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Splicing Factor SLU7 Prevents Oxidative Stress-Mediated Hepatocyte Nuclear Factor 4α Degradation, Preserving Hepatic Differentiation and Protecting From Liver Damage

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BACKGROUND AND AIMS: Hepatocellular dedifferentiation is emerging as an important determinant in liver disease progression. Preservation of mature hepatocyte identity relies on a set of key genes, predominantly the transcription factor hepatocyte nuclear factor 4α (HNF4α) but also splicing factors like SLU7. How these factors interact and become dysregulated and the impact of their impairment in driving liver disease are not fully understood.

APPROACH AND RESULTS: Expression of *SLU7* and that of the adult and oncofetal isoforms of *HNF4α*, driven by its promoter 1 (P1) and P2, respectively, was studied in diseased human and mouse livers. Hepatic function and damage response were analyzed in wild-type and *Slu7*-haploinsufficient/heterozygous (*Slu7*$^{+/-}$) mice undergoing chronic (CCl_4) and acute (acetaminophen) injury. SLU7 expression was restored in CCl_4-injured mice using SLU7-expressing adeno-associated viruses (AAV-SLU7). The hepatocellular SLU7 interactome was characterized by mass spectrometry. Reduced *SLU7* expression in human and mouse diseased livers correlated with a switch in *HNF4α* P1 to P2 usage. This response was reproduced in *Slu7*$^{+/-}$ mice, which displayed increased sensitivity to chronic and acute liver injury, enhanced oxidative stress, and marked impairment of hepatic functions. AAV-SLU7 infection prevented liver injury and hepatocellular dedifferentiation. Mechanistically we demonstrate a unique role for SLU7 in the preservation of HNF4α1 protein stability through its capacity to protect the liver against oxidative stress. SLU7 is herein identified as a key component of the stress granule proteome, an essential part of the cell's antioxidant machinery.

CONCLUSIONS: Our results place SLU7 at the highest level of hepatocellular identity control, identifying SLU7 as a link between stress-protective mechanisms and liver differentiation. These findings emphasize the importance of the preservation of hepatic functions in the protection from liver injury. (Hepatology 2021;74:2791-2807).

The global burden of chronic liver diseases is rising worldwide mainly due to changing trends in alcohol abuse, excessive caloric intake, and the sedentary lifestyle.[1] Importantly, regardless of the etiology, chronic liver damage progression is associated with the loss of hepatic functions, a strong determinant in patients' prognosis.[2] Accumulating evidence

Abbreviations: AAV, adeno-associated virus; ALT, alanine aminotransferase; APAP, acetaminophen; CYP2E1, cytochrome P450 2E1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3BP1, Ras GTPase-activating protein-binding protein 1; Gys2, glycogen synthase 2; H&E, hematoxylin–eosin; HK2, hexokinase 2; HNF4α, hepatocyte nuclear factor 4α; MS, mass spectrometry; MSGP, Mammalian Stress Granules Proteome; NAC, N-acetylcysteine; P1/P2, promoters 1 and 2; P-JNK, phospho-c-Jun N-terminal kinase; PKC, protein kinase C; PKM2, pyruvate kinase M2; RT-PCR, real-time PCR; SG, stress granule; si-, small interfering; SLU7, splicing factor SLU7; α-SMA, alpha-smooth muscle actin; Sod2, manganese-superoxide dismutase 2; TP53, tumor suppressor protein P53; UDP, uridine diphosphate; USP10, Ubiquitin-specific protease 10.

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demonstrates that progressive hepatic dysfunction is due not only to the death and loss of hepatocytic parenchyma. Dedifferentiation of the remaining hepatocytes associated with transcriptional reprogramming including reduced expression of hepatospecific genes and reactivation of fetal isoforms is emerging as a central pathogenic component.[3-7] Nevertheless, most of the molecular mechanisms implicated in this process of dedifferentiation remain unknown.

Hepatocellular identity depends on the correct expression and activity of key genes belonging to different functional networks. These comprise transcription factors, such as the master regulator hepatocyte nuclear factor 4 alpha (HNF4α), HNF1α, HNF6, CCAAT/enhancer-binding protein α and β, and forkhead box A,[8] as well as a growing complement of splicing factors including serine/arginine-rich splicing factor 3 (SRSF3),[9] the spliceosome component splicing factor SLU7,[10] and epithelial splicing regulatory protein-2.[11] Disruption of these transcription and splicing factor networks is increasingly recognized to occur in liver injury. Decreased or mislocalized expression of HNF4α is observed in the liver of patients with cirrhosis, a preneoplastic condition, and in animal models of liver damage.[4,5,7,12] Moreover, an *HNF4*α promoter 1 (P1) to P2 promoter switch has been described in human HCC.[13] Importantly, P2-derived isoforms, expressed in the fetal liver, lack an N-terminal transactivation domain present in P1-derived isoforms characteristic of the adult well-differentiated liver and differentially regulate gene expression.[7] Although the precise mechanisms implicated in HNF4α disruption remain unknown, reintroduction of the P1-derived HNF4α1 isoform limits adult-to-fetal reprogramming in models of cirrhosis and HCC, restoring liver differentiation and function.[5,14]

Regarding the splicing factors network, we demonstrated that down-regulation of hepatic SLU7 expression in mice results in rewiring of the mature hepatic transcriptional program to a fetal one, including the *HNF4*α P1 to P2 switch.[10] Remarkably, reduced SLU7 expression leads to the loss of liver metabolic and synthetic functions, disruption of hepatocellular

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quiescence, and induction of genome instability.[10,15] Moreover, we observed that *SLU7* mRNA levels are decreased in the liver of patients with cirrhosis and in HCC tissues.[16] Altogether, these findings suggest that SLU7 down-regulation in human liver injury can participate in hepatocellular dedifferentiation and the loss of hepatic functions observed along the process of hepatocarcinogenesis.[4,8]

Here we confirm that SLU7 protein is down-regulated in the human cirrhotic liver and in mouse models of acute and chronic liver damage. Moreover, we demonstrate that SLU7 down-regulation during liver injury certainly mediates the loss of hepatic functions and contributes to damage progression. Mechanistically we uncover a hierarchical relationship between key determinants of hepatocellular identity, unraveling an unexpected mechanism of HNF4α1 regulation by SLU7. We show that HNF4α1 protein stability and consequently its functions directly depend on the capacity of SLU7 to protect the liver against oxidative stress. Altogether our results place SLU7 at the highest level of hepatocellular identity control, identifying SLU7 as a link between stress-protective mechanisms and liver differentiation. Moreover, our findings emphasize the importance of the preservation of hepatic functions in the protection against acute and chronic injury, uncovering targets for intervention.

Material and Methods

ANIMAL EXPERIMENTS

Animal care and experimental protocols were approved (CEEA 062-16) and performed according to the guidelines of the Ethics Committee for Animal Testing of the University of Navarra. Animal models were developed as described in the Supporting Information.

HUMAN SAMPLES

The use of human samples was approved by the Human Research Review Committee of the University of Navarra (CEI 47/2015). Patients' samples were provided by the Biobank of the University of Navarra and processed following standard operating procedures approved by the ethical and scientific committees. Liver tissue samples from patients with cirrhosis, patients with acute liver failure, and HCC tissues were from individuals undergoing partial hepatectomy or liver transplantation. Healthy liver tissues were obtained from individuals with normal or minimal changes in the liver or healthy living liver donors. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software. Data are presented as mean ± SEM. Normally distributed data were compared among groups using a two-tailed Student test. Nonnormally distributed data were analyzed using the Mann-Whitney test. Statistical significance was considered as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Additional methods are provided as Supporting Information.

