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Caspases compromise SLU7 and UPF1 stability and NMD activity during hepatocarcinogenesis

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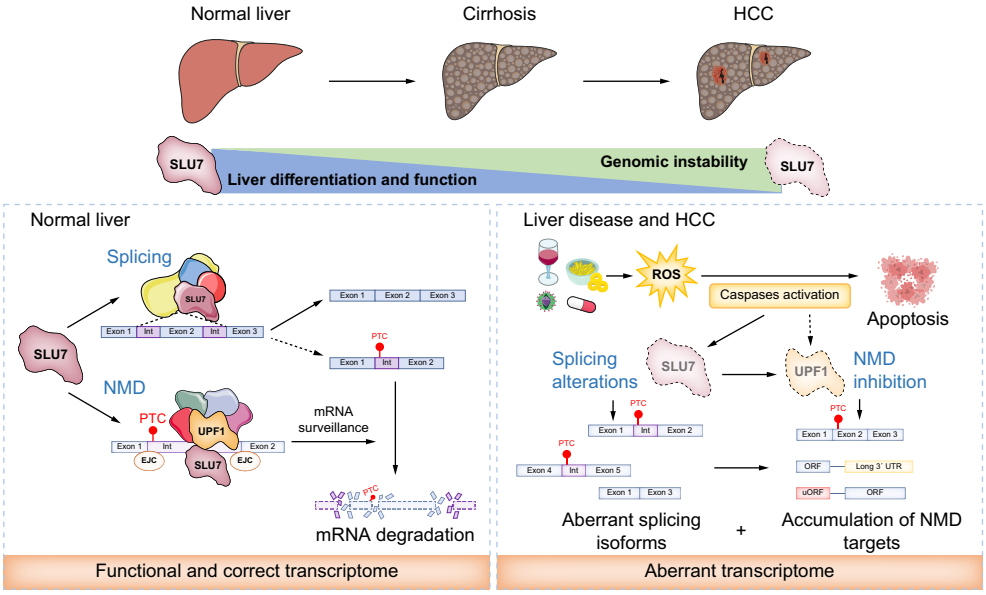
Graphical abstract



Highlights:

- The NMD mechanism for gene expression fidelity is inhibited in animal models of hepatic damage and in human cirrhosis and HCC.
- The downregulation of SLU7 during liver damage can be explained by its caspase-mediated cleavage.
- SLU7 downregulation is implicated in the cleavage of UPF1 and the inhibition of NMD.
- SLU7 represents a new link between caspase activation during liver damage and carcinogenesis.

Impact and implications:

The mechanisms involved in reshaping the hepatocellular transcriptome and thereby driving the progressive loss of cell identity and function in liver disease are not completely understood. In this context, we provide evidence on the impairment of a key mRNA surveillance mechanism known as nonsense-mediated mRNA decay (NMD). Mechanistically, we uncover a novel role for the splicing factor SLU7 in the regulation of NMD, including its ability to interact and preserve the levels of the key NMD factor UPF1. Moreover, we demonstrate that the activation of caspases during liver damage mediates SLU7 and UPF1 protein degradation and NMD inhibition. Our findings identify potential new markers of liver disease progression, and SLU7 as a novel therapeutic target to prevent the functional decay of the chronically injured organ.

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Research article

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Caspases compromise SLU7 and UPF1 stability and NMD activity during hepatocarcinogenesis

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Background & Aims: The homeostasis of the cellular transcriptome depends on transcription and splicing mechanisms. Moreover, the fidelity of gene expression, essential to preserve cellular identity and function is secured by different quality control mechanisms including nonsense-mediated RNA decay (NMD). In this context, alternative splicing is coupled to NMD, and several alterations in these mechanisms leading to the accumulation of aberrant gene isoforms are known to be involved in human disease including cancer.

Methods: RNA sequencing, western blotting, qPCR and co-immunoprecipitation were performed in multiple silenced culture cell lines (replicates n ≥4), primary hepatocytes and samples of animal models (Jo2, APAP, *Mdr2*$^{-/-}$ mice, n ≥3).

Results: Here we show that in animal models of liver injury and in human HCC (TCGA, non-tumoral = 50 *vs*. HCC = 374), the process of NMD is inhibited. Moreover, we demonstrate that the splicing factor SLU7 interacts with and preserves the levels of the NMD effector UPF1, and that SLU7 is required for correct NMD. Our previous findings demonstrated that SLU7 expression is reduced in the diseased liver, contributing to hepatocellular dedifferentiation and genome instability during disease progression. Here we build on this by providing evidence that caspases activated during liver damage are responsible for the cleavage and degradation of SLU7.

Conclusions: Here we identify the downregulation of UPF1 and the inhibition of NMD as a new molecular pathway contributing to the malignant reshaping of the liver transcriptome. Moreover, and importantly, we uncover caspase activation as the mechanism responsible for the downregulation of SLU7 expression during liver disease progression, which is a new link between apoptosis and hepatocarcinogenesis.

