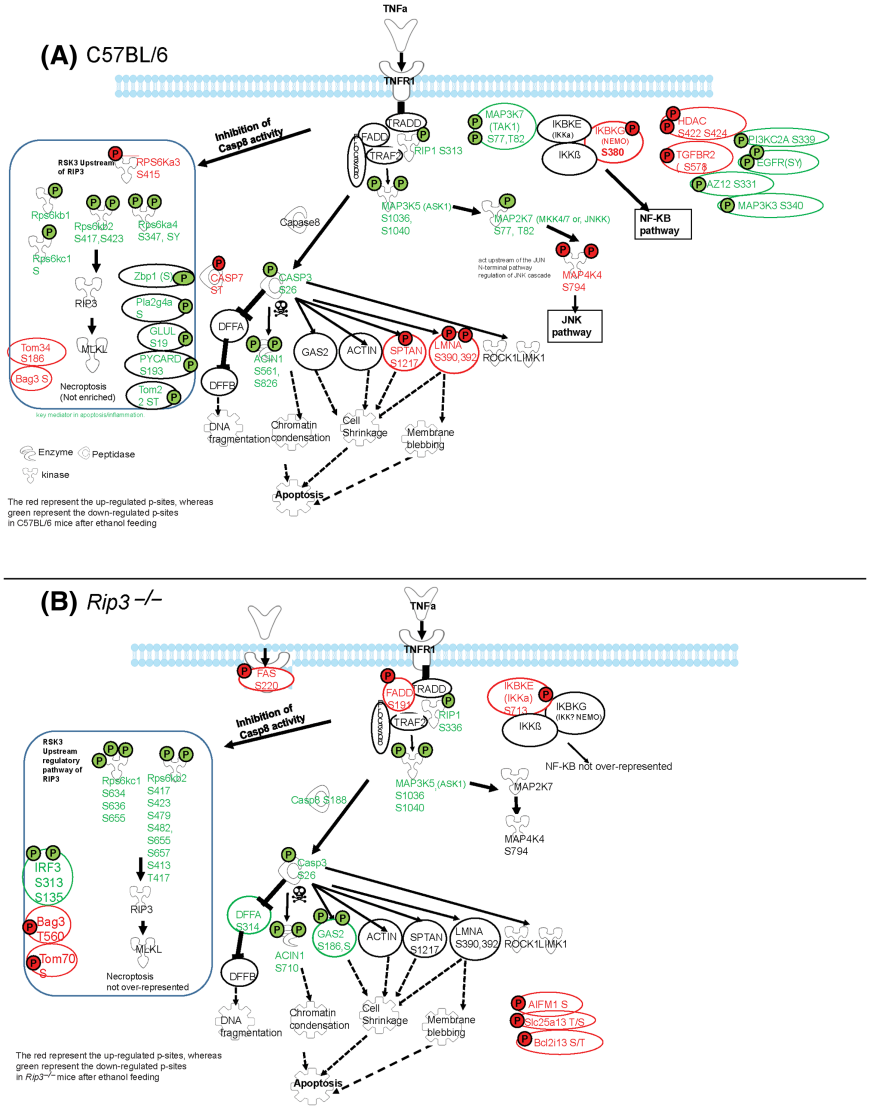


FIGURE 5 Site-specific protein phosphorylation map of death receptor signaling pathways in response to chronic ethanol in C57BL/6 and *Rip3*-deficient mice. A regulated phosphorylation pathway/network of TNFα/TNFR1 signaling was constructed based on Ingenuity Pathway Analysis and literature searches to illustrate the effects of chronic ethanol on the major pathways of DR signaling in (A) C57BL/6 and (B) *Rip3*$^{-/-}$ mice. The map includes DR signaling pathways affected by ethanol (Figure 4), including apoptosis, NF-κB, p38 MAPK, SAPK/JNK, and necroptosis. Phosphoproteins containing phosphorylation sites displaying statistical significance $p < 0.05$ changes in abundance ranging from 1.2-fold to 8-fold were included and annotated with their phosphorylation sites, color-coded to indicate significant increases (red), decreases (green), or no change (black) in phosphorylation responses. DR, death receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; Rip3, receptor-interacting protein kinase 1; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor

to C57BL/6 mice (Figure 8A). Histologic analysis of liver sections from C57BL/6 mice indicated that chronic ethanol feeding increased phospho-p38 primarily in hepatocytes, with some staining observed in nonparenchymal cells (Figure 8A). Phospho-p38 was localized to centrilobular areas with clear cytoplasmic staining in pair-fed mice, with minimal staining observed in the periportal region (Figure 8A1). In contrast, phospho-p38 immunoreactivity shifted to predominantly periportal regions in liver of chronic ethanol-fed mice, with minor staining in the centrilobular zone (Figure 8A2,A3). Phospho-p38 immunoreactivity was very low in *Rip3*$^{-/-}$ mice but was minimally detectable in periportal regions after chronic ethanol (Figure 8A).