Results

EXPRESSION OF SLU7 IS REDUCED IN THE CHRONICALLY DAMAGED LIVER IN PARALLEL WITH A SWITCH IN *HNF4α* P1/P2 USAGE

Immunohistochemical analysis of SLU7 in liver tissue samples from controls and patients with cirrhosis confirmed its reduced hepatocellular levels in the diseased organs (Fig. 1A). Reduced SLU7 immunostaining was also found in hepatocytes of mice with CCl_4-induced chronic liver injury (Fig. 1B). In these mice *SLU7* down-regulation was paralleled by a switch in *Hnf4*α promoter usage, with a significant down-regulation of *Hnf4*α P1 activity and a robust activation of *Hnf4*α P2, characteristic of fetal and transformed liver cells[17] (Fig. 1C). As we recently reported in alcoho-associated hepatitis,[18] we observed a significant activation of *HNF4*α P2 in the liver of patients with cirrhosis (Fig. 1D). A concomitant reduction in *HNF4*α P1-driven transcription was also observed, particularly in patients with more advanced disease (Child-Pugh B-C cirrhosis) (Fig. 1D; Supporting Fig. S1A). Importantly, *SLU7* down-regulation was significantly associated with the Child-Pugh index (Supporting Fig. S1B).

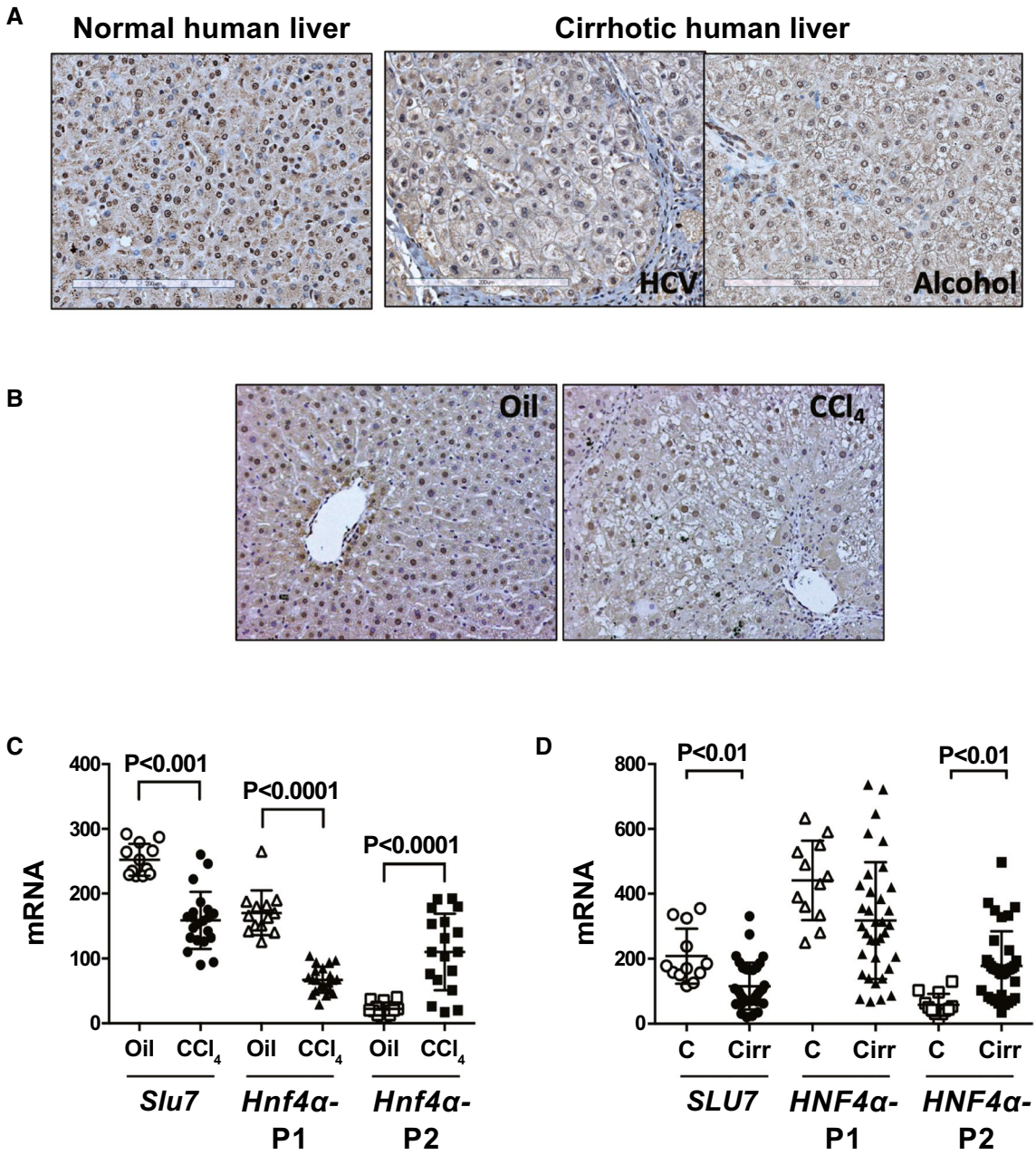

FIG. 1. Reduction of SLU7 expression in chronically injured liver in parallel to a switch in *HNF4*α promoter usage. (A) Representative SLU7 immunostainings in livers from healthy controls and patients with cirrhosis. (B) Representative SLU7 immunostainings in livers from chronically CCl_4-treated mice and controls (oil). (C) Real-time PCR (RT-PCR) analysis of *Slu7* and *Hnf4*α P1 and P2 mRNAs in livers from mice treated as in (B). (D) RT-PCR analysis of *SLU7* and *HNF4α* P1 and P2 mRNAs in livers from controls and patients with cirrhosis. Data are means ± SEM. Abbreviations: C, control; Cirr, cirrhosis.

SLU7 HAPLOINSUFFICIENCY EXACERBATES CCl_4-INDUCED CHRONIC LIVER DAMAGE

Next, we tested if SLU7 down-regulation could participate in liver damage development. To this end we employed *Slu7*-haploinsufficient/heterozygous mice (C57BL/6NTac-Slu7$^{tm1a(KOMP)Wtsi/Wtsi}$, *Slu7*$^{+/-}$ mice) (Supporting Fig. S2A,B). To induce chronic liver damage, *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice received CCl_4 for 6 weeks. *Slu7* mRNA levels were significantly reduced in both genotypes upon CCl_4 treatment (Supporting

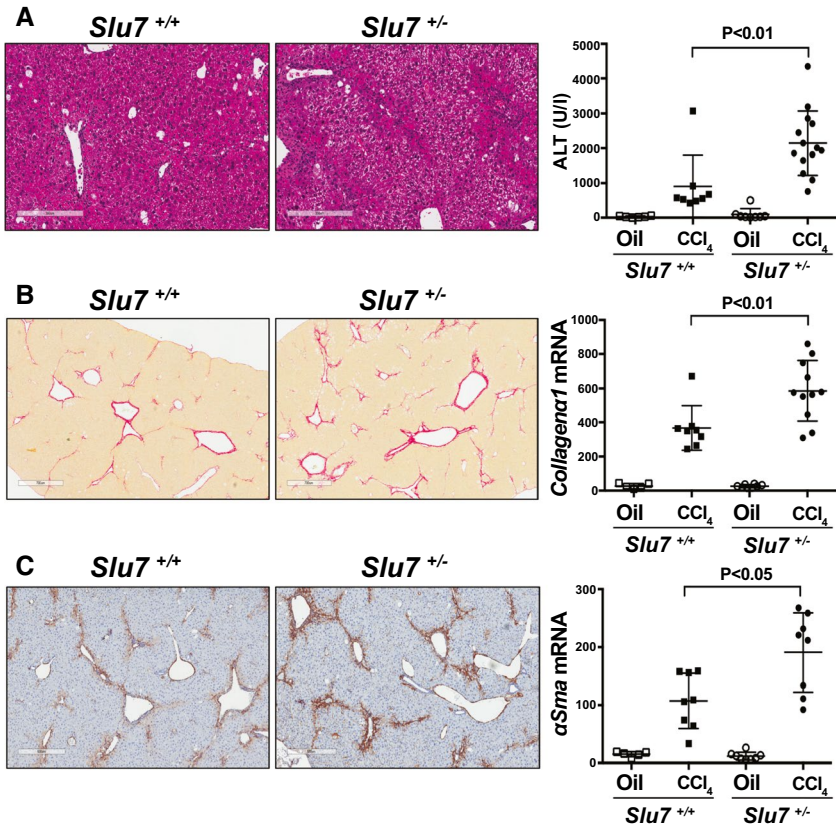

FIG. 2. SLU7-haploinsufficient mice are more sensitive to CCl_4-induced chronic liver damage. *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice were treated as in Fig. 1B. (A) Hematoxylin–eosin (H&E) stainings and significant induction of serum aminotransferases (alanine aminotransferase [ALT]) in *Slu7*$^{+/-}$ mice. (B) Sirius red staining showing bridging fibrosis in *Slu7*$^{+/-}$ mice and RT-PCR analysis of *collagen α1* mRNA expression. (C) αSMA immunohistochemistry and mRNA expression showing enhanced activation of HSCs in *Slu7*$^{+/-}$ mice. Data are means ± SEM. Abbreviation: ALT, alanine aminotransferase.