Introduction

The correct synthesis, processing and degradation of mRNAs is essential for maintaining gene expression fidelity and the functional cellular transcriptome and protein endowment on which cell identity and survival ultimately depends. An extensive machinery of interacting protein complexes involving transcription factors, splicing factors, exon-exon junction complex (EJC) proteins and proteins implicated in mRNA quality control such as nonsense-mediated RNA decay (NMD), participates in the activation of transcription, the maturation and splicing of pre-mRNAs, and the recognition of aberrant RNA species that must be degraded before accumulation.[1,2]

NMD regulates steady state levels not only of aberrant transcripts, but also of about 10% of the normal human transcriptome.[3–5] NMD substrates include mRNAs that contain upstream open reading frames and long 3′-untranslated regions, although the best-characterized NMD targets are mRNAs that harbor premature termination codons (PTCs) located 50–55 nucleotides upstream of an exon-exon junctions. Aberrant splicing together with mutations and transcriptional errors are responsible for introducing PTCs in mRNAs.[2,6] Therefore, the coupling of alternative splicing (AS) to NMD is considered an important and dynamic mechanism of gene expression regulation, playing roles in multiple processes including tissue-specific differentiation and adaptation to stress.[3,4] Moreover, through autoregulatory feedback loops, NMD is also essential for the homeostasis of NMD factors and RNA-binding proteins (RBPs) including splicing regulators.[2,3]

Therefore, aberrant splicing and changes in NMD efficiency influence different cellular functions and participate in the pathogenesis of multiple diseases including cancer.[2,4,7] In fact, although it has been proposed that NMD inhibition by the microenvironment may allow stress adaptation and tumor promotion,[8,9] NMD may operate as a bipolar modulator of malignancy[5,10,11] and pro- and anti-tumorigenic roles of NMD have been described depending on the origin and genetic background of tumor cells.[5,10–12] For example, mutations in

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NMD factors or their reduced expression may promote tumorigenesis, including hepatocellular carcinoma (HCC) where two independent studies have shown the downregulation of the core NMD effector UPF1 in HCC tumors and its role as a tumor suppressor gene.[13,14]

Alterations in AS have also been shown to be involved in liver tumorigenesis[15–17] and HCC single-molecule real-time long-read RNA sequencing has enabled identification of unannotated tumor-specific isoforms harboring PTCs mostly derived from intron retention.[18] Interestingly, the increased stability of these NMD targets generated through aberrant splicing could be associated with the inhibition of NMD.

Our work has demonstrated a relevant role for the splicing factor SLU7[19] in maintaining the hepatic transcriptome and differentiation status,[20,21] as well as hepatocellular genome stability,[22] suggesting that the observed downregulation of SLU7 in the cirrhotic liver and in HCC[21,23] contributes to the process of hepatocarcinogenesis.

Mechanistically we have demonstrated that during liver damage SLU7 is required to protect the master transcription factor hepatocyte nuclear factor 4α (HNF4α) from oxidative stress degradation[21] or to preserve the stability of DNA methyltransferase 1 (DNMT1) and secure correct DNA methylation.[24] SLU7 is also required to prevent the aberrant splicing and the generation of NMD substrates of several essential serine/arginine rich splicing factors (SRSFs) and proteins implicated in chromosome segregation and mitosis such as sororin,[22] not only in the liver but in cell lines of multiple origins. Indeed, the accumulation of PTC-containing isoforms upon SLU7 knockdown suggest a role in NMD. Here we show that many transcripts stabilized by NMD inhibition are induced in HCC and in mouse models of liver damage and we provide experimental evidence demonstrating that SLU7 interacts with UPF1 and is required for correct NMD activity. Our results therefore suggest that SLU7 downregulation contributes to liver carcinogenesis also through NMD inhibition. Importantly, we uncover a mechanism which would explain SLU7 downregulation during liver damage and hepatocarcinogenesis, as we have found that both SLU7 and UPF1 are substrates of caspases. All in all, our results contribute to a better understanding of the molecular mechanisms implicated in gene expression reshaping during the process of hepatocarcinogenesis, highlighting SLU7 as a relevant hub integrating epigenetic regulation, transcription, splicing and NMD.

Materials and methods

See supplementary information for further details.

Animal experiments

Protocols for animal experimentation were approved and performed according to the guidelines of the Ethics Committee for Animal Testing of the University of Navarra (CEEA-062/16) and Navarra Government (GN 2016/325511). Models of liver damage in C57BL/6J male mice and *Mdr2*$^{+/+}$ and *Mdr2*$^{-/-}$ mice are described in the supplementary information.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Experiments were performed at least twice with biological replicates. Data were represented as mean ± SEM. Normally distributed data were compared among groups using two-tailed Student's *t* test. Non-normally distributed data were analyzed using the Mann-Whitney *U* test. Statistical significance was considered according to the following *p* values: **p* <0.05, ***p* <0.01, ****p* <0.001.

