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Activation of LKB1-Akt pathway independent of PI3 Kinase plays a critical role in the proliferation of hepatocellular carcinoma from NASH

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Abstract

LKB1, originally considered as a tumor suppressor, plays an important role in hepatocyte proliferation and liver regeneration. Mice lacking *Methionine adenosyltransferase 1A* (MAT1A-KO mice) gene show chronic reduction in hepatic SAMe levels, basal activation of LKB1 and spontaneously develop non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC). These results are relevant for human health since patients with liver cirrhosis, who are at risk to develop HCC, have a marked reduction in hepatic MAT1A expression and SAMe synthesis. In our current work we have isolated a cell line, SAMe-D (SAMe-Deficient) from the HCC of MAT1A-KO mice to examine the role of LKB1 in the development of liver tumors derived from metabolic disorders. We find that LKB1 is required for cell survival in SAMe-D cells. LKB1 regulates Akt-mediated survival independently of PI3K, AMPK and mTORC2. In addition, LKB1 controls the apoptotic response through phosphorylation and retention of p53 in the cytoplasm, and the regulation of HAUSP and HuR nucleo-cytoplasmic shuttling. We identify 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) ASH and NASH etiology.

Conclusions—SAMe-D cell line is a relevant model of HCC derived from NASH disease where LKB1 is the principal conductor of a new regulatory mechanism and could be considered as a practical tool to uncover new therapeutic strategies.

Keywords

NASH; HCC; LKB1; AMPK; HuR

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by triglyceride accumulation in hepatocytes (hepatic steatosis) and steatosis with inflammation (NASH), which may progress to cirrhosis and hepatocellular carcinoma (HCC) (1). It has been recently demonstrated 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 germ-line or somatic mutations increases the risk of cancer development (5). However, LKB1 can also play an anti-apoptotic 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 (HGF) induces LKB1-mediated AMPK activation, which controls the nucleo-cytoplasmic shuttling of HuR, an RNA-binding protein, that leads to an increased half-life of target 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 HGF-induced LKB1/AMPK/HuR activation and cyclin D1 and A2 expression (8,9). Methionine adenosyltransferase (MAT), encoded by two genes, *MAT1A* (expressed in the adult liver), and *MAT2A* (expressed in extrahepatic tissues and during liver proliferation), catalyzes SAME synthesis (10,11). *MAT1A*-KO mice show a chronic deficiency in hepatic SAME levels and spontaneously develop NASH and HCC resembling the human pathology where the HCC are characterized by low levels of SAME and reduced *MAT1A* expression (12,13). Hepatic LKB1 and AMPK are also activated, cytoplasmic localization of HuR is increased and levels of several mRNAs involved in cell proliferation are elevated (6,8).

In the current work, we have isolated a cell line from the *MAT1A*-KO mice HCC (SAME-D) as a model of a NASH-derived tumor cell. The results obtained in these cells, and in human HCC from ASH (alcoholic steatohepatitis) and NASH etiologies, suggest that p53 inactivation by cytoplasmic sequestration and activation of LKB1 can contribute to HCC development.

Methods

SAME-D cells isolation

Procedures performed in *MAT1A*-KO and wild-type (WT) mice were done in compliance with the institutional guidelines of 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%-FBS DMEM.

Isolation of hepatocytes

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

RNA isolation and real-time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen). 1.5 µg of total RNA was retrotranscribed into cDNA using SuperScriptIII retrotranscriptase (Invitrogen). PCRs were performed using BioRad iCycler Thermalcycler.

Protein isolation and Western blot

Extraction of total protein from cultured cells has been described previously (9). Cytosolic and membrane lysates from cultured cells were prepared with the subcellular proteome extraction kit (Calbiochem). 30 µg of protein were electrophoresed on SDS-polyacrylamide gels and transferred onto membranes. Membranes were incubated as described in Table SI.

Human Samples

Surgically resected specimens of 12 liver cirrhosis patients with HCC were examined. Patients gave informed consent to all clinical investigations performed in accordance with the principles embodied in the Declaration of Helsinki. The data and type of bio-specimen used were provided by the Basque Biobank for Research with appropriate ethics approval. The etiology 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 100x objective from an epifluorescence microscope AXIO Imager.D1 (Zeiss).

Immunocytochemistry

Cells fixed with ethanol or methanol, were blocked for 30 minutes with PBS containing, 0.1% BSA, 10% horse serum and 0.1% Triton and incubated overnight at 4°C with primary antibodies in blocking solution without Triton (Table SII). Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated to rabbit or mouse IgG and Hoescht nuclear dye. Cells were examined under Leica TCS-SP (UV) confocal laser microscope using a 60x objective.

Gene Silencing

SAMe-D cells were transfected with 100 nM siRNA constructs (Quiagen) (Table SIII) using Lipofectamine 2000 (Invitrogen) twice, every 24 hours during 48 hours. Silencing mix was left overnight and afterwards medium was replaced with fresh 10%-FBS DMEM.