Fig. S2C). Importantly, *Slu7*$^{+/-}$ mice showed exacerbated liver injury (Fig. 2A). Moreover, while *Slu7*$^{+/+}$ mice showed sparse fibrosis, bridging fibrosis was appreciated in *Slu7*$^{+/-}$ animals, consistent with increased collagen α1 expression and activated (α-smooth muscle actin [αSMA]–positive) HSCs (Fig. 2B,C). Altogether these results indicate that reduced SLU7 expression sensitizes the liver to chronic damage.

SLU7 HAPLOINSUFFICIENCY EXACERBATES LIVER DAMAGE–ASSOCIATED SWITCH IN *HNF4α* PROMOTER USAGE, FOSTERING HEPATIC DEDIFFERENTIATION AND LIVER DYSFUNCTION

We hypothesized that the reduced SLU7 expression in chronic liver injury may be associated with the loss of hepatic functions characteristic of this condition. Interestingly, we found that the *Hnf4*α P1/P2 promoter switch occurring in CCl_4-treated mice (Fig. 1C) was significantly exacerbated in *Slu7*$^{+/-}$ animals (Fig. 3A,B). In fact, *Hnf4*α P1 mRNA expression and HNF4α1 protein isoform expression were reduced in *Slu7*$^{+/-}$ mice (Fig. 3A), while *Hnf4*α P2 mRNA and protein isoforms were induced (Fig. 3B).

The expression of HNF4α1 targets, including the transcription factor *Hnf1α*, the plasma proteins albumin (*Alb*) and transthyretin (*Ttr*), the enzyme glycogen-synthase 2 (*Gys2*), and the antioxidant enzyme manganese-superoxide dismutase 2 (*Sod2*), was markedly reduced upon CCl_4 treatment (Supporting Fig. S3A). Remarkably, these genes were already down-regulated in the normal liver of *Slu7*$^{+/-}$ mice, having functional consequences. For instance, hepatic glycogen storage was reduced in

Slu7$^{+/-}$ mice under both fasting and feeding conditions (Fig. 3C); and as reported for *Gys2*$^{-/-}$ mice,[19] *Slu7*$^{+/-}$ mice showed insulin resistance, evidenced by impaired phosphorylation of glycogen synthase kinase 3 (Supporting Fig. S3B). Moreover, consistent with *Sod2*[20] and glycogen[21] reduction, SLU7

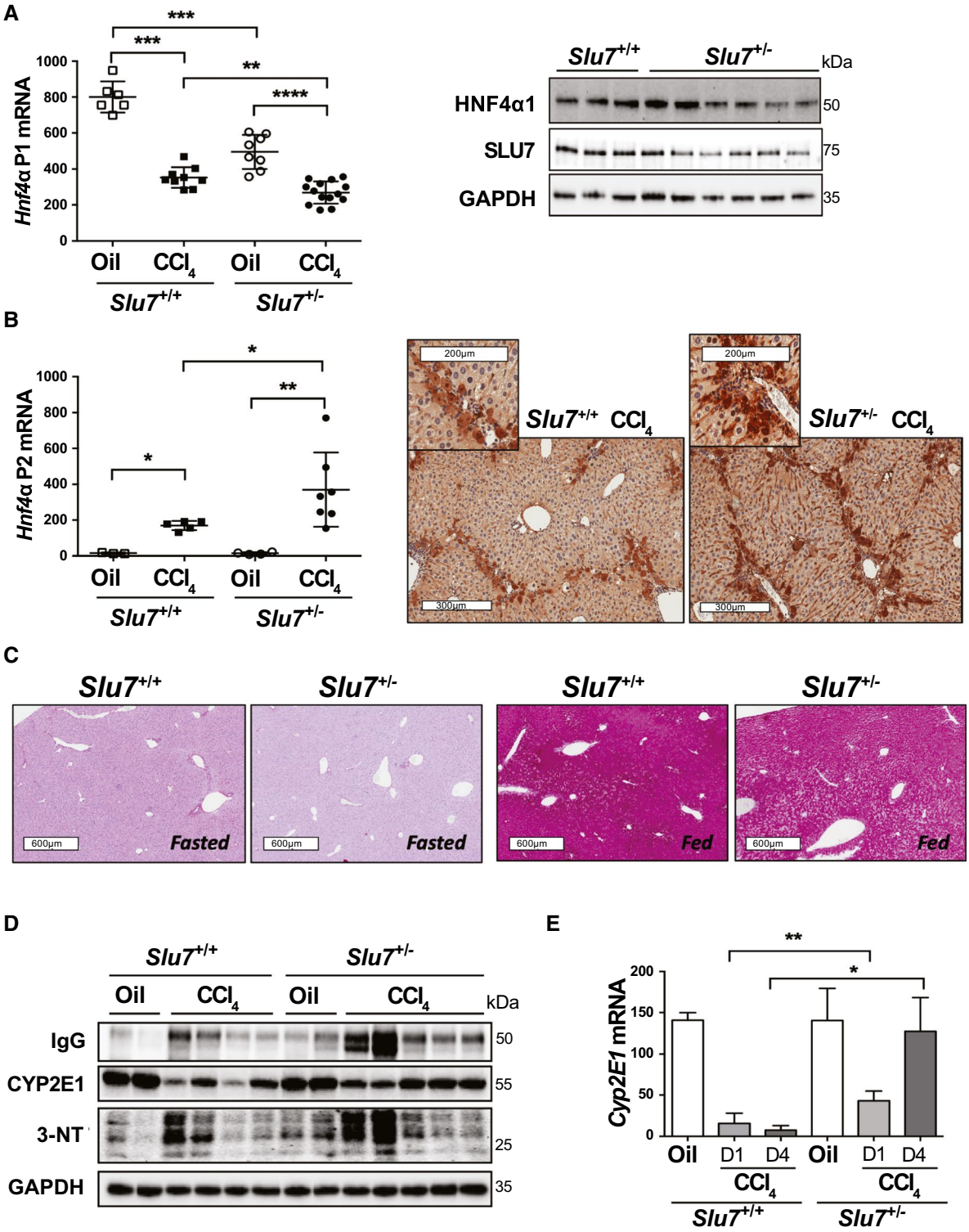

FIG. 3. SLU7 haploinsufficiency exacerbates *Hnf4α* promoter switch and liver damage–associated dysfunction and dedifferentiation. (A) RT-PCR analysis of P1-derived *Hnf4α* isoforms in the liver of *Slu7*$^{+/-}$ and *Slu7*$^{+/+}$ mice after CCl_4-induced chronic liver damage. (Right panel) HNF4α1, SLU7, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins in *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. (B) P2-derived *Hnf4α* isoforms analyzed as in (A). Immunohistochemical analysis of HNF4α P2 isoforms in the liver of chronically injured *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. Higher magnification highlights HNF4α P2 staining of hepatic nuclei in *Slu7*$^{+/-}$ mice. (C) Glycogen staining in the liver of *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice in fasting and feeding conditions. (D) Western blot analysis of heavy-chain IgG, CYP2E1, nitrosylated proteins (3-nitrotyrosine), and GAPDH in chronically injured *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mouse livers. (E) RT-PCR analysis of hepatic *Cyp2E1* mRNA in *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice treated as in (D) and sacrificed at day 1 or 4 after last CCl_4 administration. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: D, day; 3-NT, 3-nitrotyrosine.

haploinsufficiency sufficed to induce hepatic oxidative stress (Supporting Fig. S3C), promoting DNA damage (Supporting Fig. S3D) and protein nitration, which was exacerbated by CCl_4 (Fig. 3D).