Results

SLU7 silencing induces the accumulation of transcripts with NMD-inducing features

Our previous results have demonstrated that SLU7 silencing in cancer cells results in the accumulation of NMD-regulated isoforms such as *SRSF3* isoform 2[20,22] and intron containing isoforms of sororin.[22] Therefore, to further characterize the role of SLU7 in the regulation of the transcriptome we performed genome-wide RNA sequencing analyses at the transcript level using Kallisto and SLEUTH[25] packages in control PLC/PRF/5 cells (siGL) compared to SLU7 silenced cells (siSLU7). This analysis revealed that SLU7 depletion induces the accumulation of NMD-targeted isoforms and intron retention events (Fig. 1A). Introns often contain PTCs, so that intron-retaining isoforms are often rapidly degraded by the EJC-dependent NMD pathway, which recognizes PTCs at least 50 nucleotides upstream of the final exon-exon junction.[6] The significant accumulation of intron-retaining isoforms and NMD targets upon SLU7 silencing reflects both defects in splicing (incomplete splicing) together with inefficient NMD activity. AS coupled to NMD (AS-NMD) represents a post-transcriptional mechanism to control the proper expression of a protein by degrading some fraction of the already-transcribed mRNA. In fact, this mechanism is used as an autoregulatory negative feedback loop by many RBPs, including splicing factors, core spliceosome factors and ribosome proteins.[2] Accordingly, upon NMD inhibition those transcripts will accumulate.[26] Interestingly, upon KEGG enrichment analysis, genes upregulated after SLU7 silencing are enriched in ribosome, spliceosome and RNA transport categories (Fig. S1A). Moreover, it has been described that NMD perturbations result in the upregulation of NMD factors as a feedback autoregulatory response.[4,27] Therefore we performed a gene set-enrichment analysis with protein coding transcripts, and we found a significant enrichment of induced NMD factors upon SLU7 knockdown (Fig. 1B). Corroborating reduced NMD activity, our results also show that SLU7 silencing induces the accumulation of PTC-containing isoforms of *SRSF1*, *SRSF2* and *SRSF3* (Fig. 1C) at the same level as NMD inhibition by the protein synthesis inhibitor cycloheximide and the accumulation of known physiological NMD targets belonging to different molecular classes known to activate NMD, including PTC-containing isoforms degraded through the EJC-dependent NMD pathway and regulators of the integrated stress response degraded through upstream open reading frame-directed decay (Fig. 1D). The correlation between SLU7 downregulation and NMD inhibition was also confirmed in cancer cell lines of non-hepatic origin (Fig. S1B).

Given the downregulation of SLU7 expression in HCC[21,23] we decided to explore the status of NMD activity in HCC. To this end we obtained access to the TCGA database and we analyzed the sequencing data at the transcript level. Our results show that, in agreement with the results obtained upon SLU7