ASK1 and p38 MAPK expression and phosphorylation in liver of patients with AH

The relevance of inhibitory phosphorylation to ALD in patients was evaluated in liver explants from patients with AH undergoing liver transplant compared to healthy donors. Inhibitory phosphorylation at S1033/S1034 (corresponding to the murine S1040) as well

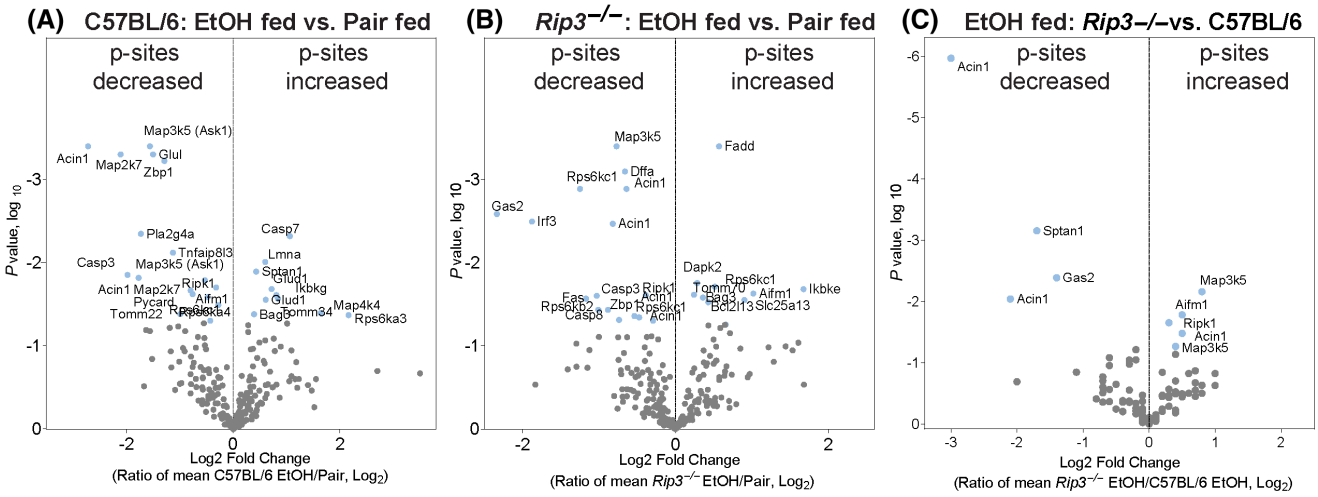


FIGURE 6 Volcano plots of dysregulated phosphosites associated with DR signaling. Volcano plots of changes in phosphosites of proteins in the DR signaling pathway after chronic ethanol feeding in (A) C57BL/6 and (B) *Rip3*$^{-/-}$ mice and (C) between chronic ethanol-fed *Rip3*$^{-/-}$ and C57BL/6 mice. *P* values (–log base 10) are plotted as a function of phosphosite ratio (log base 2) for (A,B) ethanol-fed compared to pair-fed mice or (C) between genotypes. DR, death receptor; EtOH, ethanol; Rip3, receptor-interacting protein kinase 1

as expression of ASK1 protein were decreased in liver of patients with AH compared to healthy controls (Figure 8B), consistent with previous data on expression of ASK1 transcripts.[28] In contrast, another inhibitory phosphorylation site, S1029 human (corresponding to the murine S1036), was higher in liver of patients with AH compared to healthy controls. Phosphorylation of Thr838 (corresponding to the murine Thr845), the phosphorylation site in the activation loop of the kinase domain,[29] was also higher in liver from patients with AH compared to healthy controls (Figure 8B). Of note, phosphorylation at Thr845 on ASK1 was detected in the murine MS data set, probably due to sample complexity, ion suppression, and/or background interference.

To understand the integrated effects of these multiple changes in the inhibitory and activating phosphorylation sites on ASK1, we assessed expression and phosphorylation of p38 MAPK, a downstream target of ASK1. Western blot analysis revealed that while p38 expression was lower in liver of patients with AH compared to healthy controls, phosphorylation of p38 at its activation site (Thr180/Tyr182) was higher in AH compared to healthy controls (Figure 8C). These data suggest that the balance of changes in inhibitory (S1029/S1033) and activating (Thr838) phosphorylation of ASK1 leads to enhanced ASK1 activity in the liver of patients with AH (Figure 8).