Albeit less well known, catabolism of Igs is one relevant hepatic function in systemic homeostasis. Accordingly, hyperglobulinemia is found in patients and models of severe and chronic liver dysfunction.[17,22] Importantly, we observed that the hepatic accumulation of IgGs was higher in CCl_4-treated *Slu7*$^{+/-}$ mice (Fig. 3D).

Another major liver function is the metabolism of xenobiotics through P450 cytochromes. In fact, toxins like CCl_4 are metabolized by cytochrome P450 2E1 (CYP2E1), and *Cyp2e1*$^{-/-}$ mice are resistant to CCl_4-induced hepatotoxicity.[23] Consistent with previous findings,[24] CCl_4 administration induced CYP2E1 degradation and inhibited its transcription in *Slu7*$^{+/+}$ mice livers (Fig. 3D,E). Conversely, *Slu7*$^{+/-}$ mice displayed sustained CYP2E1 expression (Fig. 3D,E), which was reproduced in liver parenchymal cells upon SLU7 knockdown (Supporting Fig. S3E,F). These results demonstrate that SLU7 is central to preserving hepatic differentiation and to protecting the liver against damage.

SLU7 CONTROLS HNF4α1 PROTEIN STABILITY THROUGH THE REGULATION OF OXIDATIVE STRESS

Given the fundamental role of *HNF4α* in liver differentiation and function, we decided to elucidate the mechanisms through which SLU7 mediates its regulation. First, we demonstrated that SLU7 is required to maintain expression of the HNF4α P1 isoform HNF4α1 in a cell-autonomous manner (Fig. 4A). A careful chronological analysis of the effect of SLU7 knockdown on HNF4α1 expression demonstrated that HNF4α1 protein down-regulation occurs as soon as 24 hours after small interfering (si-) SLU7 transfection, a time point at which no effects are observed on *HNF4α* P1 transcription (Fig. 4B,C). Moreover, the relatively mild effect on *HNF4α* P1 mRNA observed after 36 and 48 hours of SLU7 silencing did not justify the level of protein reduction observed at these time points (Fig. 4B,C), suggesting a role for SLU7 in maintaining HNF4α1 protein stability. To rule out an effect of SLU7 on HNF4α1 translation, we analyzed the effect of SLU7 silencing on HNF4α1 when protein synthesis is inhibited with cycloheximide. We found that SLU7 knockdown reduced HNF4α1 independently of protein translation inhibition (Fig. 4D).

We next evaluated whether SLU7 regulates HNF4α1 stability through proteasome activity. Indeed, the proteasome inhibitor MG-132 significantly blunted SLU7 knockdown–mediated degradation of HNF4α1 in PLC/PRF/5 and HepG2 cells (Fig. 4E). Moreover, we found that HNF4α1 degradation depended on the development of oxidative stress, a consequence of SLU7 inhibition,[25] as it was prevented by the antioxidant *N*-acetylcysteine (NAC) (Fig. 4E). It has been demonstrated that protein kinase C (PKC) activation induces HNF4α1 cytoplasmic retention[26] and degradation[27] also in response to oxidative stress.[26] Interestingly, we found that PKCα and PKCδ knockdown averted the effect of SLU7 inhibition on HNF4α1 degradation (Fig. 4F).

SLU7 REGULATES THE EXPRESSION AND ANTIOXIDANT ACTIVITY OF THE STRESS GRANULE COMPONENT UBIQUITIN-SPECIFIC PROTEASE 10

To identify the mechanisms underlying the antioxidant activity of SLU7, we undertook an unbiased approach based on the characterization of the

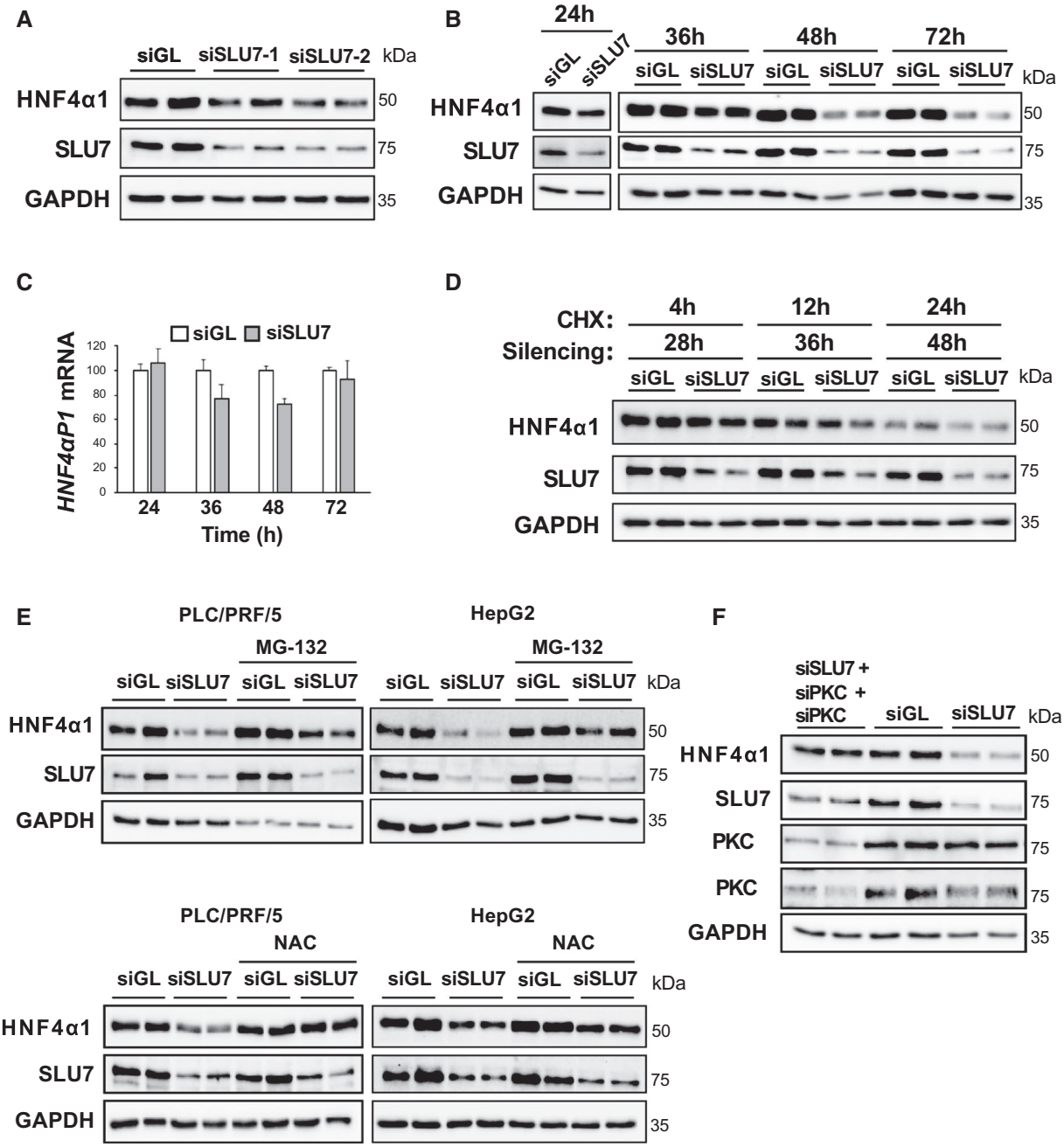

FIG. 4. SLU7 regulates HNF4α1 stability, preventing oxidative stress and PKC activation. (A) HNF4α1, SLU7, and GAPDH protein levels in HepG2 cells after SLU7 knockdown. (B) HNF4α1, SLU7, and GAPDH protein levels in PLC/PRF/5 cells at different time points after SLU7 knockdown. (C) RT-PCR analysis of *HNF4*α P1 isoforms in samples described in (B). (D) HNF4α1, SLU7, and GAPDH protein levels in PLC/PRF/5 cells upon SLU7 knockdown and cycloheximide treatment. (E) HNF4α1, SLU7, and GAPDH protein levels in PLC/PRF/5 and HepG2 cells upon SLU7 knockdown and treatment with the proteasome inhibitor MG-132 (upper) or the antioxidant NAC (lower). (F) PKCα and PKCδ knockdown prevents HNF4α1 down-regulation induced by SLU7 silencing in PLC/PRF/5 cells. SLU7, PKCα, PKCδ, and GAPDH proteins are analyzed as control. Abbreviations: CHX, cycloheximide; GL, control siRNA firefly luciferase.