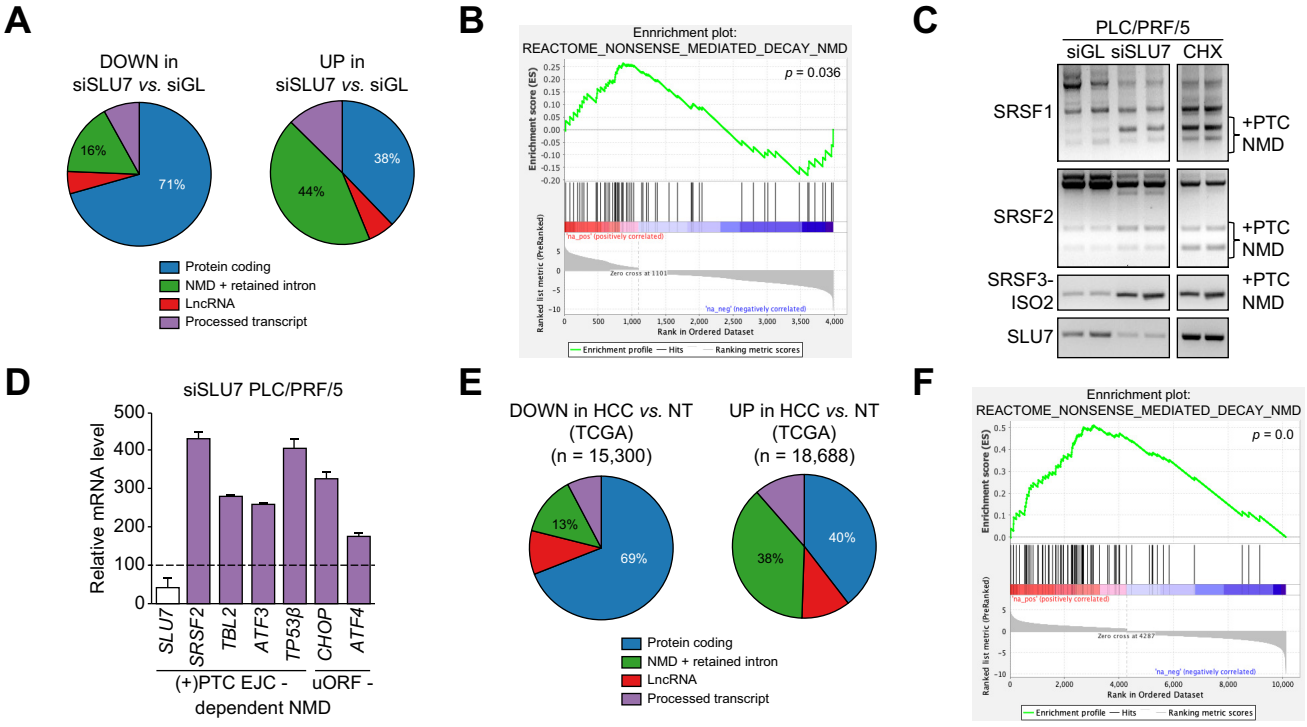

Fig. 1. Intron retained mRNAs, NMD targets and mRNAs encoding NMD factors accumulate in HCC and upon SLU7 silencing. (A) Transcript level Kallisto and SLEUTH genome-wide RNA sequencing analysis in SLU7-silenced (siSLU7) PLC/PRF/5 cells compared to control siGL cells. (B) Enrichment plot of the NMD gene set after GSEA of siSLU7 *vs.* siGL PLC/PRF/5 cells. (C) PCR analysis of *SRSF1*, *SRSF2* and *SRSF3* mRNA isoforms in siGL and siSLU7 PLC/PRF/5 cells. Positive control: Cells treated with the protein synthesis inhibitor CHX. The accumulation of PTC-containing isoforms, NMD targets, is indicated. (D) Real-time PCR relative expression of *SLU7* and different NMD targets in siSLU7 PLC/PRF/5 *vs*. siGL cells established as 100%. (E) Transcript level Kallisto and SLEUTH genome-wide RNA sequencing analysis of HCC and NT liver samples in the TCGA database. (F) Enrichment plot of the NMD gene set after GSEA of HCC and NT liver samples in the TCGA database. CHX, cycloheximide; GSEA, gene set-enrichment analysis; NMD, nonsense-mediated RNA decay; NT, non-tumoral; PTC, premature termination codon.

silencing, 13% of the downregulated transcripts in HCC correspond to NMD targets and IRI; however, this group of transcripts represents 38% of the transcripts upregulated in HCC (Fig. 1E). Moreover, gene set-enrichment analysis demonstrated an induction of the NMD factors category (Fig. 1F). Therefore, the increased accumulation of all these isoforms and genes suggests that NMD is inhibited in HCC.

SLU7 silencing inhibits NMD activity

We next decided to confirm whether SLU7 is required to secure a correct NMD activity using a chemiluminescence-based NMD reporter assay developed, and kindly provided, by Dr. Kulozik.[28] PLC/PRF/5 cells were transfected with *Renilla* constructs fused at the 3' end to the human wild-type β-globin gene (WT) or a mutated β-globin carrying a nonsense (PTC) substitution at position 39 (NS39) that is degraded by NMD (Fig. 2A). As expected, we found that the stability of the NS39 β-globin transcript was reduced to 45% compared to WT stability in control siGL cells. However, upon SLU7 silencing the stability of the NS39 β-globin transcript resembled more that of the WT, reaching 92% (Fig. 2A), and suggesting the inhibition of NMD activity. Accordingly, we observed a significant increase in *Renilla* activity when the NS39 β-globin transcript is expressed in SLU7-silenced PLC/PRF/5 and HepG2 cells (Fig. 2B and Fig. S2A). These results were also confirmed in HCT116 and H358 cells (Fig. S2B), where the increase in NS39 *Renilla* activity observed when SLU7 expression was silenced was similar or even superior to the one observed when UPF1 was silenced (Fig. S2C) suggesting that SLU7 regulates NMD.

This idea was reinforced by analyzing a genome-wide siRNA screen performed with a library of siRNAs targeting ~21,000 human genes and a bioluminescent gain-of-signal output to identify new NMD factors.[29] We found that when those genes are ranked by median seed-corrected Z-score of the corresponding siRNAs, SLU7 is ranked among the top hits only after the NMD pathway component SMG1 and the core EJC members RBM8A and EIF4A3, and with a higher score than canonical members of the NMD pathway such as UPF1 (see Supplementary File 1 in[29]). Altogether these results show that SLU7 plays a relevant role in regulating NMD activity.

SLU7 interacts with UPF1

At this point given the implication of SLU7 in NMD activity and the general connection between splicing and NMD, we decided to explore whether SLU7 interacts with NMD regulators. To this end, we performed a KEGG enrichment analysis of the immunoprecipitated proteins identified with more than five peptides by mass spectrometry (MS) after SLU7 co-immunoprecipitation in PLC/PRF/5 cells.[21] This analysis revealed a significant enrichment of the category corresponding to the mRNA surveillance pathway right after the categories of spliceosome and RNA transport (Fig. 2C). In fact, we found that 17 out of the selected 145 top SLU7 interacting proteins belong to the KEGG category of NMD proteins, including the key NMD regulator