DISCUSSION

Cell death is a major driver of the progression of ALD. However, the role of different cell death pathways is understudied.[30] While the contributions of *Rip3*, a mediator of necroptosis, to liver injury varies depending on the insult,[17,31,32] *Rip3* is known to contribute to chronic ethanol-induced liver injury.[11,12] To better understand the mechanisms of *Rip3*-dependent ethanol-induced liver injury, the current study compared changes in the global phosphoproteome after chronic ethanol feeding in liver of C57BL/6 (Figures 2B,D and 4B,C) and *Rip3*$^{-/-}$ (Figures 2B,E and 4B–D) mice and revealed major differences in the phosphorylation patterns and associated pathways between genotypes. Our study highlights the dysregulation in TNF-induced DR signaling pathways (Figure 5) after chronic ethanol and identifies differences in phosphorylation of ASK1 at S1036 (S1029 human) and S1040 (S1029 human), sites that negatively control ASK1 activity. Chronic ethanol feeding lowered the frequency of this inhibitory phosphorylation event in both C57BL/6 and *Rip3*$^{-/-}$ mice compared to pair-fed controls, with less reduction in *Rip3*$^{-/-}$ compared to C57BL/6. No differences in inhibitory phosphorylation of ASK1 were observed between genotypes in pair-fed C57BL/6 and *Rip3*$^{-/-}$ mice, indicating that differences in inhibitory phosphorylation of ASK1 were due to chronic ethanol feeding rather than a direct effect of genotype. ASK1 appears to be an important target of chronic ethanol as the phosphorylation of both inhibitory and activating sites on ASK1 were different in liver from patients with AH compared to healthy controls. The net impact of these changes in ASK1 phosphorylation was associated with increased phosphorylation of p38, a downstream target of ASK1 implicated in the progression of ALD,[24] in patients with AH and mice after chronic ethanol feeding (Figure 8C).

Our study first determined the impact of chronic ethanol on global dysregulation of protein phosphorylation (Figure 2D) in liver of C57BL/6 mice. Kinases and phosphatases were among the most affected classes