SLU7 protein interactome. SLU7 interacting partners were identified by mass spectrometry (MS) after SLU7 immunoprecipitation. SLU7 is predominantly bound to chromatin (Supporting Fig. S4A); to enrich complexes outside this compartment, immunoprecipitations were performed on cytoplasmic and

soluble nuclear fractions. We selected 145 proteins (Supporting Table S2) identified by at least five different peptides in SLU7 immunoprecipitates but absent from control IgG immunoprecipitates, and we performed a gene ontology cellular component analysis. The expected category of spliceosome components ranked third, but surprisingly, the first and second categories identified were proteins associated with cytoplasmic stress granules (SG) (Fig. 5A). Moreover, we found that 82 out of the 145 (56%) SLU7-interacting proteins were present in the Mammalian Stress Granules Proteome (MSGP) and/or the RNA Granule Databases (Fig. 5A). Immunoprecipitation assays confirmed SLU7 interaction with the most common SG components, such as Ras GTPase-activating protein-binding protein 1 (G3BP1) and ubiquitin-specific protease 10 (USP10) in unstressed PLC/PRF/5 cells (Fig. 5B), consistent with the existence of pre-SG protein complexes that facilitate rapid coalescence into SGs during stress.[28,29] Likewise, although >20% of SG diversity is stress-dependent or cell type–dependent,[28] we confirmed these SLU7 interactions in normal mouse liver (Fig. 5B). Moreover, we found that SLU7 is required for stress-induced SG formation in $NaAsO_2$-stressed PLC/PRF/5 cells[28] (Fig. 5C). Remarkably, this effect was accompanied by a significant reduction in G3BP1 and USP10 protein, but not mRNA, levels upon SLU7 silencing (Fig. 5D; Supporting Fig. S4B). G3BP1 is essential for SG assembly,[29] and both G3BP1[30] and, more specifically, USP10[31] depletions induce oxidative stress. Accordingly, USP10 knockdown triggered reactive oxygen species (ROS) accumulation to a similar extent than SLU7 knockdown (Supporting Fig. S4C). Moreover, HNF4α1 protein levels were down-regulated upon USP10 silencing (Fig. 5E; Supporting Fig. S4D), and this effect involved oxidative stress as it was prevented by NAC (Fig. 5E). Additionally, we detected the physical interaction of USP10 with HNF4α1 (Supporting Fig. S4E), suggesting its possible role as a ubiquitin-specific protease.[32] Therefore, we demonstrate that SLU7 is required to maintain USP10 protein levels, protecting cells against oxidative stress and preventing HNF4α1 down-regulation. Furthermore, these interactions may bear clinical significance as we found that USP10 expression is reduced in the liver of patients with cirrhosis and HCC tissues (Fig. 5F).

SLU7 HAPLOINSUFFICIENCY EXACERBATES ACETAMINOPHEN-INDUCED ACUTE LIVER DAMAGE

Acetaminophen (APAP) overdose is a leading cause of acute DILI.[33] At therapeutic doses, most APAP is conjugated in the liver with glucuronic acid and sulfate to be excreted in urine and around 5% is oxidized mainly by CYP2E1 to the toxic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI), eventually detoxified by glutathione (GSH) conjugation.[34] Upon APAP overdose, glucuronidation and sulfation pathways are saturated, leading to increased NAPQI formation, hepatic GSH depletion, and extensive oxidative stress.[35] In view of our current findings, we evaluated the role of SLU7 in APAP-mediated DILI. We observed a progressive reduction in SLU7 protein in APAP-overdosed mice (Fig. 6A). Enhanced cytoplasmic SLU7 staining 3 hours post-APAP paralleled the activation of phospho-c-Jun N-terminal-kinase (P-JNK) (Supporting Fig. S5A), which is involved in stress-mediated cytoplasmic translocation of SLU7.[36] *Slu7* transcription remained unaltered (Supporting Fig. S5B). We then compared the sensitivity of $Slu7^{+/+}$ and $Slu7^{+/-}$ mice to an APAP overdose. Serum levels of hepatic enzymes and parenchymal injury were significantly higher in $Slu7^{+/-}$ mice (Fig. 6B,C). Consistent with our findings in the CCl_4 model, expression of *Hnf4*α P1 isoforms was significantly reduced in $Slu7^{+/-}$ mice 6 hours after APAP administration (Supporting Fig. S5C), and HNF4α1 was depleted 12 hours after APAP intoxication (Fig. 6D). Importantly, we found that USP10 and G3BP1 proteins were significantly reduced in APAP-treated $Slu7^{+/-}$ mice (Fig. 6D). Moreover, these responses were paralleled by enhanced protein nitration, ROS production, heme-oxygenase-1 expression, and tumor suppressor protein (TP53) stabilization (Fig. 6D; Supporting Fig. S5D,E) in $Slu7^{+/-}$ mice. Reduced expression of the HNF4α1 target *Sod2* (Supporting Fig. S5F) may also contribute to enhanced oxidative stress. Remarkably, HNF4α1, USP10, and SLU7 protein levels were dramatically down-regulated in the liver of patients with acute liver failure (Fig. 6E), in parallel with P-JNK activation.[37]

Importantly, the expression of other HNF4α1 targets such as *Gys2* and uridine diphosphate (UDP)–glucuronosyl-transferase-1A1 (Ugt1α),[38,39]