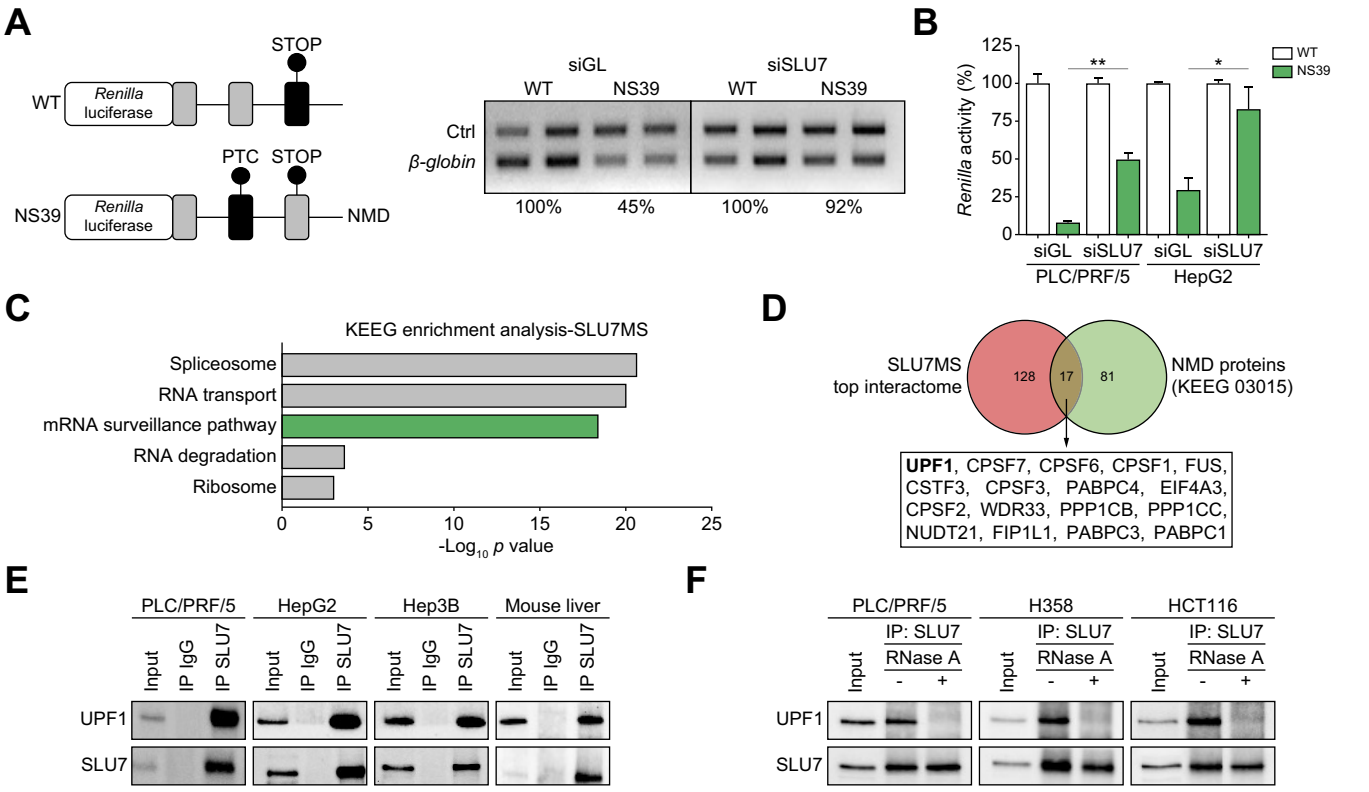

Fig. 2. SLU7 regulates NMD and interacts with UPF1. (A) Left panel: luciferase plasmid system used to study NMD activity. *Renilla* construct is fused at the 3' end to the human WT β-globin gene or a mutated β-globin carrying a PTC substitution at position 39 (NS39) that is degraded by NMD. Right panel: PCR amplification of *β-globin* and control (pCtrol) plasmid mRNAs in siGL and siSLU7 PLC/PRF/5 cells transfected with WT and NS39 plasmids. (B) *Renilla* activity of siGL and siSLU7 PLC/PRF/5 and HepG2 cells transfected with WT and NS39 β-globin plasmids. (C) Molecular pathways revealed by KEGG enrichment analysis associated with the proteins co-immunoprecipitated with SLU7 and identified by mass spectrometry. (D) Venn diagram comparing the top 145 proteins interacting with SLU7 with the 98 proteins included in KEGG category 03015 corresponding to NMD proteins. (E) UPF1 and SLU7 western blot after immunoprecipitation in PLC/PRF/5, HepG2, Hep3B and mouse liver protein extracts with anti-SLU7 antibody or control IgG. Input is shown. (F) SLU7 and UPF1 western blot after immunoprecipitation of PLC/PRF/5, H358 and HCT116 protein extracts treated or not with RNase A with anti-SLU7 antibody. Input is shown. Data are means±SEM. *p <0.05, **p <0.01 Mann-Whitney test. MS, Mass Spectrometry; NMD, nonsense-mediated RNA decay; PTC, premature termination codon; WT, wild-type.

UPF1 (Fig. 2D). Accordingly, we subsequently demonstrated that SLU7 interacts with UPF1 in several liver cancer cell lines including PLC/PRF/5, HepG2 and Hep3B (Fig. 2E) and importantly also in the normal mouse liver (Fig. 2E). In agreement with the MS data, we were unable to detect the interaction of SLU7 with other NMD members such as UPF2 and UPF3 (Fig. S3A), although in some cell lines the low levels of UPF2 detected do not make it possible to draw a clear conclusion. Moreover, our data showed that the SLU7/UPF1 interaction also occurs in cancer cells of different origin, and importantly it depends on the presence of RNA, as it was abolished upon RNase A treatment in PLC/PRF/5, H358 and HCT116 cell lines (Fig. 2F).