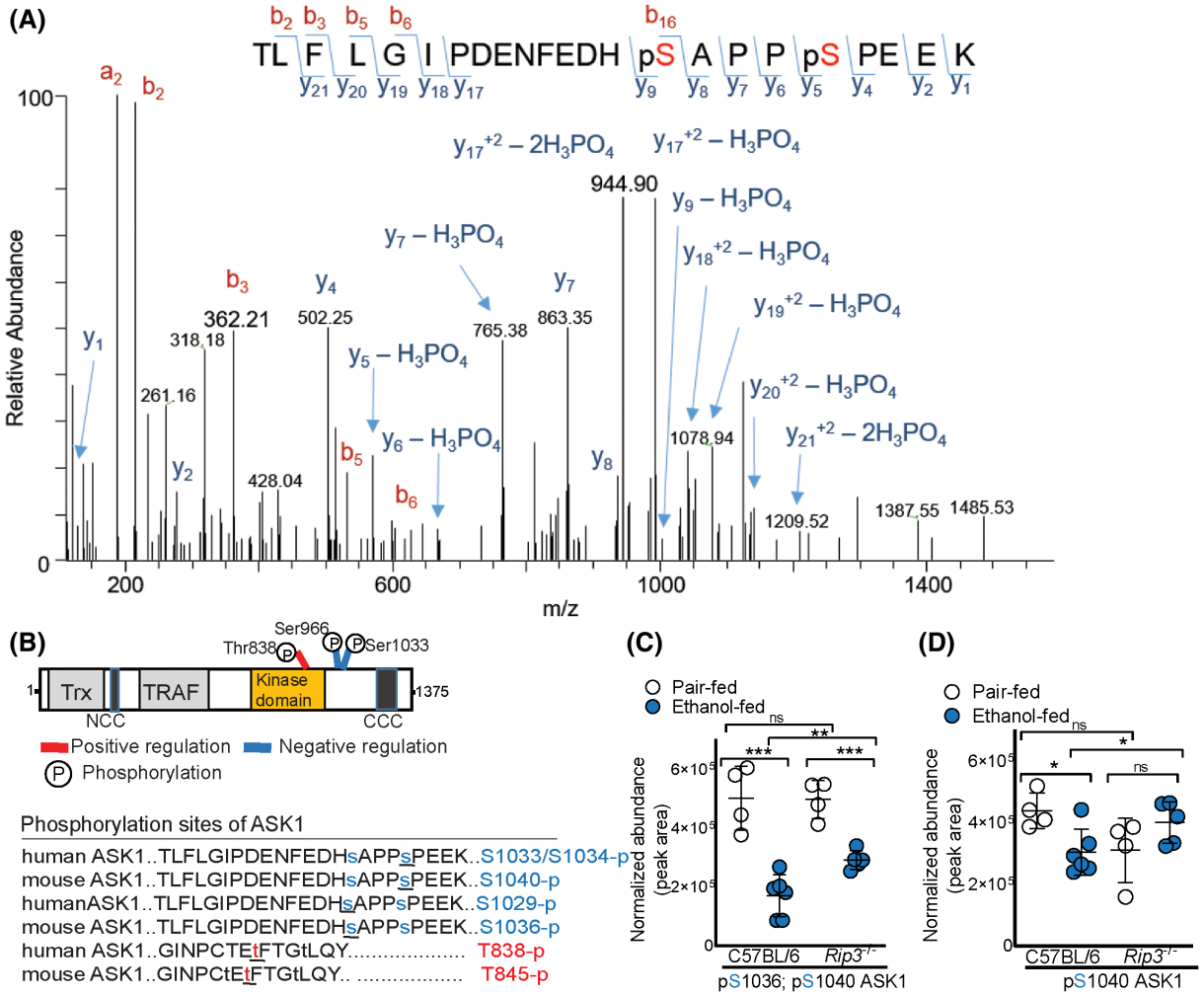


FIGURE 7 Characterization of hepatic ASK1 phosphorylation in response to chronic ethanol in C57BL/6 and *Rip3*-deficient mice. (A) Representative MS-MS spectrum of phosphorylated ASK1 peptide; MS-MS spectrum of the S1036 and S1040 ASK1 phosphorylation sites containing the m/z values and abundances of peptide ion products. A longer series of contiguous y and b ions shows a higher probability of correct identification. Phosphorylated residues are depicted in red for S1036 and S1040. (B) Schematic representation of ASK1 structure and regulation by phosphorylation. The kinase domain is represented in the central region of the molecule. Phosphorylation of Thr838 human (Thr845 mouse) activates ASK1 (positive regulation shown in red), and phosphorylation of the serine residues at position S1033/S1034 is associated with inhibition of ASK1 activity (negative regulation shown in blue). (C,D) Quantity of individual ASK1 phosphopeptides containing (C) S1036 and S1040 and (D) S1040 in response to chronic ethanol in C57BL/6 and *Rip3*-deficient mice. Values represent means + SD; *P* values were assessed by two-way analysis of variance as described in Materials and Methods. $p < 0.05$ for ethanol-fed compared to pair-fed within each genotype. $p < 0.05$ for ethanol-fed between genotypes. *P* value not significant for pair-fed controls between genotypes; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ASK1, apoptosis signal-regulating kinase 1; H_3PO_4, orthophosphoric acid; MS-MS, tandem mass spectrometry; ns, not significant; Rip3, receptor-interacting protein kinase 1; S/Ser, serine; Thr, threonine; TRAF, tumor necrosis factor receptor–associated factor; Trx, thioredoxin

of phosphoproteins (Figure 3F), consistent with a critical role for the regulation of protein phosphorylation in the response to chronic ethanol. These changes in the phosphoproteome influenced multiple pathways, including metabolism and oxidative stress-related effects (Figure 4C). Many of the identified pathways, such as pregnane X receptor, AMPK, peroxisome proliferator-activated receptor/retinoic X receptor, and endoplasmic reticulum stress signaling, are linked with ethanol-induced injury and/or ALD in previous studies.[33–36] Multiple DR signaling pathways, also associated with chronic ethanol-induced liver injury,[31] were dysregulated in response to chronic ethanol feeding in C57BL/6 mice (Figure 4C).

The impact of chronic ethanol feeding on *Rip3*-deficient mice was distinct from C57BL/6 mice. For example, the SAPK/JNK pathway was impacted by chronic ethanol in C57BL/6 (Figure 4C) but not in *Rip3*$^{-/-}$ mice (Figure 4D), consistent with our previous report that ethanol-induced JNK activation was reduced in *Rip3*$^{-/-}$ mice compared to C57BL/6 mice.[11] Multiple distinct pathways of apoptosis (a more benign pathway of cell death) were overrepresented in *Rip3*$^{-/-}$ mice (Figure 4D), consistent with a previous study