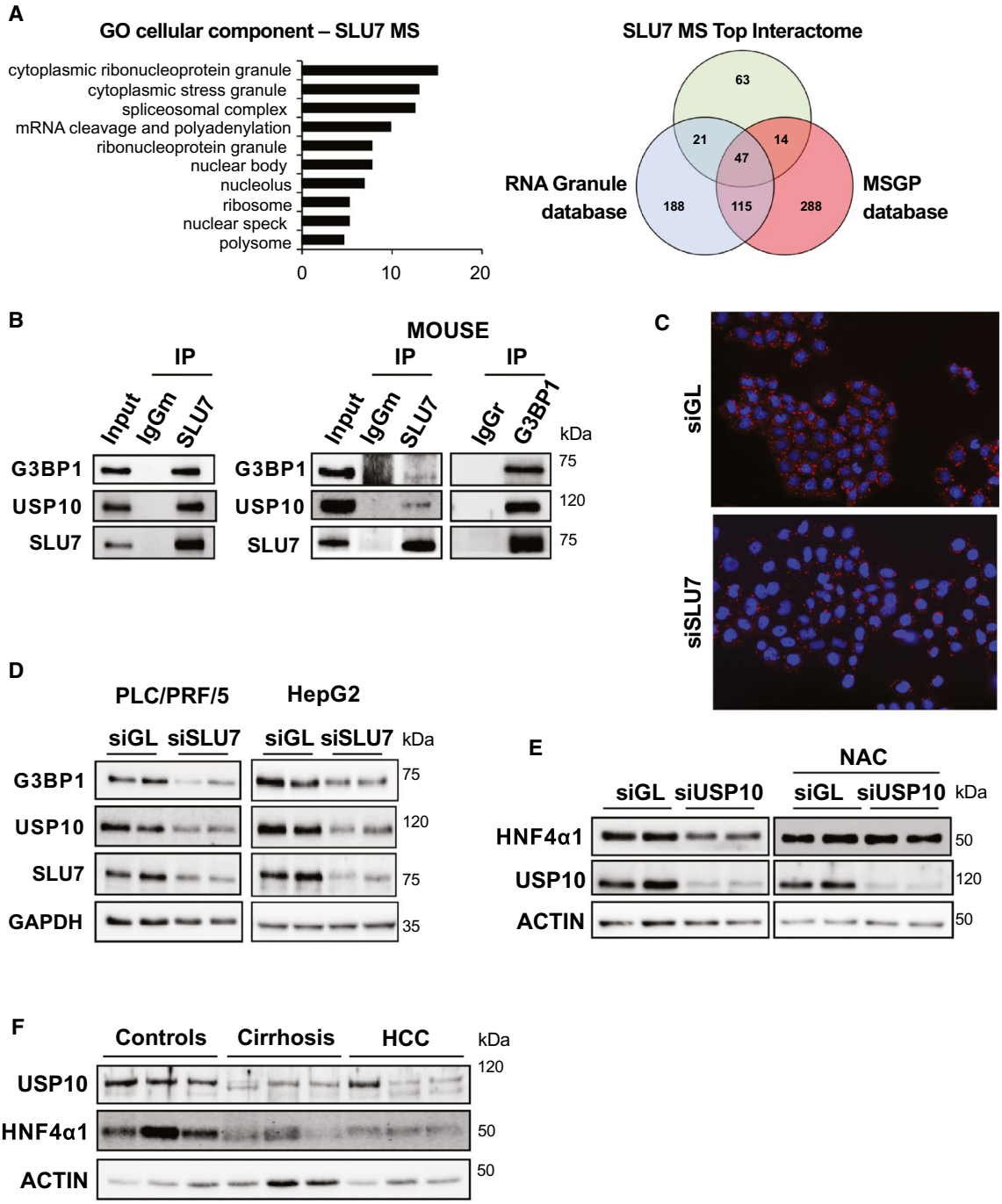

FIG. 5. SLU7 regulates HNF4α1 stability through the antioxidant activity of the SG component USP10. (A) Gene Ontology cellular component analysis of SLU7-interacting proteins identified by MS in PLC/PRF/5 cells. (Right panel) Venn diagram comparing the 145 top proteins identified in the SLU7 MS interactome, with the proteins present in the RNA Granule and the MSGP databases. (B) G3BP1, USP10, and SLU7 proteins in SLU7 immunoprecipitates from PLC/PRF/5 cells and in SLU7 and G3BP1 immunoprecipitates from mouse liver extracts. Input and immunoprecipitates with control mouse or rabbit IgGs are shown as control. (C) Immunofluorescence detection of SGs with anti-G3BP1 antibodies in siGL or siSLU7 transfected PLC/PRF/5 cells treated with $NaAsO_2$ (1 hour). (D) G3BP1, USP10, SLU7, and GAPDH protein levels in PLC/PRF/5 and HepG2 cells upon SLU7 knockdown. (E) HNF4α1, USP10, and actin protein levels in PLC/PRF/5 cells upon USP10 knockdown and effect of NAC. (F) USP10, HNF4α1, and actin protein levels in the liver of controls, patients with cirrhosis, and HCC tissues. Data are means ± SEM. Abbreviations: GL, control siRNA firefly luciferase ; GO, Gene Ontology; IP, immunoprecipitated; m, mouse; r, rabbit.

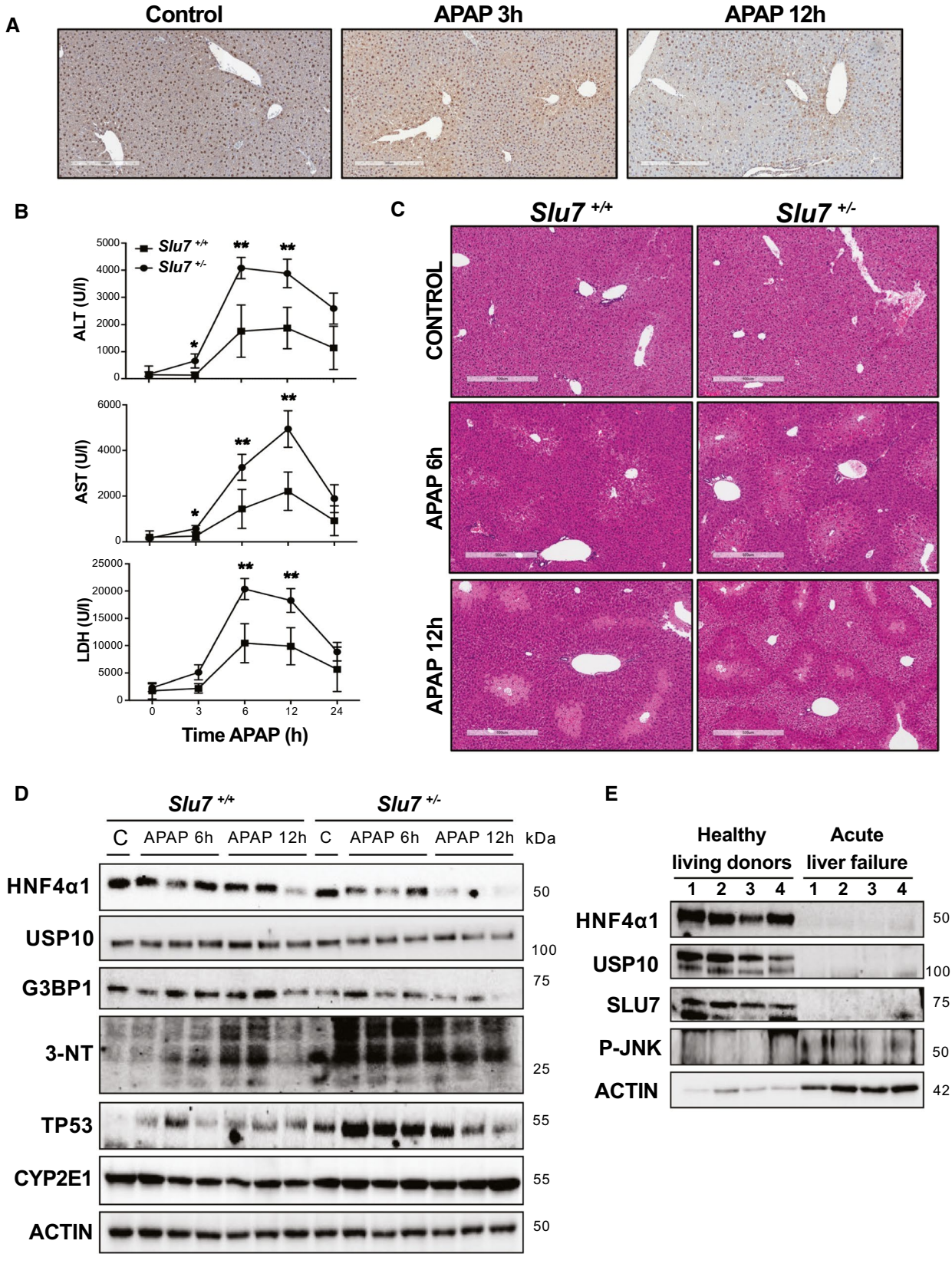
A
Control
APAP 3h
APAP 12h
B
Slu7 +/+
Slu7 +/-
ALT (U/l)
AST (U/l)
LDH (U/l)
Time APAP (h)
C
Slu7 +/+
Slu7 +/-
CONTROL
APAP 6h
APAP 12h
D
Slu7 +/+
Slu7 +/-
C
APAP 6h
APAP 12h
kDa
HNF4α1
USP10
G3BP1
3-NT
TP53
CYP2E1
ACTIN
E
Healthy living donors
Acute liver failure
HNF4α1
USP10
SLU7
P-JNK
ACTIN

FIG. 6. SLU7 haploinsufficiency exacerbates APAP-induced acute liver damage. (A) SLU7 immunohistochemistry in control and APAP-treated mice. (B) Serum aminotransferases (ALT and aspartate aminotransferase) and lactate dehydrogenase levels in APAP-treated *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. (C) H&E stainings of liver sections from APAP-treated *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. (D) Hepatic HNF4α1, USP10, G3BP1, TP53, CYP2E1, and actin protein expression and protein nitrosylation (3-nitrotyrosine) in APAP-treated *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. (E) HNF4α1, USP10, SLU7, P-JNK, and actin protein levels in healthy liver tissues and in liver tissues from patients with acute liver failure. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; 3-NT, 3-nitrotyrosine.

the enzyme responsible for APAP glucuronidation,[34] also decreased in APAP-treated *Slu7*$^{+/-}$ mice (Supporting Fig. S5G). Glycogenolysis is the source of UDP-glucuronic acid for APAP glucuronidation and hepatoprotection.[40] Therefore, we measured serum APAP-glucuronide conjugate levels and found them significantly reduced in *Slu7*$^{+/-}$ animals, suggesting a lower capacity for nontoxic APAP metabolism (Supporting Fig. S5H).

