Activation of LKB1-Akt Pathway Independent of Phosphoinositide 3-Kinase Plays a Critical Role in the Proliferation of Hepatocellular Carcinoma from Nonalcoholic Steatohepatitis

Nuria Martínez-López,[1]* Marta Varela-Rey,[1]* David Fernández-Ramos,[1] Ashwin Woodhoo,[1] Mercedes Vázquez-Chantada,[1] Nieves Embade,[1] Luis Espinosa-Hevia,[1] Francisco Javier Bustamante,[2] Luis A. Parada,[1] Manuel S. Rodriguez,[1] Shelly C. Lu,[3] José M. Mato,[1] and Maria L. Martínez-Chantar[1]

LKB1, originally considered a tumor suppressor, plays an important role in hepatocyte proliferation and liver regeneration. Mice lacking the methionine adenosyltransferase (MAT) gene *MAT1A* exhibit a chronic reduction in hepatic *S*-adenosylmethionine (SAMe) levels, basal activation of LKB1, and spontaneous development of nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC). These results are relevant for human health because patients with liver cirrhosis, who are at risk to develop HCC, have a marked reduction in hepatic *MAT1A* expression and SAMe synthesis. In this study, we isolated a cell line (SAMe-deficient [SAMe-D]) from *MAT1A* knockout (MAT1A-KO) mouse HCC to examine the role of LKB1 in the development of liver tumors derived from metabolic disorders. We found that LKB1 is required for cell survival in SAMe-D cells. LKB1 regulates Akt-mediated survival independent of phosphoinositide 3-kinase, adenosine monophosphate protein–activated kinase (AMPK), and mammalian target of rapamycin complex (mTORC2). In addition, LKB1 controls the apoptotic response through phosphorylation and retention of p53 in the cytoplasm and the regulation of herpesvirus-associated ubiquitin-specific protease (HAUSP) and Hu antigen R (HuR) nucleocytoplasmic shuttling. We identified HAUSP as a target of HuR. Finally, we observed cytoplasmic staining of p53 and p-LKB1(Ser428) in a NASH-HCC animal model (from MAT1A-KO mice) and in liver biopsies obtained from human HCC derived from both alcoholic steatohepatitis and NASH. *Conclusion:* The SAMe-D cell line is a relevant model of HCC derived from NASH disease in which LKB1 is the principal conductor of a new regulatory mechanism and could be a practical tool for uncovering new therapeutic strategies. (HEPATOLOGY **2010;52:1621-1631**)

Nonalcoholic fatty liver disease is characterized by triglyceride accumulation in hepatocytes (hepatic steatosis) and steatosis with inflammation (nonalcoholic steatohepatitis [NASH]), which may progress to cirrhosis and hepatocellular carcinoma (HCC).[1] It has been demonstrated recently that tumor suppressor genes such as p53, pRb, M6P/IGF2 receptor, and E-cadherin are involved in the development and progression of HCC.[2-4] LKB1 (serine/threonine protein kinase 11) is a tumor suppressor whose inactivation by germline or somatic mutations increases the risk of cancer development.[5] However, LKB1 can also play an

Abbreviations: AMPK, adenosine monophosphate protein–activated kinase; ASH, alcoholic steatohepatitis; HAUSP, herpesvirus-associated ubiquitin-specific protease; HCC, hepatocellular carcinoma; HuR, Hu antigen R; IgG, immunoglobulin G; MAT, methionine adenosyltransferase; MAT1A-KO, MAT1A *knockout; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NASH, nonalcoholic steatohepatitis; PARP, poly(ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; SAMe,* S*-adenosylmethionine; SAMe-D, SAMe-deficient; siRNA, small interfering RNA; UTR, untranslated region; UVC, ultraviolet C; WT, wild-type.*

From the [1]CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), Technology Park of Bizkaia, Bizkaia, Spain; [2]Cruces Hospital (Hospital de Cruces), University of Basque Country, Bizkaia, Spain; and the [3]Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, CA.

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antiapoptotic role in tumor cells with constitutively active Akt[6] and can mediate phosphorylation of p53 by decreasing its nuclear import,[7] suggesting that LKB1 may be potentially oncogenic. During hepatocyte proliferation and regeneration after partial hepatectomy, hepatocyte growth factor induces LKB1-mediated adenosine monophosphate protein–activated kinase (AMPK) activation, which controls the nucleocytoplasmic shuttling of Hu antigen R (HuR), an RNA-binding protein that leads to an increased half-life of target messenger RNAs (mRNAs) involved in cell cycle progression.[8,9]

S-adenosylmethionine (SAMe), the main biological methyl donor and a precursor of glutathione synthesis,[10,11] blocks hepatocyte proliferation and liver regeneration by inhibiting hepatocyte growth factor–induced LKB1/AMPK/HuR activation and cyclin D1 and A2 expression.[8,9] Methionine adenosyltransferase (MAT), which is encoded by the genes *MAT1A* (expressed in the adult liver) and *MAT2A* (expressed in extrahepatic tissues and during liver proliferation), catalyzes SAMe synthesis.[10,11] *MAT1A* knockout (MAT1A-KO) mice show a chronic deficiency in hepatic SAMe levels and spontaneously develop NASH and HCC; this resembles the human pathology, in which HCC is characterized by low levels of SAMe and reduced *MAT1A* expression.[12,13] In addition, hepatic LKB1 and AMPK are activated, cytoplasmic localization of HuR is increased, and levels of several mRNAs involved in cell proliferation are elevated.[6,8]

In the present study, we isolated a cell line (SAMe-deficient [SAMe-D]) from MAT1A-KO mouse HCC as a model of a NASH-derived tumor cell. The results obtained in these cells and in human HCC derived from alcoholic steatohepatitis (ASH) and NASH suggest that p53 inactivation by cytoplasmic sequestration and activation of LKB1 can contribute to HCC development.

Patients and Methods

SAMe-D Cell Isolation. Procedures performed in MAT1A-KO and wild-type (WT) mice were in compliance with institutional guidelines for laboratory animal use. Fresh tumor specimens with primary HCC from 15-month-old MAT1A-KO mice were enzymatically dissociated,[14] and several cell clones of SAMe-D cells were maintained in 10% fetal bovine serum plus Dulbecco's modified Eagle's medium.

Isolation of Hepatocytes. Hepatocytes were isolated from 3-month-old male WT mice by way of collagenase perfusion (Gibco-BRL).[8,15] Hepatocytes were serum-deprived for 16 hours before ultraviolet C (UVC) treatment at 20 J with a CL-1000UV Crosslinker (254 nm).

RNA Isolation and Real-Time Polymerase Chain Reaction. Total RNA was isolated using an RNeasy Mini Kit (Qiagen); 1.5 μg of total RNA was retrotranscribed into complementary DNA using SuperScriptIII retrotranscriptase (Invitrogen). Polymerase chain reaction was performed using a BioRad iCycler Thermalcycler.

Protein Isolation and Western Blot Analysis. Extraction of total protein from cultured cells has been described.[9] Cytosolic and membrane lysates from cultured cells were prepared with the subcellular proteome extraction kit (Calbiochem). Thirty micrograms of protein was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels and transferred onto membranes. Membranes were incubated as described in Supporting Table 1.

Human Samples. Surgically resected specimens of 12 patients with liver cirrhosis and HCC were examined. Patients gave informed consent for all clinical investigations, which were performed in accordance with the Declaration of Helsinki. The data and type of biospecimen used were provided by the Basque Biobank for Research with appropriate ethics approval. The cause of liver cirrhosis was considered to be ASH in 10 patients and NASH in 2 patients.