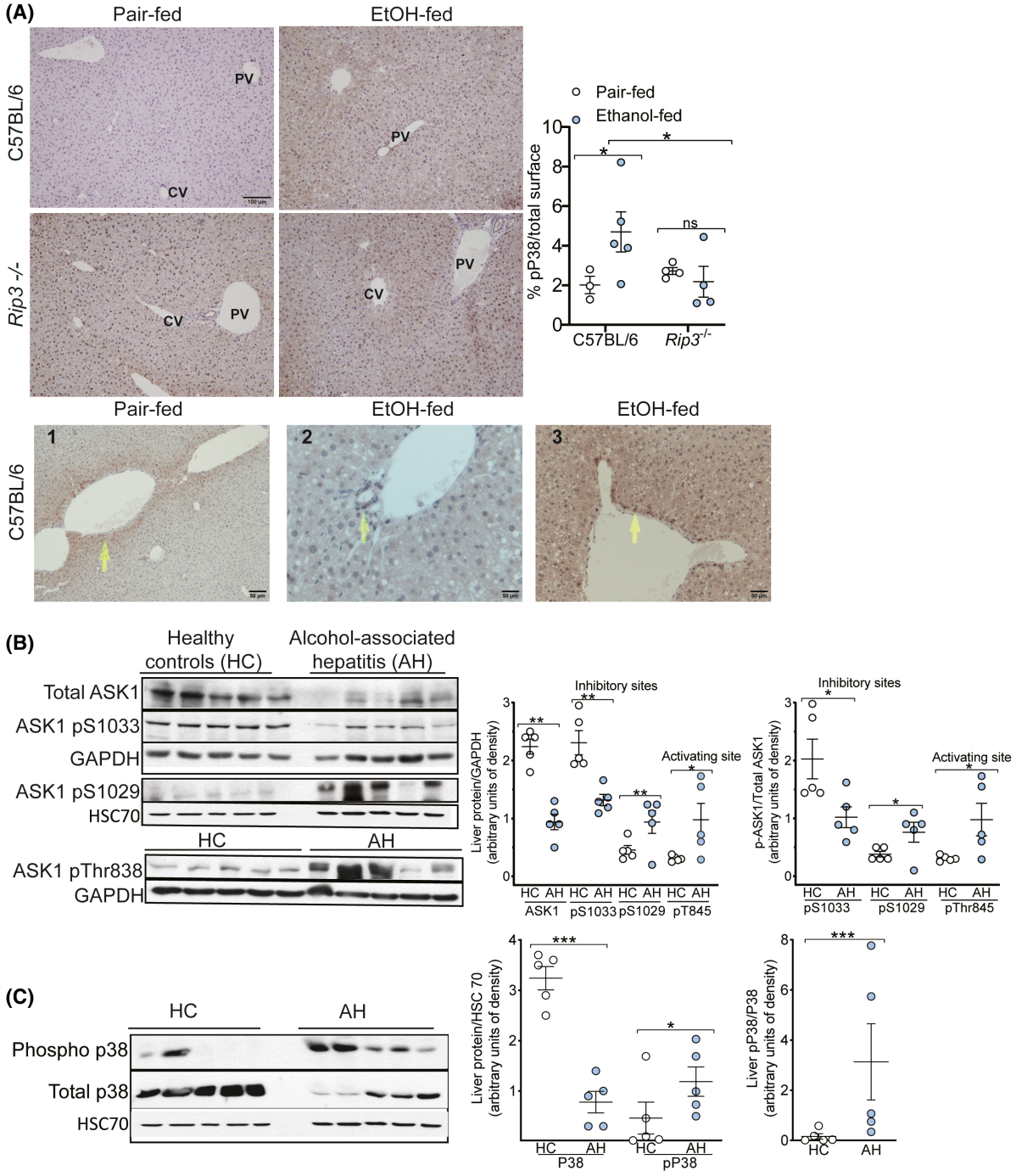


FIGURE 8 ASK1 and its downstream target p38 in liver from C57BL/6 and *RIP3*-deficient mice exposed to a chronic ethanol diet and in patients with AH compared to healthy controls. (A) C57BL/6 and *Rip3*-deficient mice were allowed free access to ethanol or pair-fed control diets. Paraffin-embedded livers were deparaffinized, followed by immunodetection of phosphorylation of p38 at Thr180/Tyr182, a phosphorylation site leading to activation of p38. Scale bar is shown at 100 μm. Localization of positive staining was assessed by a certified pathologist and quantified using Image Pro-Plus software, excluding any tissue edges from the quantification. Zoomed images (scale bar shown at 50 μm) from (A1) pair-fed C57BL/6 mice, immunoreactivity around central vein hepatocytes (arrow); (A2) ethanol-fed C57BL/6 mice, immunoreactive phospho-p38 in periportal area (arrow); and (A3) a few localized inflammatory cells, centrilobular hepatocytes (arrow). (B,C) Liver from patients undergoing liver transplant for severe AH or liver explants from healthy controls were used for western blot analysis of (B) expression and phosphorylation (activation and inhibitory) of hepatic ASK1 and (C) its downstream target p38 MAPK (Thr180/Tyr182). GAPDH and HSC70 were used as loading controls. Representative images are shown. Values represent means ± SD, n = 5 per group; *P* value was assessed by the Student *t* test. Statistical evaluation was performed by the Student *t* test or analysis of variance with post hoc test for multiple comparisons. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. AH, alcohol-associated hepatitis; ASK1, apoptosis signal-regulating kinase 1; HC, healthy control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSC70, heat shock cognate protein 70; MAP, mitogen-activated protein; ns, not significant; Phospho, phosphorylated; Rip3, receptor-interacting protein kinase 1; S/Ser, serine; Thr, threonine; Tyr, tyrosine

TABLE 4 Function of the phosphorylation sites associated with cell death signaling and effect of phosphorylation on the activity of proteins and function