SLU7 is required to preserve UPF1 protein stability

Having demonstrated that SLU7 is required to preserve correct NMD function, and that SLU7 interacts with UPF1 in an RNA-dependent manner, we decided to investigate the fate of UPF1 upon SLU7 silencing. Interestingly, we observed a significant reduction in UPF1 protein levels (Fig. 3A and Fig. S3B), without significant modifications of UPF2 and UPF3 proteins (Fig. S3B), in different cell lines after transfection with siSLU7. Remarkably, UPF1 protein downregulation was not accompanied by a reduction in *UPF1* mRNA levels (Fig. 3B), suggesting a post-transcriptional regulation. On the contrary in many cases, and perhaps as a compensatory mechanism, in agreement with the reported transcriptional induction of NMD factors upon NMD inhibition,[27] an induction of *UPF1* mRNA levels was observed upon SLU7 silencing (Fig. 3B). These results demonstrate that SLU7 is required to maintain the expression of UPF1 protein and would justify why NMD is inhibited upon SLU7 silencing. In fact, we found that UPF1 overexpression rescues the accumulation of NMD targets induced upon SLU7 silencing in PLC/PRF/5 cells (Fig. 3C).

The oxidative stress and apoptosis induced upon SLU7 silencing are responsible for the degradation of UPF1 and NMD inhibition

We next evaluated the mechanisms implicated in the down-regulation of UPF1 observed upon SLU7 silencing. It has been shown that UPF1 is cleaved by caspases during apoptosis.[30,31] Our previous results have also demonstrated that SLU7 silencing induces apoptosis in cancer cells[32] preceded by the accumulation of reactive oxygen species (ROS) (Fig. S4A) and the induction of oxidative stress.[21,32] Importantly, it has been shown that cellular stresses, including oxidative stress, inhibit NMD through the phosphorylation of eIF2α.[9] Therefore, we