Immunohistochemistry. Immunohistochemistry of paraffin sections of formalin-fixed liver samples were performed as described before.[9] Images were taken with a 100× objective from an epifluorescence microscope AXIO Imager.D1 (Zeiss).

Immunocytochemistry. Cells fixed with ethanol or methanol were blocked for 30 minutes with phosphate-buffered saline containing 0.1% bovine serum albumin, 10% horse serum, and 0.1% Triton and incubated overnight at 4°C with primary antibodies in blocking solution without Triton (Supporting Table 2). Cells were incubated with fluorescein isothiocyanate conjugated to rabbit or mouse immunoglobulin G

*These authors contributed equally to this study.
Address reprint requests to: Maria L. Martínez-Chantar, CIC bioGUNE, Technology Park of Bizkaia, 48160 Derio, Bizkaia, Spain. E-mail: mlmartinez@cicbiogune.es; fax: (34)-944-061301.

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(IgG) and Hoescht nuclear dye. Cells were examined under a Leica TCS-SP confocal laser microscope using a 60× objective.

Gene Silencing. SAMe-D cells were transfected with 100 nM small interfering RNA (siRNA) constructs (Qiagen) (Supporting Table 3) using Lipofectamine 2000 (Invitrogen) twice during a 48-hour period (once every 24 hours). Silencing mix was left overnight, and afterward the medium was replaced with fresh 10% fetal bovine plus serum Dulbecco's modified Eagle's medium.

Protein Immunoprecipitation. Five hundred micrograms of total cellular protein was immunoprecipitated overnight at 4°C with 10 μg of IgG1 (BD Pharmingen), herpesvirus-associated ubiquitin-specific protease (HAUSP) or Akt (Cell Signaling) antibodies, and protein A Sepharose (Sigma).

RNA Immunoprecipitation. RNA immunoprecipitation was performed as described.[14] mRNA bound to HuR was measured by way of real-time polymerase chain reaction and normalized with glyceraldehyde 3-phosphate dehydrogenase.

Biotin Pull-down. Synthesis of biotinylated transcripts and analysis of HuR bound to biotinylated RNA were performed as described.[9,16]

Statistical Analysis. Experiments were performed in triplicate; data are expressed as the mean ± SEM. Statistical significance was estimated using a Student *t* test.

Results

Characterization of SAMe-D Cells. In SAMe-D cells, we observed several kinases [p-Akt(Ser473), p-AMPK(Thr172), and p-LKB1(Ser428)], proteins involved in p53 signaling [Mdm2, p-Mdm2(Ser166), Bax, PUMA, and HAUSP], and apoptotic (cleaved caspase-3) and antiapoptotic markers (Bcl-2, HuR) highly expressed compared with WT hepatocytes, which correlated with positive levels of proliferative markers such as cyclin D1 and proliferating cell nuclear antigen (Supporting Fig. 1). Similar results were observed in another SAMe-D cell clone (clone 2) (Supporting Fig. 2A).

In normal cells, expression of p53 is maintained at low levels through control of the ubiquitin proteasome system.[17] In SAMe-D cells, however, the p53 gene and protein were overexpressed compared with WT hepatocytes (Supporting Table 4 and Supporting Fig. 1A). Fluorescence in situ analysis in SAMe-D cells revealed p53 genomic amplification with four copies of chromosome 11, each harboring one p53 signal (Supporting Fig. 3), which was not affected by any mutation after sequencing analysis (Supporting Table 5).

Cellular localization can regulate the apoptotic function of p53, and in several human tumors p53 accumulates in the cytoplasm.[18-20] p53 was predominantly cytoplasmic in SAMe-D cells and clone 2 (Supporting Figs. 2C, 4A); p53 silencing confirmed the specificity of the staining (Supporting Fig. 4B). Treatment with the nuclear export inhibitor leptomycin B (20 nM) induced the nuclear accumulation of HuR[21] but not of p53 (Supporting Fig. 4C), indicating that a hyperactive nuclear export is not responsible for p53 cytosolic localization.

Delay in the Apoptotic Response in SAMe-D Cells. We then investigated the p53-dependent apoptotic response to short-wavelength UVC irradiation in SAMe-D cells. In SAMe-D cells, cleaved caspase-3 and cytosolic cytochrome c appeared at 12 and 24 hours, respectively, after UVC irradiation; in WT hepatocytes, this response was observed at 2 hours and 1 hour, respectively (Fig. 1A), suggesting that the apoptotic response in SAMe-D cells upon UVC treatment was substantially delayed.

In SAMe-D cells, UVC irradiation increased p53 levels (Fig. 1B), mainly in the cytoplasm (Fig. 1C). A low-level nuclear staining was observed after 6 hours of UVC stimulation, whereas in hepatocytes, p53 was detected exclusively as nuclear speckles after 30 minutes of UVC treatment (Fig. 1C).

Mdm2 is a ubiquitin-protein ligase that regulates the stability of various essential cellular factors, including p21 and p53, as well as its own degradation.[22] UVC irradiation induced a sustained decrease of p21 and Mdm2 but a large increase in p53 content despite the active p-Mdm2(Ser166) (Fig. 1B,D,E), suggesting that Mdm2 might not be able to regulate p53 degradation in SAMe-D cells. In addition, an increase of Bax and a decrease of Bcl-x_L were observed at the time when apoptosis occurs.

LKB1 and Akt in the Apoptotic Response of SAMe-D Cells. Akt is a serine/threonine kinase that promotes cell survival and anabolic processes.[23,24] SAMe-D cells expressed high levels of p-Akt(Ser473) that increased after UVC irradiation, peaked at 18 hours, and returned to basal levels after 24 hours (Fig. 2A). In hepatocytes, however, p-Akt returned to basal levels 4 hours after UVC irradiation. Levels of phosphatase and tensin homolog (PTEN) phosphatase, the major negative regulator of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway,[25] were normal in SAMe-D cells (Fig. 2A).

In WT hepatocytes, wortmannin (PI3K inhibitor) and LY294002 (dual mammalian target of rapamycin [mTOR] and PI3K inhibitor) prevented UVC-