Protein immunoprecipitation

500 µg of total cellular protein were immunoprecipitated overnight at 4°C with 10 µg of IgG1 (BD Pharmingen), HAUSP or Akt (Cell signalling) antibodies and protein A Shepharose (Sigma).

RNA immunoprecipitation

RNA immunoprecipitation was done as described previously (14) and mRNA bound to HuR was measured by real time PCR, and normalized with GAPDH.

Biotin pull-down

Synthesis of biotinylated transcripts and analysis of HuR bound to biotinylated RNA was done as described before (9,16).

Statistical analysis

Experiments were performed in triplicate with data expressed as means ± SEM. Statistical significance was estimated with Student's *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 signalling [Mdm2, p-Mdm2(Ser166), Bax, PUMA and HAUSP] as well as apoptotic (cleaved caspase-3) and anti-apoptotic markers (Bcl-2, HuR) highly expressed compared with WT hepatocytes, which correlated with positive levels of proliferative markers such as cyclin D1 and PCNA (Figure S1). Similar results were observed in another SAME-D cell clone (Clone 2) (Figure S2A).

In normal cells, expression of p53 is maintained at low levels by the control of the ubiquitin proteasome system (UPS) (17). In SAME-D cells, however, the p53 gene and protein were over-expressed compared to WT hepatocytes (Table SIV and Figure S1A). A fluorescent in situ (FISH) analysis in SAME-D cells showed p53 genomic amplification having four copies of chromosome 11, each harbouring one p53 signal (Figure S3), which was not affected by any mutation after sequencing analysis (Table SV).

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 (Figures S4A and S2C). P53 silencing confirmed the specificity of the staining (Figure S4B). Treatment with the nuclear export inhibitor leptomycin B (20 nM) induced the nuclear accumulation of HuR (21) but not of p53 (Figure S4C), 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 and 1 hours respectively (Figure 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 (Figure 1B), mainly in the cytoplasm (Figure 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 (Figure 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) (Figures 1E, 1D and 1B), 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 (Figure 2A). In hepatocytes, however, p-Akt returned to basal levels 4 hours after UVC irradiation. The levels of PTEN phosphatase, the major negative regulator of PI3K/Akt signalling pathway (25), were normal in SAME-D cells (Figure 2A).

In WT hepatocytes, wortmannin (PI3K inhibitor) and LY294002 (dual mTOR and PI3K inhibitor) prevented UVC-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 (Figure 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) and Rictor (forming mTOR complex 2) (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) (Figure 2B and S5). These data are in agreement with previous studies showing that S6K phosphorylation in response to UVC is mediated through mTOR1 and PI3K but Akt-independent (29). Rictor silencing did not change p-Akt (Ser473 and Thr308) either at basal levels or after UVC stimulation, and had no effect on wortmannin sensitivity (Figures 2C and S6). In addition, Rictor knockdown downregulated p-mTOR2 without affecting p-mTOR1 and p-S6K, confirming the high substrate specificity of the two TOR complexes (30).

Since 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 (Figure 2B). Interestingly, LKB1 knockdown reduced p-Akt(Ser473 and Thr308) after UVC treatment and, most importantly, rendered the cells responsive to wortmannin (Figures 2C and S6). 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 cells to wortmannin although this effect was not additive compared to 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 PARP cleavage even without apoptotic stimulus (Figures 2C and S6).

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 (Figure S7A). 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 signalling pathway (Figure S7B).

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 the pro-survival mechanism.

Finally, we studied the extrinsic apoptotic response in SAME-D cells by stimulation with the Fas agonist Jo2 antibody (32). While a single dose of Jo2 (2 µg/ml) was enough for detection of cleaved PARP in primary hepatocytes at 12 hours, SAME-D cells did not respond to the treatment even at 48 hours (Figure S8A). 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) (Figure S8B).

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, while untreated SAME-D cells showed a mostly basal-cytoplasmic p-LKB1 that moved to the nucleus after UVC stimulus (Figure 3A, upper

panel), and decreased 12 hours later (Figure 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 (Figure 3B and C). Akt controls the Mdm2/p53 signalling 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 (Figure 3C). Previous reports showed that LKB1-mediated phosphorylation of p53(Ser389) regulates its transcriptional activity (7), and that p53 post-translational 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 (Figure 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 (Figure 3C).

Finally, a reduction in total and active Mdm2 was found after Akt knockdown in SAME-D cells and this effect was accompanied by a slight decrease in p-LKB1 (Figure 3D). Immunoprecipitation analysis showed a complex between p-LKB1 and Akt (Figure 3E) at basal levels in SAME-D cells, suggesting a crosstalk between both kinases.