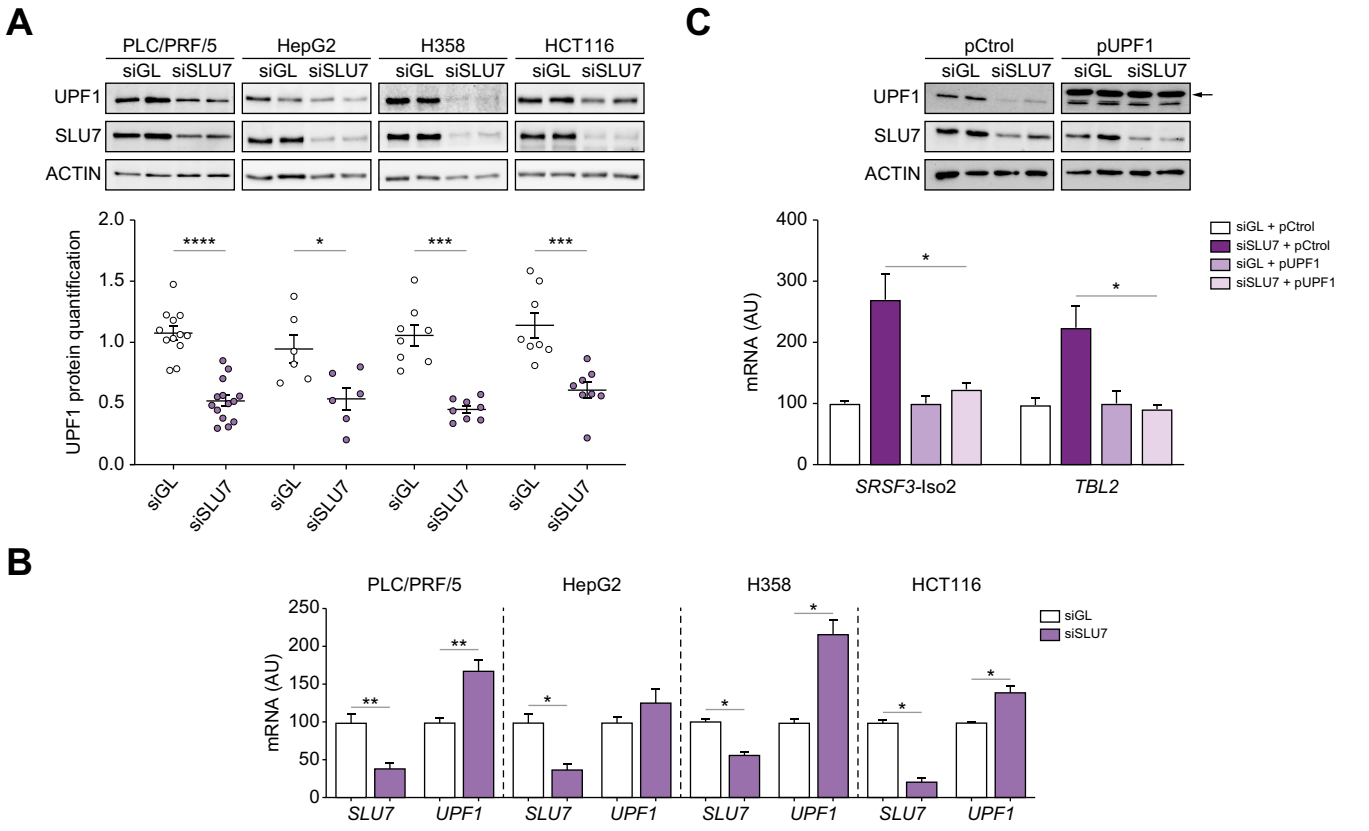

Fig. 3. SLU7 is required to preserve UPF1 protein stability and NMD function. (A) Western blots of UPF1, SLU7 and Actin (loading control) in PLC/PRF/5, HepG2, H358 and HCT116 cells 48 h after transfection with siGL or siSLU7. Lower graph: quantification of UPF1 protein expression in several experiments. (B) *SLU7* and *UPF1* mRNAs real-time PCR quantification in the experiments used in (A). (C) NMD targets *SRSF3-ISO2* and *TBL2* real-time PCR quantification in PLC/PRF/5 cells 48 h after transfection with siGL or siSLU7 and an empty control plasmid (pCtrol) or the same plasmid over-expressing GFP-UPF1 (pUPF1). Lower panels: Western blot of UPF1, SLU7 and Actin (loading control) in the same transfected PLC/PRF/5 cells. The arrowhead indicates over-expressed GFP-UPF1. Data are means ± SEM. **p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001 Mann-Whitney test. NMD, nonsense-mediated RNA decay.

decided to first examine whether SLU7 silencing induces eIF2α phosphorylation in an oxidative stress-dependent manner. As shown in Fig. S4B, SLU7 knockdown induced ROS-dependent P-eIF2α in PLC/PRF/5 and H358 cells as it was blunted upon treatment with the ROS scavenger N-acetylcysteine (NAC) (Fig. S4B). However, we found that eIF2 does not play a relevant role in SLU7 knockdown-mediated NMD inhibition, as concomitant silencing of eIF2 was unable to prevent the accumulation of NMD targets (Fig. S4C), in the context of UPF1 downregulation. Interestingly, NAC treatment was able to prevent the fall of UPF1 protein expression observed upon SLU7 silencing in PLC/PRF/5 cells (Fig. 4A). Accordingly, NAC treatment significantly restored NMD activity in SLU7-silenced PLC/PRF/5 cells, attenuating the accumulation of PTC-containing the β-globin NS39 isoform (Fig. 4B), PTC-containing *SRSF1* and *SRSF2* isoforms (Fig. 4C), and other physiological NMD targets (Fig. 4D) observed upon SLU7 knockdown.

As mentioned above, it has been described that caspases are implicated in UPF1 cleavage,[30,31] and the oxidative stress induced in cancer cells when SLU7 is silenced activates caspases and induces apoptosis.[32] Therefore, we evaluated whether the activation of caspases was implicated in the reduction of UPF1 protein levels observed after SLU7 knockdown. As shown in Fig. 4E we found that treatment of SLU7 knockdown PLC/PRF/5 cells with the pan-caspase inhibitor zVAD-fmk not only reversed apoptosis, as evidenced by the absence of PARP cleavage, but also prevented the decrease in UPF1 levels. Accordingly, as shown in Fig. 4F, the inhibition of caspase activity prevented the accumulation of NMD targets observed upon SLU7 knockdown.

UPF1 and SLU7 are substrates of caspases

In the cellular models used before, UPF1 cleavage upon SLU7 silencing is associated with caspase activation and a reduced availability of SLU7 to interact with UPF1. Therefore, we cannot evaluate the contribution of each event to UPF1 stability. As mentioned, different apoptotic insults induce UPF1 cleavage in HeLa cells[30] in conditions where SLU7 status was not evaluated. We then decided to study whether UPF1 cleavage occurs in the setting of a correct SLU7/UPF1 interaction. Overnight treatment of HeLa cells with 0.1 and 0.25 μM staurosporin induced UPF1 cleavage in a caspase activation-dependent manner as evidenced by parallel cleavage of PARP and prevention by the pan-caspase inhibitor zVAD-fmk (Fig. 5A). Unexpectedly, and importantly, we observed that SLU7 protein expression was also significantly reduced in a dose- and caspase-dependent manner (Fig. 5A), which was also observed in PLC/PRF/5 cells (Fig. 5B). Treatment with other inducers of