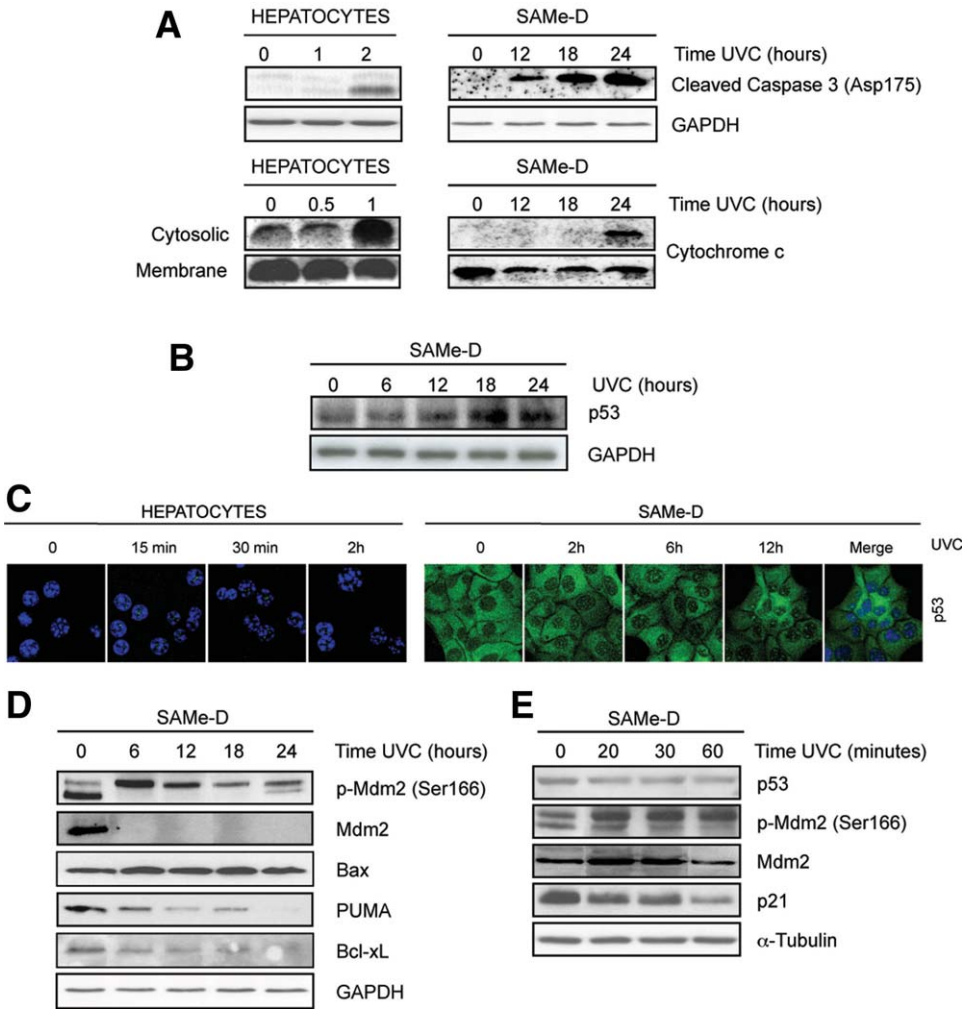

Fig. 1. SAMe-D cell response to UVC. Hepatocytes and the SAMe-D cell line were treated with UVC light. (A) Western blotting of total extract (upper) and cytosolic and membrane (bottom) fractions. (B) Western blotting of p53 in total extract. (C) Immunocytochemical analysis of p53. (D,E) Response of SAMe-D cells to UVC analyzed by way of western blotting.

mediated activation of Akt (phosphorylation at Ser473 and Thr308). Unexpectedly, only a slight attenuation of Akt activation was detected in SAMe-D cells treated with LY294002 or wortmannin (Fig. 2B), suggesting that Akt activation after UVC treatment in SAMe-D cells was PI3K-independent.

mTOR is a PI3K-related protein that regulates Akt phosphorylation by interacting with proteins such as Raptor (forming mTOR complex 1 [mTORC1]) and Rictor (forming mTOR complex 2 [mTORC2]).[26] The basal and UVC-stimulated levels of p-mTOR2(Ser2481) were inhibited by LY294002, suggesting that in SAMe-D cells, p-Akt(Ser473) was independent of mTORC2.[27] Furthermore, as observed by others,[28] wortmannin did not inhibit UVC-induced p-mTOR2(Ser2481) or p-mTOR1(Ser2448) and its downstream effector S6K(Thr389). In contrast, LY294002 inhibited p-mTOR1(Ser2448) and p-S6K(Thr389) (Fig. 2B and Supporting Fig. 5). These data are in agreement with previous studies showing that S6K phosphorylation in response to UVC is mediated through mTOR1 and PI3K but is Akt-independent.[29] Rictor silencing did not change p-Akt(Ser473) or p-Akt(Thr308) either at basal levels or after UVC stimulation, and had no effect on wortmannin sensitivity (Fig. 2C and Supporting Fig. 6). In addition, Rictor knockdown down-regulated p-mTOR2 without affecting p-mTOR1 and p-S6K, confirming the high substrate specificity of the two TOR complexes.[30]

Because SAMe-D cells expressed high levels of p-LKB1(Ser428), we examined its response after UVC treatment in this cell line. UVC-induced LKB1 phosphorylation was independent of PI3K activity (Fig. 2B). Interestingly, LKB1 knockdown reduced p-Akt(Ser473) and p-Akt(Thr308) after UVC treatment and, most importantly, rendered the cells responsive to wortmannin (Fig. 2C and Supporting Fig. 6). In addition, LKB1 silencing did not induce notable changes in p-mTOR2(Ser2481), p-S6K(Thr389) or p-PTEN (Ser370).[23,25,28] Double silencing of LKB1 and Rictor reduced p-Akt(Ser473 and Thr308) and sensitized the