Phosphorylation sites related to DRS	Protein function	Effect of phosphorylation on protein function (role in ALD)
Protein kinases		
MAP3K5 or, ASK1 S1040 S1033 human S1036 S1029 human	Activation determines cell fate	S1040 negatively regulates activity[23] of ASK1 kinase/inhibits cell death-promoting activity
Map2k7 S77; T82 S61;T66 human	Component of SAP/JNK	Not reported in ALD
MAP3K3 S340	NF-κB regulator in HCC cell lines/ inhibits signaling in EtOH-fed mice	Not reported in ALD RIP3 dependent
MAP3K7/TAK1 S77,T82 (novel phosphorylation site)	NF-κB/MAPK regulator/activates autophagy; regulates hepatic cell survival	Not reported in ALD
MAP4K4/HGK S794 S800 human	Upstream of the Jun N-terminal pathway	Not reported in ALD
RIP1 S313 N312 human	Cell survival and death	Not required for activity[43]
RIP1 S336 S335-human		S336 negatively regulates activity of RIPK1 kinase and prevents TNF-induced cell death[44]
IKBKE/IKKα S713 A712 human	Phosphorylates inhibitors of NF-κB allows NF-κB nuclear translocation	Not reported in ALD
IKBKG/IKKγ/NEMO S380 S387 human	Controls NF-κB activation/non enzymatic scaffold protein	Not reported in ALD Not required for activity/to mediate efficient NF-κB signaling[45]
FADD S191 S194 human	Essential for death receptor-induced apoptosis	S191 negatively regulates activity of FADD/inhibits protein function/ apoptosis
Peptidases and caspase 3 substrates		
CASP3 S26 same in human	Key apoptotic executer	Apoptosis-specific site, promotes cleavage of caspase-3 by caspase-8[46]
LMNA S390/S392 (novel phosphorylation site) same in human	Nuclear assembly/chromatin organization	S390/S392-induces activity and intracellular localization regulates protein degradation/cell cycle
SPTAN1 S1217 same in human	Stability and organization of organelles; necrosis/apoptosis biomarker	Not reported in ALD
ACIN1 S561 (novel phosphorylation site) same in human S826	Induces apoptotic chromatin condensation	Not reported in ALD
GAS2 S186 S185 human	Regulates microfilament alterations to respond to growth arrest induced apoptosis[47]; depleted in some HCC cell lines	Not reported in ALD
DFFA S314 A314 human	DFFA cleavage by caspase-3 activates DFF to trigger DNA fragmentation/a hallmark of apoptosis	Negatively regulates execution phase of apoptosis

(Continues)

TABLE 4 (Continued)

Phosphorylation sites related to DRS	Protein function	Effect of phosphorylation on protein function (role in ALD)
Enriched necroptosis pathways RSKs		
RPS6Ka3/p90RSK3 S415 same in human	Upstream regulator of RIP3 phosphorylation during necroptosis[48]/downstream effectors of the ERK/MAPK signaling/promotes the survival of hepatic stellate cells by phosphorylating CEBPB in response to the hepatotoxic CCl_4	Negatively regulates apoptotic process; caspase inhibitor activity, ERK-binding site
Rps6ka4/MSK 2 S347 same in human	Regulate transcription factor RELA (NF-κB p65 subunit) that regulates inflammatory genes.	Nonconserved modification, relates to persistent activation of Rps6ka4; activated through p38- and ERK-mitogen-activated protein kinases
Rps6kb2 70-kDa ribosomal protein S6 kinase 2 S417,S423 same in human	Phosphorylates the 40 S ribosomal protein S6 *in vitro*	Not reported in ALD; phosphorylation induces enzymatic activity conserved in S6K2
RPS6KC1 S634, S655 S640;S661 human S636 (novel phosphorylation site)	Sphingosine 1 phosphate signaling	Not reported in ALD
ZBP1 S	Master regulators of NLRP3 inflammasome/pyroptosis, apoptosis, and necroptosis RIPK1 deficiency/RHIM mutation triggers ZBP1-dependent necroptosis and inflammation in mice	NA
GLUL S19 same in human	Enzyme/removes ammonia in liver	Not reported in ALD
PYCARD S193 human	Key mediator in apoptosis and inflammation	Not reported in ALD
TGFBR2 S578 S553 human	TGFBR2 phosphorylation increased after EtOH treatment.	Not reported in ALD
HDAC S422, S424	NF-κB binding; binge liver injury associated with down-regulation of hepatic HDAC	Not reported in ALD
AIFM1 S	Regulator of apoptosis	NA
PIK3C2A S339	Class II member of the phosphoinositide 3-kinase family, catalyzes the phosphorylation of phosphatidylinositol (PI) into P	Not reported in ALD; site identified in liver mitochondrial phosphoproteome/ketogenesis.
IRF3 S313 (novel phosphorylation site), S135 T135-human	Key transcriptional regulator of type I interferon-dependent immune responses	S135 phosphorylation relates to intracellular localization and protein stabilization
TOM22 S		NA