As for CCl_4 intoxication, CYP2E1 activity is the major determinant in APAP-mediated hepatotoxicity.[34] Consistent with our observations in the CCl_4 model, the decrease in CYP2E1 expression observed in APAP-treated *Slu7*$^{+/+}$ mice was delayed or prevented in *Slu7*$^{+/-}$ animals (Fig. 6D; Supporting Fig. S5I). This sustained expression of CYP2E1 can contribute to the observed increase in oxidative stress[41] and, of course, to the higher APAP hepatotoxicity.[34] Altogether, we demonstrate that SLU7 is required to maintain the functional antioxidant and drug-metabolizing capacity of the liver.

SLU7 IS A KEY GENE IN PROTECTION FROM CHRONIC LIVER INJURY

To further demonstrate the important hepatoprotective role of SLU7, we evaluated the effect of preventing damage-mediated SLU7 down-regulation on the development of liver injury. To this end, mice were injected with a SLU7-expressing adeno-associated virus (AAV-SLU7) or a control virus (AAV-Ren)[10] before chronic CCl_4 treatment. While expression of SLU7 transgene was reduced upon damage, it was significantly higher than in control AAV-Ren CCl_4-treated mice and similar to that in control mice (AAV-Ren oil) (Supporting Fig. S6A,B). Importantly, restoration of SLU7 expression protected against chronic liver damage. AAV-SLU7 significantly reduced serum transaminases, improved liver histology, inhibited hepatic collagen expression and accumulation, and suppressed HSC activation (Fig. 7A-C), altogether translating into a reduced loss of body weight (Supporting Fig. S6C). Mechanistically, SLU7 expression attenuated *Hnf4α* P1 and HNF4α1 down-regulation (Fig. 7D; Supporting Fig. S6D) and inhibited *Hnf4α* P2 activity (Supporting Fig. S6D), while sustaining USP10 expression and reducing TP53 protein levels (Fig. 7D). Accordingly, expression of hepatic dedifferentiation markers such as the fetal/oncogenic enzyme isoforms hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2) as well as the hepatic accumulation of IgGs was attenuated in CCl_4-treated AAV-SLU7 mice (Fig. 7D). Therefore, preventing SLU7 down-regulation in the damaged liver preserves hepato-specific functions and protects against chronic injury.

Discussion

Impairment of liver function dictates the prognosis of patients with acute and chronic hepatic diseases irrespective of the underlying etiology.[2] Importantly, hepatic dysfunction relates not only to hepatocellular loss but also to the dedifferentiation of the remaining parenchyma in a microenvironment shaped by cytokines, growth factors, oxidative stress, and matrix remodeling.[3,4,6,8,18]

Among transcription factors, HNF4α is considered essential for preserving the normal functions of the fetal and the adult liver through the activity of *HNF4α* P2 and P1, respectively.[7] Different observations strongly support the involvement of HNF4α deregulation in the development of liver damage. First, HNF4α1 expression is significantly reduced[4,5] or mislocalized to the cytoplasm[12] in the liver of patients with cirrhosis and in animal models of liver injury, paralleling the loss of hepatic functions. Moreover, a switch from P1-driven to P2-driven *HNF4α* expression is observed in human HCC tissues.[13] Second, when HNF4α is reintroduced in models of liver cirrhosis and HCC,

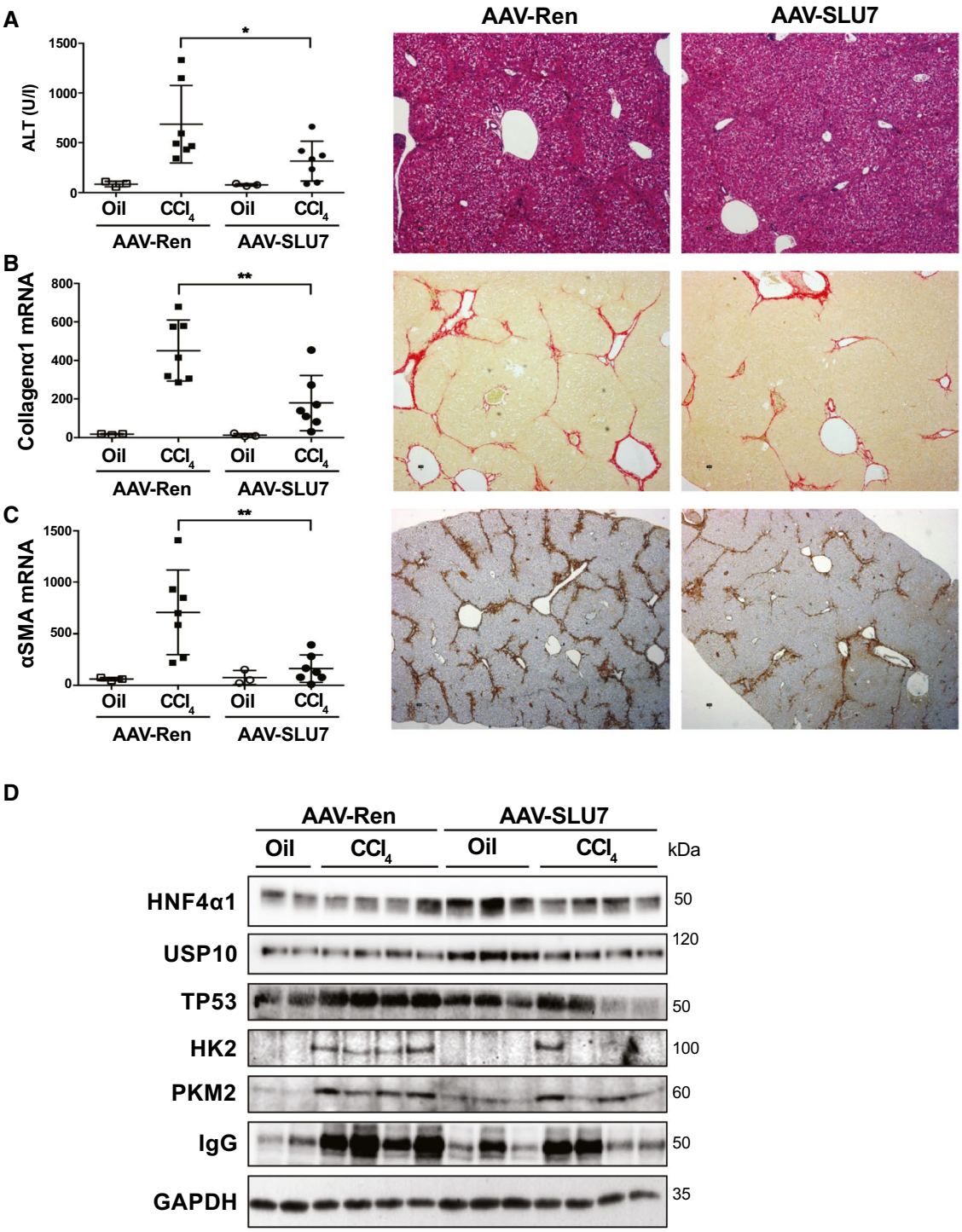

FIG. 7. Preservation of hepatic SLU7 expression protects against CCl_4-induced chronic damage. Mice were injected with AAV-SLU7 or control AAV-Ren 2 weeks before CCl_4 treatment, as in Fig 1B. (A) Serum ALT levels and representative H&E staining of liver sections. (B) RT-PCR analysis of *Collagen α1* mRNA and representative Sirius red stainings. (C) RT-PCR analysis of α-*Sma* mRNA and representative α-SMA stainings. (D) HNF4α1, USP10, TP53, HK2, PKM2, IgG, and GAPDH protein levels. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: AAV, adenoassociated virus; ALT, alanine aminotransferase.

liver functions are reestablished, and hepatocyte differentiation is recovered.[5,14] Here, we demonstrate that *HNF4*α promoter switch is an early and conserved event occurring in human liver cirrhosis and in models of chronic liver damage. Importantly, and as discussed below, we uncover a molecular mechanism underlying HNF4α disruption which involves the splicing factor SLU7.