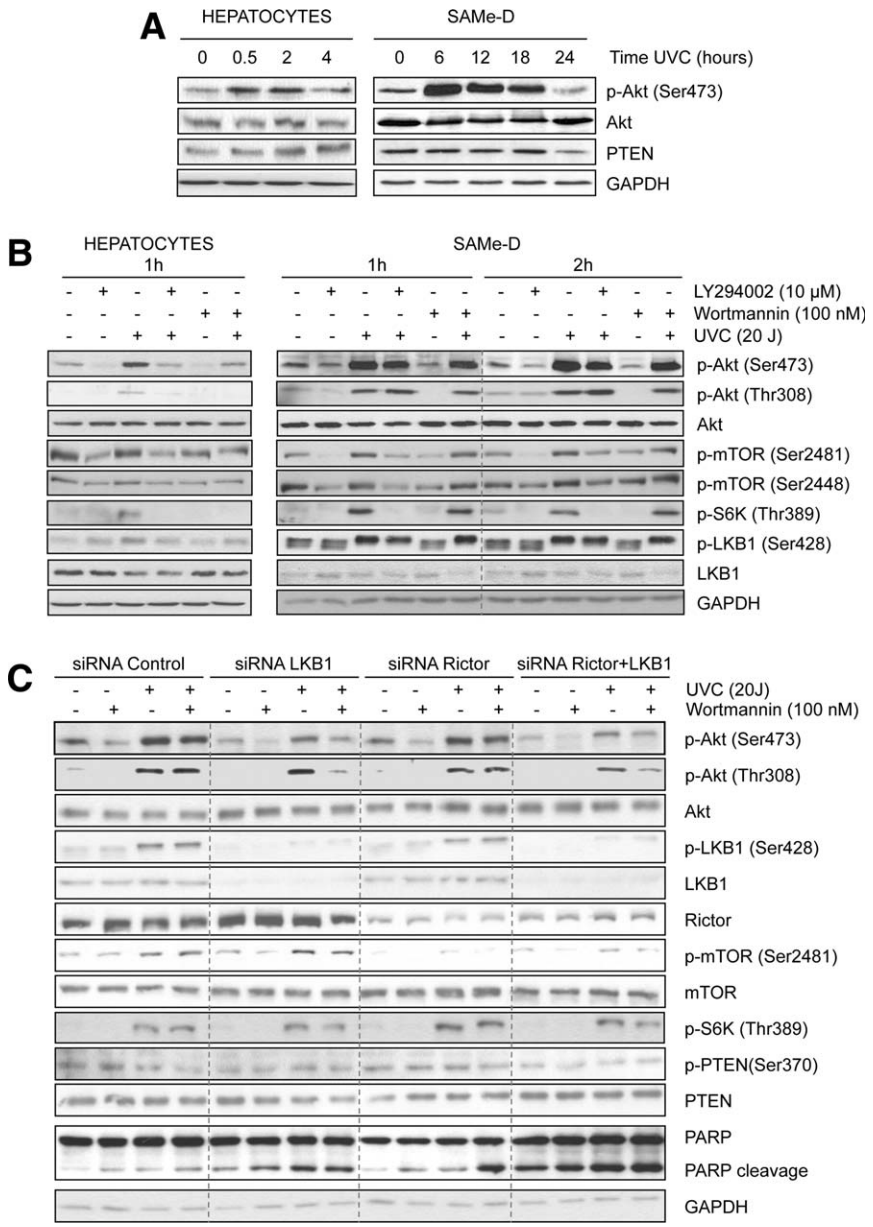

Fig. 2. UVC-induced AKT phosphorylation. (A) Hepatocytes and SAMe-D cells treated with UVC light. (B) Hepatocytes and SAMe-D cells were incubated for 1 hour with or without LY204002 (10 μM) or wortmannin (100 nM) before treatment with UVC light for 1 hour. (C) SAMe-D cells transfected with control, LKB1, Rictor, or LKB1 and Rictor siRNA were incubated for 1 hour with or without wortmannin, before treatment with UVC light for 1 hour. Protein extracts were analyzed by way of western blotting. Densitometric analysis is represented in Supporting Figs. 5 and 6.

cells to wortmannin, although this effect was not additive compared with only LKB1 silencing. These results are in agreement with the observation that Rictor silencing alone did not affect LKB1 phosphorylation. Importantly, we also observed that the decrease in p-Akt induced by LKB1 knockdown led to an increase in poly(ADP-ribose) polymerase (PARP) cleavage even without apoptotic stimulus (Fig. 2C and Supporting Fig. 6).

Even though AMPK has recently been described as a kinase of Akt,[31] UVC did not induce AMPK activation in hepatocytes or in SAMe-D cells (Supporting Fig. 7A). Furthermore, AMPK silencing did not affect Akt activation or render SAMe-D cells responsive to wortmannin, suggesting that this kinase is not involved in this signaling pathway (Supporting Fig. 7B).

Together, these results indicate that the phosphorylation of Akt at Ser473 and Thr308 in SAMe-D cells is not fully dependent of PI3K, mTORC2, or even AMPK, and suggest a parallel pathway involving LKB1 as part of the prosurvival mechanism.

Finally, we studied the extrinsic apoptotic response in SAMe-D cells through stimulation with the Fas agonist Jo2 antibody.[32] Although a single dose of Jo2 (2 μg/mL) was enough for the detection of cleaved PARP in primary hepatocytes at 12 hours, SAMe-D cells did not respond to the treatment even at 48 hours (Supporting Fig. 8A). A second dose of Jo2 treatment for an additional 24 hours was necessary to induce cleaved PARP in SAMe-D cells at 48 hours, together with a decrease in p-LKB1(Ser428) and p-Akt(Ser473) (Supporting Fig. 8B).

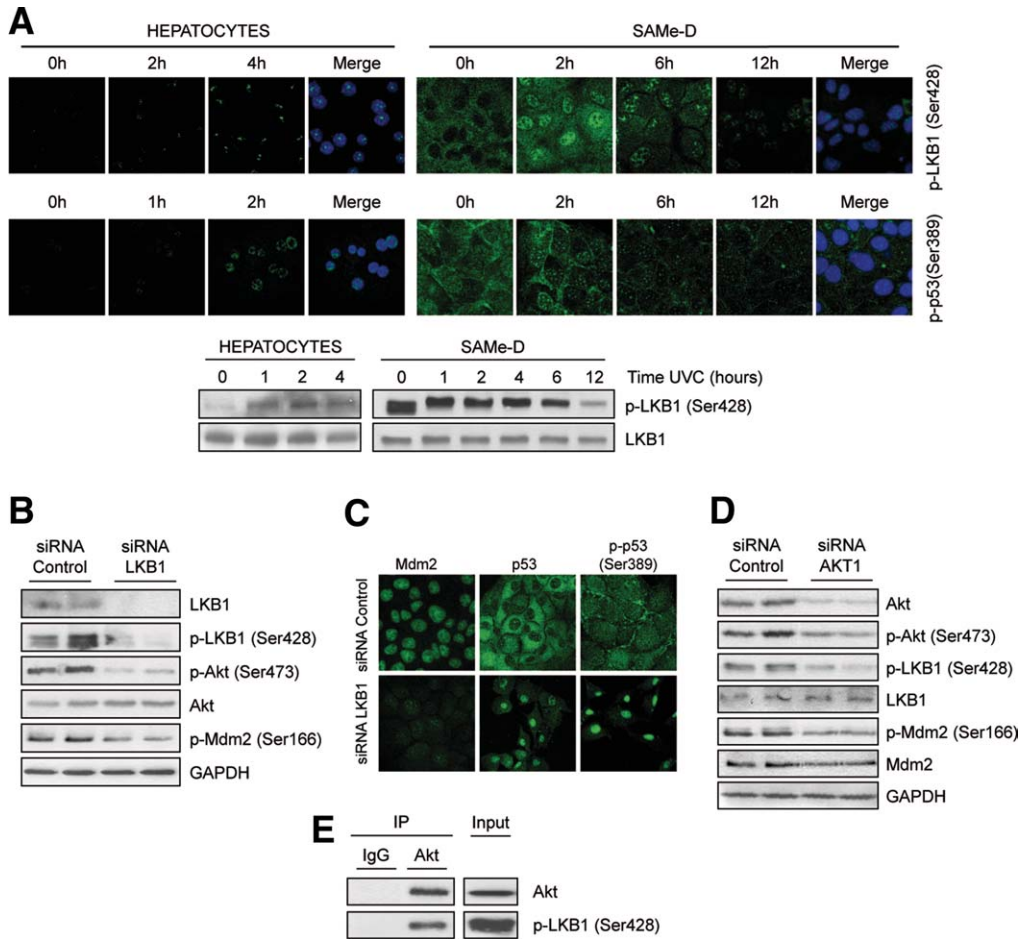

Fig. 3. LKB1 and apoptosis. (A) Hepatocytes and SAMe-D cells were treated with UVC light. p-LKB1 and p-p53(Ser389) were detected by way of immunostaining (upper), and p-LKB1 was detected by way of western blotting (bottom). (B) Western blot analysis of lysates from SAMe-D cells, transfected with control or LKB1 siRNA. (C) Immunocytochemistry of p53, p-p53(Ser389), and Mdm2 in SAMe-D cells transfected with control and LKB1 siRNA. (D) Western blot analysis of lysates from SAMe-D cells transfected with control or AKT siRNA. (E) Lysates from SAMe-D cells were immunoprecipitated with Akt antibody and a nonspecific IgG. Inmunoprecipates (IP) and lysates (input) were analyzed by way of western blotting.

LKB1 and the Apoptotic Response in SAMe-D Cells. We observed an increase in nuclear p-LKB1 (Ser428) during the apoptotic response in hepatocytes after UVC treatment, whereas untreated SAMe-D cells showed a mostly basal-cytoplasmic p-LKB1 that moved to the nucleus after UVC stimulus (Fig. 3A, upper panel) and decreased 12 hours later (Fig. 3A, lower panel). This decrease coincided with the apoptotic response observed in these cells.