Abbreviations: ACIN1, Apoptotic Chromatin Condensation Inducer 1; AIFM1, apoptosis inducing factor mitochondria associated 1; ALD, Alcohol-associated Liver Disease; CCl4, Carbon tetrachloride; CASP, Apoptosis-Related Cysteine Peptidase/cysteine-aspartic acid protease; DFFA, DNA Fragmentation Factor Subunit Alpha; DRS, death receptor signaling ; EtOH, ethanol; FADD, FAS-Associating Death Domain-Containing Protein; FDR, false discovery rate; GAS2, Growth Arrest-Specific Protein 2; GLUL, glutamate-ammonia ligase; HCC, Hepatocellular carcinoma; HDAC2, Histone deacetylase 2; IKBKG, Inhibitor Of Nuclear Factor Kappa B Kinase Regulatory Subunit Gamma/NEMO; IKBKE, Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Epsilon; IAP, inhibitor of apoptosis proteins; LMNA, Lamin A/C; MAP3K5, mitogen-activated protein kinase kinase kinase 5/Apoptosis Signal-Regulating Kinase 1 (ASK1); MAP3K7, Mitogen-Activated Protein Kinase Kinase Kinase 7/Transforming Growth Factor-Beta-Activated Kinase 1; MAP4K4, Mitogen-Activated Protein Kinase Kinase Kinase Kinase 4/Hepatocyte Progenitor Kinase-Like; MLKL, pseudokinase mixed lineage kinase-like protein; NF Kappa B, Nuclear Factor Kappa B Kinase; PYCARD, PYD and CARD domain containing; RIP1, Receptor-Interacting Protein Kinase 1; RIP3, receptor interacting protein 3; RPS6K, ribosomal protein S6 kinase; SPTAN, Spectrin Alpha, Non-Erythrocytic 1/Fodrin Alpha Chain; TGFBR2, transforming growth factor beta receptor 2; TNF-α, tumor necrosis factor α; TNFR1, TNF receptor 1; TRAD, TNFR associated death domain; Tyr, tyrosine; ZBP1, Z-DNA binding protein 1.

reporting that ethanol-induced apoptosis was independent of the *Rip3* genotype.[11] By mapping the impact of ethanol on phosphorylation events in the TNFα/TNFR1 signaling network (Figure 5), our study revealed a complex genotype-specific regulation of phosphorylation of proteins of the DR pathways in DR components, including kinases (RIP1, MAPK members, IKK components, and ribosomal s6 kinases [RSKs]), caspases (8/7 and 3), death receptors (FAS and FADD), and cytoskeletal proteins/substrates of caspase-3 downstream of caspase-3 (lamin A/C [LMNA], spectrin alpha, nonerythrocytic 1/fodrin alpha chain [SPTAN], growth arrest-specific protein 2 [GAS2], and apoptotic chromatin condensation inducer [1ACIN1]). It is likely that these site-specific phosphorylation events fine tune TNFα/DR signaling to either enhance or decrease signaling strength toward specific arms of the TNFR1 signaling network. For example, in C57BL/6 mice, chronic ethanol affected sequentially activated MAPK family members (Figure 5A); changes in the phosphorylation of MAP3K (MAP3K5/ASK1 and MAP3K7/TAK1) were associated with changes in phosphorylation of MAP2K (MAP2K7, also known as MKK4JNKK) which, in turn, activates a MAPK. Differences in MAP4K4 (MEKKK4) phosphorylation, which activates JNK, were also observed (Table 2; Figure 5A). Ma et al.[24] evaluated the ASK1/p38 pathway in alcohol-associated steatohepatitis and found increased phosphorylation of p38 in a chronic-plus-binge model and that hepatocyte-specific deletion of p38 ameliorates ethanol-induced hepatocyte injury and oxidative stress. Our data show increased phosphorylation of Thr180/Tyr182-p38 MAPK, sites that activate p38, in C57BL/6 mice after chronic ethanol (Figure 8A) and in liver from patients with AH (Figure 8C). In contrast, chronic ethanol feeding of *Rip3*$^{-/-}$ mice had only a modest effect on phosphorylation of ASK1 and no impact on the downstream kinases. Taken together, these data suggest a critical role for maintenance of homeostatic phosphorylation of ASK1 in preventing ethanol-induced liver injury.

There is accumulating evidence in the literature that proteins in the canonical pathway of necroptosis (RIP1, RIP3, and MLKL) also have additional functions outside of the necroptotic pathway.[17] For example, while *Rip3*$^{-/-}$ mice are protected from ethanol-induced liver injury, MLKL—the key effector in necroptotic cell death—is not phosphorylated or translocated to the plasma membrane in response to ethanol, and *Mlkl*$^{-/-}$ mice are not protected from chronic ethanol-induced injury.[16] In our phosphoproteomic data set, MLKL was also not phosphorylated in response to chronic ethanol (data not shown). Taken together, these data suggest the involvement in noncanonical functions of *Rip3* in ethanol-induced liver injury. Indeed, our finding that chronic ethanol-induced loss of inhibitory phosphorylation of ASK1 was muted in *Rip3*$^{-/-}$ mice suggests a possible noncanonical interaction of RIP3 with ASK1 to modulate activation of MAPK family members, including p38 and/or JNK, contributing to ethanol-induced liver injury. Our study identified decreases in phosphorylation of murine ASK1 on S1040 (corresponding to human S1033/1034), a phosphorylation site that controls the kinase activity of ASK1.[23] This is particularly relevant as inhibitory phosphorylation of human ASK1 on S1033/1034 (corresponding to murine S1040) was shown by Fujii and colleagues[23] to directly regulate apoptotic functions in COS7 cells. Given the conserved regulation of cell death pathways across cell types, these data are consistent with our conclusions regarding the role of murine S1040 inhibitory phosphorylation in ethanol-induced liver disease. Further molecular studies will be required to determine if this is a direct and/or indirect interaction between RIP3 and ASK1.