We previously demonstrated that SLU7 is required to maintain the differentiated and quiescent phenotype of the normal adult liver, regulating the hepatic transcriptome.[10] Importantly, we discovered that SLU7 controls the proper splicing and expression of *SRSF3* and the correct usage of *HNF4α* P1 promoter,[10,15] placing SLU7 upstream of two master regulators of liver differentiation.[9] Our earlier findings also evidenced that hepatic *SLU7* mRNA expression is significantly reduced in patients with cirrhosis,[16] altogether suggesting a role of SLU7 dysregulation in liver dysfunction. Now we show that SLU7 down-regulation occurs in both human and mouse liver upon injury and that reduced SLU7 expression contributes to both the loss of hepatic function and the development of parenchymal damage. In fact, we found that SLU7 haploinsufficiency is enough to exacerbate liver dysfunction and sensitivity to APAP-induced and CCl_4-induced acute and chronic liver damage, respectively.

In this context, the hepatic dysfunction concomitant with SLU7 down-regulation was associated with a maintained or faster recovery of CYP2E1 levels in the liver of CCl_4-treated and APAP-treated *Slu7*$^{+/-}$ mice. Sustained CYP2E1 expression could contribute to higher sensitivity to liver damage as it has been shown that CYP2E1 overexpression or its induction by ethanol, obesity, or diabetes[42,43] is associated with enhanced oxidative stress and liver damage susceptibility, while *Cyp2E1*$^{-/-}$ mice are resistant to CCl_4 and APAP-induced hepatotoxicity.[23] Regarding the mechanisms implicated in the regulation of CYP2E1 expression, it has been proposed that oxidative stress induces[44] and insulin inhibits *CYP2E1* transcription, and accordingly insulin resistance results in CYP2E1 up-regulation.[42] Based on these notions, the enhanced hepatic expression of CYP2E1 in *Slu7*$^{+/-}$ mice upon damage could be explained by higher levels of oxidative stress and insulin resistance observed in *Slu7*$^{+/-}$ mice, which could be attributable to an inhibition of USP10 expression.

In agreement with our previous observations on the effect of SLU7 on *Hnf4*α transcription in the normal liver,[10] we found that SLU7 haploinsufficiency led to the inhibition of *Hnf4α* P1 promoter activity. However, when we explored the mechanisms implicated in SLU7-mediated HNF4α regulation *in vitro*, we uncovered an unexpected role for SLU7 in the preservation of HNF4α1 protein stability in an oxidative stress–dependent and PKC-dependent manner. MS-based protein interactome studies demonstrated that SLU7 interacts with components of the SGs in unstressed conditions, both *in vivo* and *in vitro*. Moreover, we found that SLU7 is necessary to secure SG formation upon stress induction as SLU7 is required to maintain the expression of the SG core components G3BP1 and USP10,[28] which in turn also have antioxidant capacity.[30,31] While the specific mechanisms involved in the stabilization of SG components by SLU7 remain to be fully elucidated, we revealed that USP10 antioxidant function controls HNF4α1 stability and that upon liver damage SLU7 haploinsufficiency results in USP10 down-regulation. Moreover, we found that USP10 expression is reduced in the liver of patients with cirrhosis as well as in HCC tissues, which would be in agreement with the recent description of USP10 down-regulation in the liver of patients with NAFLD[45] and in HCC in association with poor prognosis.[46] Interestingly, these studies described mechanisms associated with USP10 function as ubiquitin-specific protease interacting and stabilizing proteins like sirtuin 6, AMP-activated protein kinase α, and phosphatase and tensin homolog. Therefore, and in view of their physical interaction, a direct role for USP10 in maintaining HNF4α stability cannot be disregarded.[32]

Interestingly, reduced HNF4α1 protein stability would explain the changes observed in P1/P2 usage *in vivo* as it has been demonstrated that HNF4α1 activates P1[47] while it inhibits P2.[48] Moreover, impaired expression of P1-driven *HNF4α* isoforms results in *Hnf4α* P2 promoter activation.[49] Importantly, and from a physiopathological perspective, impaired expression of P1-driven *HNF4α* isoforms in *Slu7*$^{+/-}$ mice would be responsible for the down-regulation of HNF4α1 target genes such as *Hnf1α*, *Alb*, *Gys2*, *Ttr*, *Ugt1*α, and *Sod2*.[50] These transcriptomic alterations not only have functional consequences but can indeed contribute to the development of liver damage. It has been demonstrated that the response to stress largely

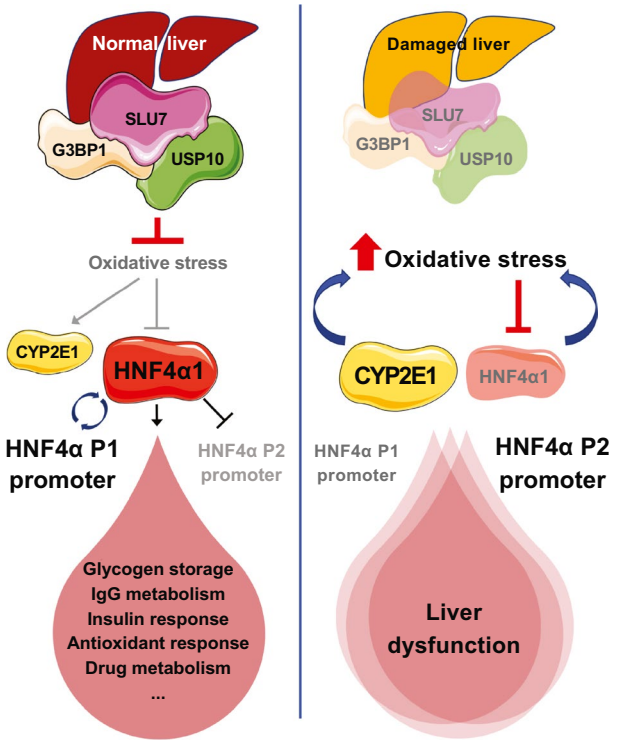

FIG. 8. Mechanisms involved in SLU7-mediated preservation of liver differentiation and function.

depends on the differentiation state of the cells. Particularly in the liver, reduced expression of *Gys2* and *Ugt1*α would explain the observed decrease in glycogen stores and APAP glucuronidation detected in *Slu*$^{+/-}$ mice, which in turn may contribute to the higher sensitivity of these animals to APAP-induced liver damage. Moreover, the down-regulation of the antioxidant enzyme *Sod2*[20] and the depleted levels of glycogen, an evolutionarily conserved antioxidant,[21] may further contribute to the increased oxidative and nitroxidative stress observed in *Slu7*$^{+/-}$ mice and therefore to their enhanced susceptibility to CCl_4 and APAP.[41]

The biological response to stress is orchestrated through multiple mechanisms which will eventually dictate cellular fate. The correct performance of such mechanisms largely depends on cellular differentiation. Our present results demonstrate that in the liver SLU7 integrates antioxidative stress responses with the maintenance of hepatocellular differentiation (Fig. 8). Moreover, we show that the loss of hepatic functions is not only a consequence of liver damage but a key player in its onset and development. Importantly, our results demonstrate that subtle changes in the expression of central homeostatic genes, such as SLU7, can drastically influence both liver function and injury progression. Finally, we uncover a hierarchical pathway linking SLU7 to HNF4α regulation.

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Author Contributions: M.G-R., M.R., M.J., M.E., M.A., I.U., M.U.L., R.U., conducted the research and provided methodology. I.B. and B.S. provided resources. C.G-R, J.C.F-C., F.J.C., A.E. and A.P-L. contributed to the acquisition of data. M.G.F-B. contributed to interpretation of data and reviewed the manuscript. M.A.A., M.A., and C.B. contributed to conceptualization, fund acquisition, project administration, supervision, and writing.

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