LKB1 silencing reduced p-Akt(Ser473) in untreated SAMe-D cells, accompanied by a decrease in p-Mdm2(Ser166) and in total Mdm2 levels (Fig. 3B,C). Akt controls the Mdm2/p53 signaling pathway by decreasing Mdm2 proteasomal degradation.[33] Consistent with the specific regulation of LKB1 on Mdm2 content, increased levels of nuclear p53 were detected after LKB1 ablation (Fig. 3C). Previous reports have shown that LKB1-mediated phosphorylation of p53(Ser389) regulates its transcriptional activity,[7] and that p53 posttranslational modifications play an important role in the stabilization and activation of p53. We observed p-p53(Ser389) in the cytoplasm of unstimulated SAMe-D cells. Hepatocytes showed a nuclear expression 2 hours after UVC treatment (Fig. 3A), whereas in SAMe-D cells, the nuclear staining was observed at 12 hours. After LKB1 knockdown, p-p53(Ser389) was mainly localized in the nuclear compartment in SAMe-D cells (Fig. 3C).

Finally, a reduction in total and active Mdm2 was observed after Akt knockdown in SAMe-D cells, and this effect was accompanied by a slight decrease in p-LKB1 (Fig. 3D). Immunoprecipitation analysis revealed a complex between p-LKB1 and Akt (Fig. 3E) at basal levels in SAMe-D cells, suggesting cross-talk between these two kinases.

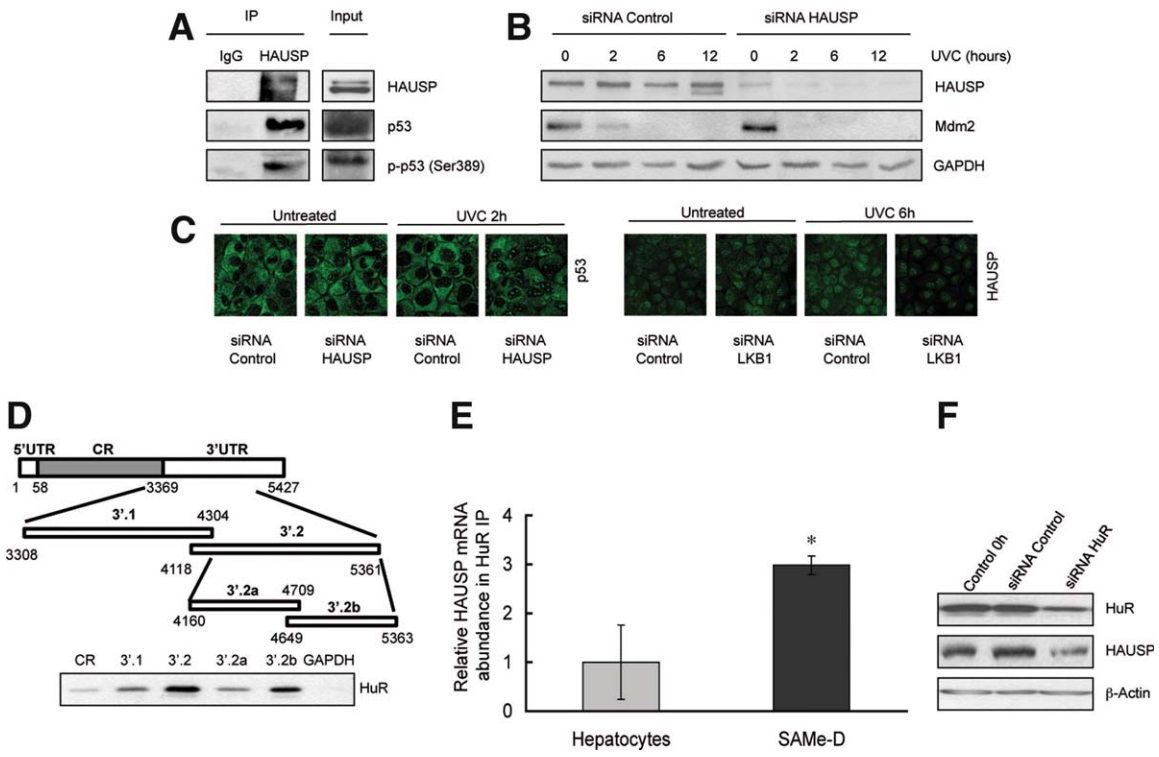

Fig. 4. HuR regulates HAUSP stability. (A) Cell lysates from SAMe-D cells were immunoprecipitated with HAUSP antibody and with a nonspecific IgG. Inmunoprecipates (IP) and lysates (input) were analyzed by way of western blotting. (B) Western blot analysis and (C) immunocytochemistry of SAMe-D cells transfected with control or HAUSP siRNA. (D) Immunocytochemistry of SAMe-D cells transfected with control or LKB1 siRNA. (E) Upper panel: Scheme of *HAUSP* mRNA showing biotinylated transcripts (5′-UTR, coding region, 3′-UTR) and the predicted HuR motifs. Lower panel: Western blots of HuR and the biotinylated HAUSP fragments. (F) Lysates from SAMe-D cells were immunoprecipitated with HuR antibody and *HAUSP* and *ACTIN* mRNA detected. (G) Western blot analysis of SAMe-D cells transfected with control and HuR siRNA.

LKB1 Regulates Herpesvirus-Associated Ubiquitin-Specific Protease (HAUSP) Localization. HAUSP, a nuclear ubiquitin-specific protease, targets p53 and Mdm2 as substrates and, in concert with Mdm2, plays a dynamic role in p53 functionality.[34] SAMe-D cells expressed higher levels of HAUSP than hepatocytes both in the cytoplasm and nucleus (Supporting Fig. 9). Hepatocytes showed predominantly nuclear HAUSP accumulation after UVC treatment (Supporting Fig. 9). A defect in interaction with HAUSP has been shown to cause cytoplasmic accumulation of p53.[34] We detected HAUSP-p53 interaction in SAMe-D cells (Fig. 4A), suggesting that this cytosolic HAUSP could be responsible for p53 cytosolic localization in these cells. In fact, we found that HAUSP silencing in SAMe-D cells induced a decrease in levels of Mdm2 (Fig. 4B), the major substrate stabilized by HAUSP,[35] and an increase in nuclear localization of p53 after UVC treatment (Fig. 4C, left panel).