LKB1 regulates 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 (Figure S9). Hepatocytes showed predominantly nuclear HAUSP accumulation after UVC treatment (Figure S9). 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 (Figure 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 Mdm2 levels (Figure 4B), the major substrate stabilized by HAUSP (35), and an increase in nuclear localization of p53 after UVC treatment (Figure 4C left panel).

LKB1 knockdown in SAME-D cells also induced a slight increase in the nuclear levels of HAUSP after UVC treatment (Figure 4C right panel). This correlated with a reduction in Mdm2 levels, nuclear accumulation of p53 and activation of apoptosis (Figure 3C). Our findings suggest that localization of HAUSP is dynamically regulated and it has previously 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 anti-apoptotic and apoptotic proteins (21,37). High HuR levels characterize SAME-D cells (Figure S1), correlating with its pro-survival function during cell division (37), and the promotion of a malignant phenotype (38). Computer analysis of *HAUSP* mRNA revealed a large 3' UTR of 2,000 bp (Figure 4D upper panel) suggesting that HAUSP could be a target of HuR. We performed a biotin pull down with the cDNAs corresponding to either the 5' UTR, the coding region (CR) or the 3' UTR of *HAUSP*. We found that HuR was predominantly bound to the 3' UTR transcript in the specific regions indicated (Figure 4D lower panel). IP assays of HuR-bound mRNAs from hepatocytes and SAME-D cell lysates revealed a 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 to hepatocytes (Figure 4E). Finally, HuR knockdown induced a clear decrease in HAUSP levels, emphasizing that HAUSP is a target of HuR (Figure 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 to hepatocytes after UVC treatment (Figure 5A). LKB1 knockdown promoted faster cytoplasmic localization of HuR after UVC treatment (Figure 5B). In summary, our results suggest a novel signalling network between LKB1, HuR, HAUSP and p53. We identify LKB1 as a key regulator of HuR and HAUSP localization and, consequently, of their functionality, while HuR regulates HAUSP mRNA stability.

p53 and p-LKB1 in liver from MAT1A-KO mice and human HCC

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

Discussion

Although LKB1 has been traditionally considered as a tumor suppressor (6) the results presented here suggest that LKB1 might play a role in cell survival in liver tumors originated from metabolic disorders, such as NASH.

HCC are characterized by low levels of SAME and reduced expression of *MAT1A*. Although MAT1A-KO mice is a chronic model of SAME deficiency, the mice develop steatosis, NASH and HCC in a spontaneous way (8). Liver LKB1 and AMPK are hyperphosphorylated in those animals during the progression of the disease (8). LKB1 localization is predominantly nuclear and its activation takes place mainly in the cytoplasm (39). In HCC from MAT1A-KO mice, p-LKB1(Ser428) was found to be mostly cytoplasmic. SAME-D is a cell line isolated from the HCC of MAT1A-KO mice 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 the 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 a crosstalk 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 regulating 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, the 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 a crosstalk 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 previously

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 had been previously 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 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 have identified a translational mechanism involved in this process. For the first time we have demonstrated that HuR, 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 (Figure 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 by the transactivation of its gene. In addition, Mdm2 levels are controlled by post-translational 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: (i) phosphorylating Akt that upregulates p-Mdm2(Ser166) inhibiting its binding to p53, and (ii) phosphorylating p53(Ser389), making p53 protein more stable; (iii) regulating the cytoplasmic localization of HuR and therefore stabilizing the mRNA of *HAUSP*; and (iv) 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, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SAmE-D cells response to UVC

Hepatocytes and SAmE-D cell line were treated with UVC light. (A) Western blot of total extract (upper) and cytosolic and membrane (bottom) fractions. (B) Western blot of p53 in total extract, and (C) immunocytochemical analysis of p53. (D, E) Response of SAmE-D cells to UVC analyzed by Western blot.

Figure 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 μ M) 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 via western blotting. Densitometric analysis is represented in Figures S5 and S6.

Figure 3. LKB1 and apoptosis

(A) Hepatocytes and SAME-D cells were treated with UVC light, and p-LKB1 and p-p53(Ser389) detected by immunostaining (upper), and p-LKB1 by Western blot (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 of lysates from SAME-D cells transfected with control or AKT siRNA. (E) Lysates from SAME-D cells were immunoprecipitated with Akt antibody and with a non-specific IgG. Immunoprecipitates (IP) and lysates (input) were analyzed by western blot.

Figure 4. HuR regulates HAUSP stability

(A) Cell lysates from SAMe-D cells were immunoprecipitated with HAUSP antibody and with a nonspecific IgG. Immunoprecipitates (IP) and lysates (input) were analyzed by western blot. (B) Western blot 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 (CR), 3' UTR] and the predicted HuR motifs. Bottom: 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.

Figure 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.

Figure 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 of ASH and NASH etiology.

Figure 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 poly-ubiquitinates p53 for proteasomal degradation. The de-ubiquitinating enzyme HAUSP contributes to the stability of Mdm2, impairing its self-ubiquitination 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.