ASK1 phosphorylation was also dysregulated in liver of patients with severe AH compared to healthy controls. Phosphorylation of the inhibitory S1033 site was lower in patients with AH, following the same pattern observed in our murine proteomics data (murine S1040). In contrast to the decrease in phosphorylation of murine S1036, phosphorylation of S1029 in patients was increased compared to controls. However, the net impact of these differences in inhibitory site phosphorylation was associated with an increase in phosphorylation of the activating Thr838 site on ASK1 in patients with AH. Taken together with a previous report,[23] these data suggest that S1033 may independently mediate inhibition of ASK1. Importantly, the changes in inhibitory site phosphorylation of ASK1 in both patients with AH and mice after chronic ethanol exposure were associated with increased phosphorylation of p38, a critical downstream target of ASK1, implicated in the progression of ethanol-induced liver injury.[24] Differences in the phosphorylation of ASK1 in patients with AH compared to mice exposed to chronic ethanol may be due to species differences and/or differences in disease severity. One limitation of the current study is our inability to determine what specific cell types in the liver exhibit changes in phosphorylated ASK1 in patients with AH as we only had access to whole liver lysates.

Taken together, our data indicate that changes in inhibitory phosphorylation of ASK1 are an important target of chronic ethanol; however, additional mechanisms may also contribute to dysregulation of ASK1 in AH. ASK1 is widely expressed and evolutionarily conserved in mediating cell death, fibrosis, and inflammation[37] in various hepatic conditions, including ALD.[24,38] The activation of ASK1 and its downstream target p38 MAPK is involved in ALD. Inhibition of ASK1 and p38 reduces ethanol-induced liver injury.[24] Mechanistically, ASK1 is maintained in an inactive state through formation of ASK1 homodimers. Dimerization status is subject to redox regulation mediated through thioredoxin, with reduced thioredoxin keeping ASK1 in its inactive form. Chronic ethanol increases oxidative

stress in the liver, and provision of exogenous thioredoxin reduced oxidative stress.[38] ASK1 is also regulated by protein–protein interactions, with the C-terminal region of ASK1 important for signalosome formation and activity. Of the multiple ASK1-interacting proteins, chronic ethanol only affected phosphorylation of 14-3-3 epsilon in our analysis (Table S1). The 14-3-3 protein-binding site is in the C-terminal region of ASK1, followed by a region proposed to promote constitutive oligomerization of ASK1. The interaction of 14-3-3 proteins with ASK1 is phosphorylation dependent; the 14-3-3/ ASK1 complex is induced following phosphorylation of S967 (another inhibitory phosphorylation site) in ASK1 and suppresses ASK1 activity.[39] The interaction of 14-3-3 with ASK1 may allow functional crosstalk between signaling pathways, which is likely to be critical for signal integration.

ASK1 is considered an important therapeutic target in both nonalcohol-associated steatohepatitis (NASH)[40] and AH.[41] Selonsertib/GS-4997 is an ASK1 kinase inhibitor that targets the kinase domain, thereby preventing ASK1 phosphorylation and activation of the effector kinases p38 and JNK. ASK1 inhibition with selonsertib had promising results in phase II clinical trials in patients with NASH,[41] but phase III trials failed to demonstrate clinical improvements.[42] Selonsertib in combination with prednisolone also did not show improvement in liver function in patients with severe AH in a phase II clinical study (NCT02854631). Although there was no therapeutic response with selonsertib in NASH or severe AH, alternative strategies targeting ASK1 are still of potential interest. Our data suggest that, in particular, the role of dual inhibitory phosphorylation on ASK1 in the C-terminal region needs to be further explored. Moreover, non-kinase regulation of ASK1 should be investigated.

In summary, the use of MS phosphoproteomics identified multiple novel phosphorylation sites in DR signaling that are affected by chronic ethanol and *Rip3* genotype (Table 4). The functional consequences of many of these phosphosites have not yet been investigated. Our findings highlight the importance of specific phosphorylation events in TNF/DR signaling and provide new molecular insights into the mechanism of *Rip3* regulation in chronic ethanol-induced liver disease. Given the limited availability of site-specific phospho-antibodies, our work provides a valuable resource for future in-depth studies of ethanol-dependent phosphorylation events and illustrates the utility of MS-based methodologies for identifying novel changes in the phosphoproteome in response to ethanol.

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None.

CONFLICT OF INTEREST

Daniela Allende advises Incyte.

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SUPPORTING INFORMATION

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