LKB1 knockdown in SAMe-D cells also induced a slight increase in the nuclear levels of HAUSP after UVC treatment (Fig. 4C, right panel). This correlated with a reduction in Mdm2 levels, nuclear accumulation of p53, and activation of apoptosis (Fig. 3C). Our findings suggest that localization of HAUSP is dynamically regulated, and it has been shown that phosphorylation in Ser18 and Ser963 could be responsible for this.[35]

Identification of HAUSP as a Novel Target of HuR. HuR binds to and regulates many mRNAs that encode stress response, proliferative proteins, and antiapoptotic and apoptotic proteins.[21,36,37] High HuR levels characterize SAMe-D cells (Supporting Fig. 1), correlating with its prosurvival function during cell division,[37] and the promotion of a malignant phenotype.[38] Computer analysis of *HAUSP* mRNA revealed a large 3′ untranslated region (UTR) of 2,000 bp (Fig. 4D, upper panel), suggesting that HAUSP could be a target of HuR. We performed a biotin pull-down with the complementary DNAs corresponding to either the 5′-UTR, the coding region, or the 3′-UTR of *HAUSP*. We found that HuR was predominantly bound to the 3′-UTR transcript in the specific regions indicated (Fig. 4D, lower panel). Immunoprecipitation assays of HuR-bound mRNAs from hepatocytes and SAMe-D cell lysates revealed an HuR–*HAUSP* mRNA interaction in both cell types, although the amount of *HAUSP* mRNA and HuR–*HAUSP* mRNA complex was clearly elevated in SAMe-D cells compared with hepatocytes (Fig. 4E). Finally, HuR

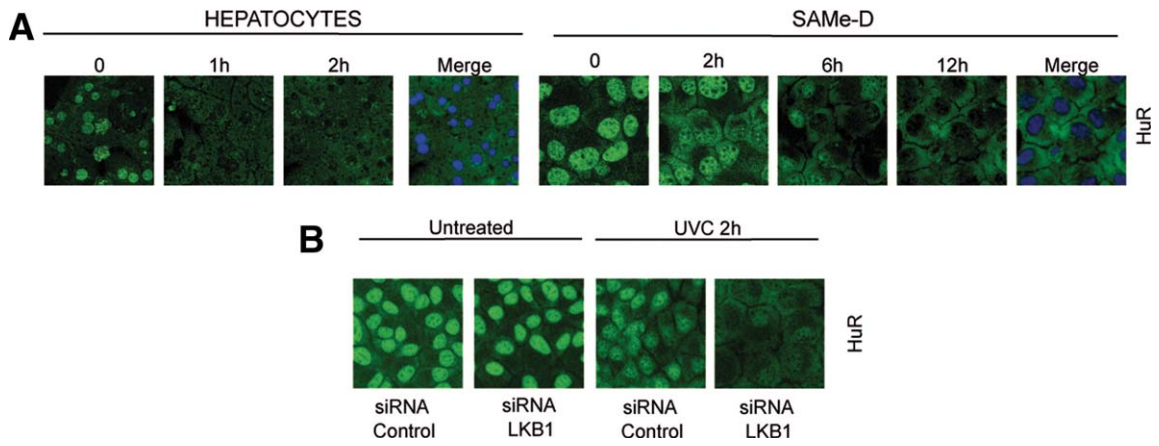

Fig. 5. HuR activity is regulated by LKB1. Immunocytochemical analysis of (A) hepatocytes and SAMe-D or (B) SAMe-D cells transfected with control or LKB1 siRNA and treated with UVC light.

knockdown induced a clear decrease in HAUSP levels, emphasizing that HAUSP is a target of HuR (Fig. 4F).

HuR Activity Is Regulated by LKB1 in SAMe-D Cells. HuR is predominantly nuclear, and the translocation to the cytoplasm has been linked to its mRNA-stabilizing function.[21] We observed a delay in HuR translocation to the cytoplasm in SAMe-D cells compared with hepatocytes after UVC treatment (Fig. 5A). LKB1 knockdown promoted faster cytoplasmic localization of HuR after UVC treatment (Fig. 5B). In summary, our results suggest a novel signaling network between LKB1, HuR, HAUSP, and p53. We identify LKB1 as a key regulator of HuR and HAUSP localization and, consequently, of their functionality, whereas HuR regulates HAUSP mRNA stability.

p53 and p-LKB1 in Livers from MAT1A-KO Mice and Human HCC. Liver tumors from MAT1A-KO mice highly expressed cytoplasmic p53 and p-LKB1(Ser428) compared with WT mice (Fig. 6A). In HCC samples surgically resected from patients with ASH and NASH, we observed cytoplasmic staining of p53 and p-LKB1(Ser428) (Fig. 6B), suggesting that this localization and activation may be characteristic of HCC in patients with steatohepatitis.

Discussion

Although LKB1 has been traditionally considered a tumor suppressor,[6] the results of this study suggest that LKB1 might play a role in cell survival in liver tumors that have originated from metabolic disorders, such as NASH.

HCC is characterized by low levels of SAMe and reduced expression of *MAT1A*. Although MAT1A-KO

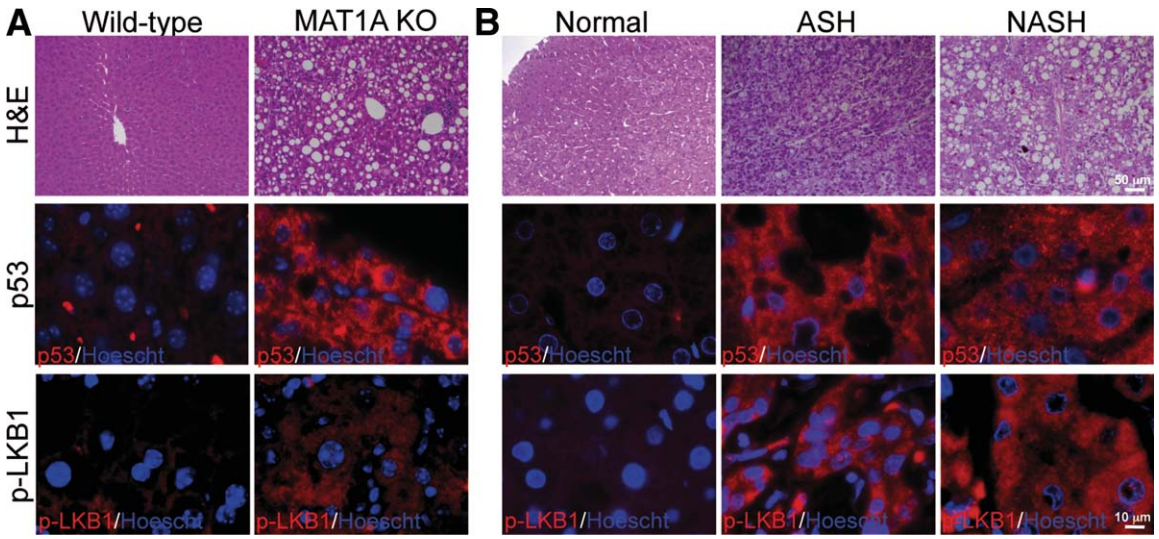

Fig. 6. p53 and p-LKB1 in HCC. Hematoxylin-eosin staining and immunohistochemical analysis of p53 and p-LKB1 protein in liver samples from (A) MAT1A-KO tumors and (B) human HCC derived from ASH and NASH.

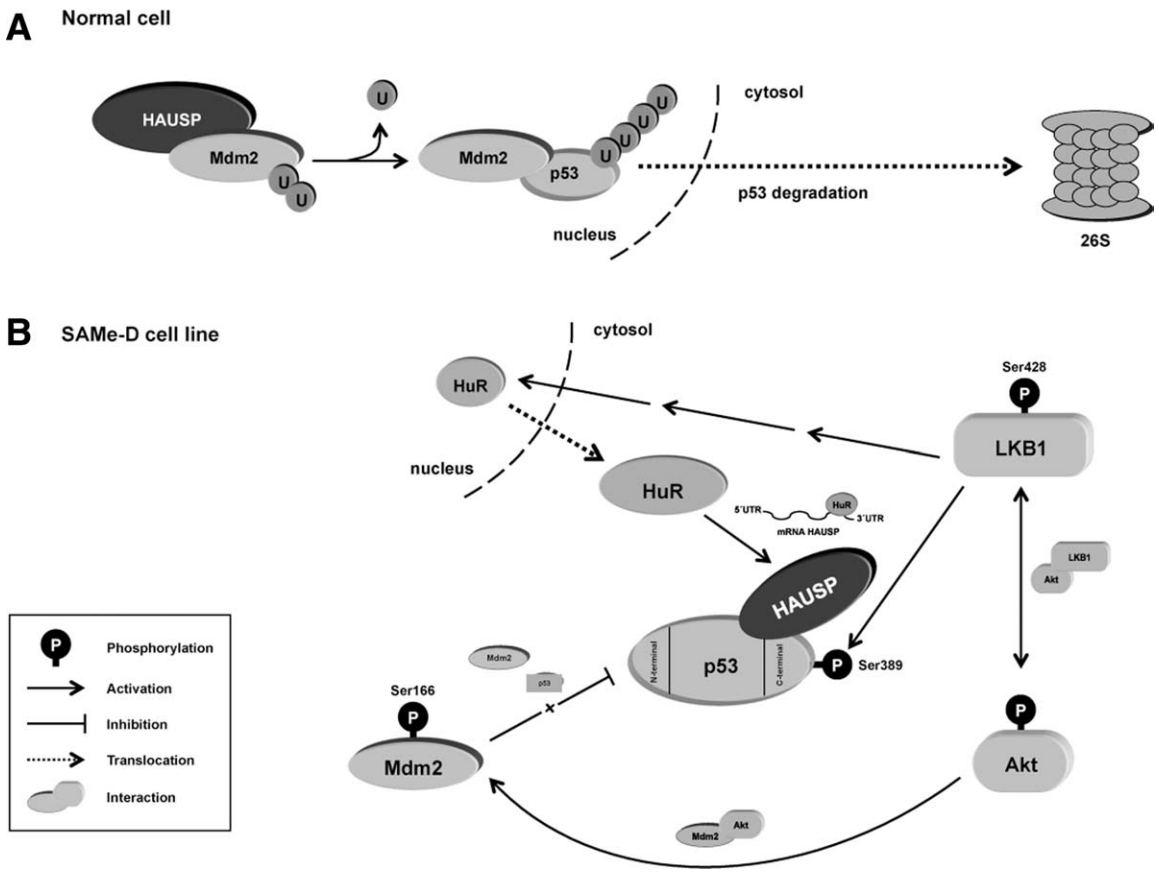

Fig. 7. Schematic representation of p53 behavior in SAMe-D cells. (A) In normal cells, p53 is kept at low levels due to the activity of its negative regulator Mdm2. Mdm2 binds and polyubiquitinates p53 for proteasomal degradation. The deubiquitinating enzyme HAUSP contributes to the stability of Mdm2, impairing its self-ubiquitilation and degradation. (B) In SAMe-D cells, p53 is mostly cytosolic and hyperphosphorylated by several kinases, such as p-LKB1, which is basally hyperactivated. The hyperphosphorylation of p53 and its interaction with HAUSP blocks the negative regulation by Mdm2. p-LKB1 is responsible for two more processes: (1) the activation of survival Akt, which leads to the phosphorylation of Mdm2 decreasing the HAUSP-Mdm2 interaction, and (2) the cytosolic translocation of HuR, which stabilizes *p53* and *HAUSP* mRNA.

mice represent a chronic model of SAMe deficiency, they develop steatosis, NASH, and HCC in a spontaneous way.[8] Liver LKB1 and AMPK are hyperphosphorylated in these animals during the progression of the disease.[8] LKB1 localization is predominantly nuclear, and its activation takes place mainly in the cytoplasm.[39] In MAT1A-KO mice with HCC, p-LKB1(Ser428) was found to be mostly cytoplasmic. SAMe-D is a cell line isolated from MAT1A-KO mice with HCC as a model of a NASH-derived tumor cell. In this model, the cytoplasmic hyperphosphorylation of LKB1 (Ser 428) prevents UVC-induced apoptosis partially through the Akt survival pathway. In SAMe-D cells, the PI3K inhibitors LY294002 and wortmannin did not affect Akt phosphorylation, and LKB1 depletion was necessary to induce Akt inhibition after wortmannin treatment. We observed an interaction between LKB1 and Akt proteins. In addition, UVC-induced Akt phosphorylation was independent of mTORC2, AMPK, or PTEN activity. Furthermore, LKB1 knockdown induced an increase in PARP cleavage, even without any apoptotic stimuli. These results provide the first evidence of cross-talk between LKB1 and Akt in response to an apoptotic signal, leading us to consider this pathway as a compensatory and salvage mechanism in SAMe-D global response.

It is well known that LKB1 plays a dual role in the regulation of cell death and proliferation through functions linked to the tumor suppressor p53. LKB1 binds to and phosphorylates p53 at Ser389, reducing the efficiency in its nuclear import.[39] In SAMe-D cells, total p53 and its phosphorylated form (Ser389) were mostly present in the cytoplasm, although a nuclear accumulation of p-p53 was observed 12 hours after UVC treatment. LKB1 knockdown decreased the amount of p-p53 in SAMe-D cells and increased p53 nuclear accumulation, confirming the existence of cross-talk between these two proteins.

p53 cytoplasmic staining was observed in the HCC of MAT1A-KO mice and in ASH- and NASH-derived

human HCC. A defect in HAUSP-p53 interaction has been shown to be a cause of p53 accumulation.[40] However, in SAMe-D cells, the levels of HAUSP were higher, and there was a functional interaction between p53 and HAUSP.

LKB1 could play a critical role in the control of the cellular localization of HAUSP in SAMe-D cells. It has been reported that phosphorylation of HAUSP is sufficient to achieve changes in the localization of the enzyme.[35] In SAMe-D cells, the partial reduction of LKB1 levels induces nuclear accumulation of the HAUSP compartment after UVC treatment. Thus, LKB1 could be regulating HAUSP localization in SAMe-D cells directly by phosphorylation, or indirectly by regulating interactions with its partners, Mdm2 and p53.[34] Consistent with the latter, LKB1 knockdown reduced Mdm2 levels. This could cause a modification in Mdm2 partners and, in this case, alter the localization of HAUSP. In addition, we investigated the influence of HAUSP on the apoptotic response in SAMe-D cells. HAUSP silencing destabilized Mdm2, which is constitutively self-ubiquitinated and degraded *in vivo*, and led to nuclear accumulation of p53 in SAMe-D cells.

Following the analysis of HAUSP regulation, we identified a translational mechanism involved in this process. For the first time, we have demonstrated that HuR, which is highly expressed in SAMe-D, can specifically bind the 3′-UTR of *HAUSP*, stabilizing the mRNA and increasing *HAUSP* levels.

Finally, our findings are summarized in a representative model (Fig. 7). In normal cells, p53 is maintained at low levels, and Mdm2 is responsible for its ubiquitination and degradation. The levels of Mdm2 are limited by its own partner p53 through the transactivation of its gene. In addition, Mdm2 levels are controlled by posttranslational modifications and through the deubiquitinase HAUSP that modulates its stability. In SAMe-D cells, low SAMe levels are due to loss of *MAT1A* expression, as occurs in different types of HCC. In SAMe-D cells, cytoplasmic LKB1 phosphorylation is maintained at high levels and acts in different ways: (1) phosphorylating Akt that up-regulates p-Mdm2(Ser166), inhibiting its binding to p53; (2) phosphorylating p53(Ser389), making the p53 protein more stable; (3) regulating the cytoplasmic localization of HuR and therefore stabilizing the mRNA of *HAUSP*; and (4) regulating the cytoplasmic accumulation of HAUSP, allowing HAUSP–p53 interaction and leading to a more stable p53 in the cytoplasm. In summary, LKB1 seems to control the survival pathway through Akt activation and the apoptotic response through p53 and HuR.

Taken together, the SAMe-D cell line is a new model of NASH-derived HCC with a chronic deficiency in *MAT1A*, in which LKB1 plays a crucial role as principal conductor of a new regulatory mechanism. Our findings might represent a useful tool to uncover new therapeutic strategies for HCC.

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References

1. Varela-Rey M, Embade N, Ariz U, Lu SC, Mato JM, Martínez-Chantar ML. Non-alcoholic steatohepatitis and animal models: understanding the human disease. Int J Biochem Cell Biol 2009;41:969-976.
2. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339-346.
3. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307-310.
4. Yahagi N, Shimano H, Matsuzaka T, Sekiya M, Najima Y, Okazaki S, et al. p53 involvement in the pathogenesis of fatty liver disease. J Biol Chem 2004;279:20571-20575.
5. Mehenni H, Gehrig C, Nezu J, Oku A, Shimane M, Rossier C, et al. Loss of LKB1 kinase activity in Peutz-Jeghers syndrome, and evidence for allelic and locus heterogeneity. Am J Hum Genet 1998;63: 1641-1650.
6. Zhong D, Liu X, Khuri FR, Sun SY, Vertino PM, Zhou W. LKB1 is necessary for Akt-mediated phosphorylation of proapoptotic proteins. Cancer Res 2008;68:7270-7277.
7. Zeng PY, Berger SL. LKB1 is recruited to the p21/WAF1 promoter by p53 to mediate transcriptional activation. Cancer Res 2006;66: 10701-10708.
8. Vázquez-Chantada M, Ariz U, Varela-Rey M, Embade N, Martínez-Lopez N, Fernández-Ramos D, et al. Evidence for LKB1/AMP-activated protein kinase/ endothelial nitric oxide synthase cascade regulated by hepatocyte growth factor, S-adenosylmethionine, and nitric oxide in hepatocyte proliferation. HEPATOLOGY 2009;49:608-617.
9. Martínez-Chantar ML, Vázquez-Chantada M, Garnacho M, Latasa MU, Varela-Rey M, Dotor J, et al. S-adenosylmethionine regulates cytoplasmic HuR via AMP-activated kinase. Gastroenterology 2006; 131:223-232.
10. Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. HEPATOLOGY 2007;45:1306-1312.
11. Mato JM, Martinez-Chantar ML, Lu SC. Methionine metabolism and liver disease. Annu Rev Nutr 2008;28:273-293.
12. Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, et al. Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. Proc Natl Acad Sci USA 2001;98:5560-5565.
13. Martínez-Chantar ML, Corrales FJ, Martínez-Cruz LA, García-Trevijano ER, Huang ZZ, Chen L, et al. Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. FASEB J 2002;16:1292-1294.
14. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res 1988;48: 6863-6871.
15. Leffert HL, Koch KS, Moran T, Williams M. Liver cells. Methods Enzymol 1979;58:536-544.
16. Vázquez-Chantada M, Fernández-Ramos D, Embade N, Martínez-Lopez N, Varela-Rey M, Woodhoo A, et al. HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation and carcinogenesis.2010;138:1943-1953.

17. Von Mikecz A, Chen M, Rockel T, Scharf A. The nuclear ubiquitin-proteasome system: visualization of proteasomes, protein aggregates, and proteolysis in the cell nucleus. Methods Mol Biol 2008;463:191-202.
18. Moll UM, Riou G, Levine AJ. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A 1992;89:7262-7266.
19. Moll UM, LaQuaglia M, Benárd J, Riou G. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci U S A 1995;92: 4407-4411.
20. Bosari S, Viale G, Bossi P, Maggioni M, Coggi G, Murray JJ, et al. Cytoplasmic accumulation of p53 protein: an independent prognostic indicator in colorectal adenocarcinomas. J Natl Cancer Inst 1994;86: 681-687.
21. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci 2001;58:266-277.
22. Alarcon-Vargas D, Ronai Z. p53-Mdm2—the affair that never ends. Carcinogenesis 2002;23:541-547.
23. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905-2927.
24. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86-90.
25. Yang Z, Yi J, Li X, Long W. Correlation between loss of PTEN expression and PKB/AKT phosphorylation in hepatocellular carcinoma. J Huazhong Univ Sci Technolog Med Sci 2005;25:45-47.
26. Bhaskar PT, Hay N. The two TORCs and Akt. Dev Cell 2007;12: 487-502.
27. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098-1101.
28. Kristof AS, Marks-Konczalik J, Billings E, Moss J. Stimulation of signal transducer and activator of transcription-1 (STAT1)-dependent gene transcription by lipopolysaccharide and interferon-gamma is regulated by mammalian target of rapamycin. J Biol Chem 2003;278: 33637-33644.
29. Huang C, Li J, Ke Q, Leonard SS, Jiang BH, Zhong XS, et al. Ultraviolet-induced phosphorylation of p70(S6K) at Thr(389) and Thr(421)/Ser(424) involves hydrogen peroxide and mammalian target of rapamycin but not Akt and atypical protein kinase C. Cancer Res 2002;62:5689-5697.
30. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signalling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell 2006;11:859-871.
31. Levine YC, Li GK, Michel T. Agonist-modulated regulation of AMP-activated protein kinase (AMPK) in endothelial cells. Evidence for an AMPK -> Rac1 -> Akt -> endothelial nitric-oxide synthase pathway. J Biol Chem 2007;282:20351-20364.
32. González-Rodriguez A, Alba J, Zimmerman V, Kozma SC, Valverde AM. S6K1 deficiency protects against apoptosis in hepatocytes. HEPATOLOGY 2009:50:216-229.
33. Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, et al. Akt enhances Mdm2-mediated ubiquitination and degradation of p53. J Biol Chem 2002;277:21843-21850.
34. Stevenson LF, Sparks A, Allende-Vega N, Xirodimas DP, Lane DP, Saville MK. The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2. EMBO J 2007;26:976-986.
35. Fernández-Montalván A, Bouwmeester T, Joberty G, Mader R, Mahnke M, Pierrat B, et al. Biochemical characterization of USP7 reveals post-translational modification sites and structural requirements for substrate processing and subcellular localization. FEBS J 2007;274:4256-4270.
36. Westmark CJ, Bartleson VB, Malter JS. RhoB mRNA is stabilized by HuR after UV light. Oncogene 2005;24:502-511.
37. Wang W, Caldwell MC, Lin S, Furneaux H, Gorospe M. HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. EMBO J 2000;19:2340-2350.
38. Lopez de Silanes I, Lal A, Gorospe M. HuR: post-transcriptional paths to malignancy. RNA Biol 2005;2:11-13.
39. Ullrich SJ, Sakaguchi K, Lees-Miller SP, Fiscella M, Mercer WE, Anderson CW, et al. Phosphorylation at Ser-15 and Ser-392 in mutant p53 molecules from human tumors is altered compared to wild-type p53. Proc Natl Acad Sci U S A 1993;90:5954-5958.
40. Becker K, Marchenko ND, Maurice M, Moll UM. Hyperubiquitylation of wild-type p53 contributes to cytoplasmic sequestration in neuroblastoma. Cell Death Differ 2007;14:1350-1360.