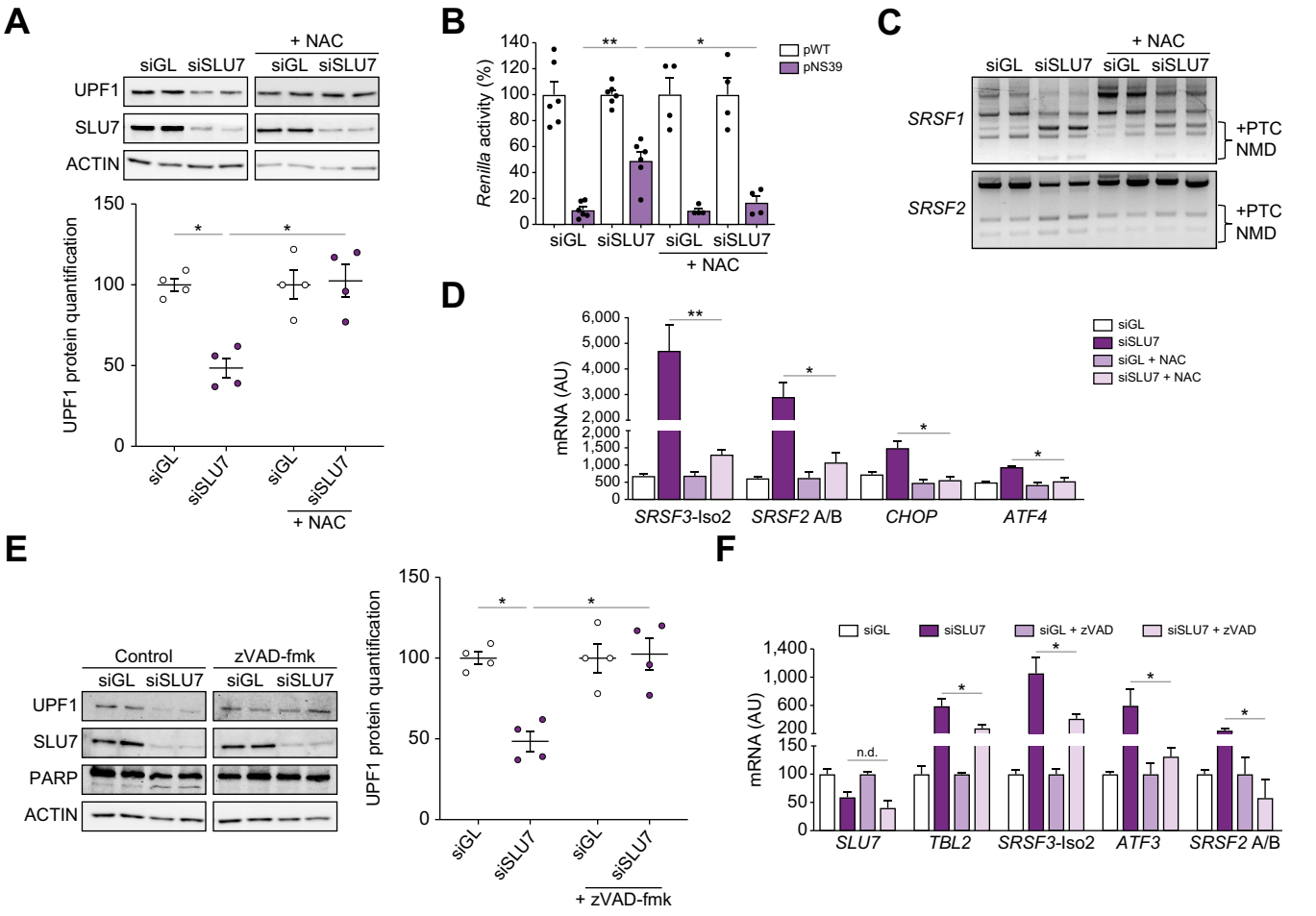

Fig. 4. Oxidative stress and caspase activation are implicated in SLU7 knockdown-mediated UPF1 instability and NMD inhibition. (A) UPF1, SLU7 and Actin (loading control) western blots in PLC/PRF/5 cells transfected with siGL or siSLU7 in the absence or presence of the antioxidant NAC. Lower graph: quantification of UPF1 protein in two independent experiments. (B) *Renilla* activity of siGL and siSLU7 PLC/PRF/5 cells transfected with WT and NS39 β-globin plasmids and treated or not with NAC. (C) PCR analysis of *SRSF1* and *SRSF2* mRNA isoforms in siGL and siSLU7 PLC/PRF/5 cells treated as in (A). (D) Real-time PCR analysis of different NMD targets in siGL and siSLU7 PLC/PRF/5 cells treated as in (A). (E) UPF1, SLU7, PARP and Actin (loading control) western blots in PLC/PRF/5 cells transfected with siGL or siSLU7 in the absence or presence of the pan-caspase inhibitor zVAD-fmk. The graph on the right represents the quantification of UPF1 protein in two independent experiments. (F) Real-time PCR analysis of *SLU7* and different NMD targets in siGL and siSLU7 PLC/PRF/5 cells treated or not with zVAD-fmk. Data are means ± SEM. **p* <0.05, ***p* <0.01 Mann-Whitney test. NAC, N-acetylcysteine; NMD, nonsense-mediated RNA decay; PTC, premature termination codon; WT, wild-type.

apoptosis such as the apoptosis activator 2 and cisplatin (Fig. 5B), revealed that UPF1 and SLU7 cleavage occurs upon general caspase activation. Importantly, this result was also confirmed in mouse primary hepatocytes when Fas receptor-mediated apoptosis, a relevant pathophysiological mechanism,[33] was induced upon Jo2 antibody and actinomycin D treatment (Fig. S5A). Moreover, reduced expression of SLU7 and UPF1 was induced in mouse primary hepatocytes treated with acetaminophen (APAP) (Fig. S5B), which was confirmed in the well-differentiated hepatic cell line HepaRG (Fig. S5C) and was prevented by treatment with zVAD-fmk (Fig. S5B,C). In parallel to the induction of apoptosis (Fig. S5D), APAP was able to significantly inhibit NMD activity (Fig. S5E) and induce the accumulation of different NMD targets including the corresponding isoforms of *SRSF2* and *SRSF3* and multiple transcripts (Fig. S5F,G) in HepaRG cells.

In the DegraBase[34] database we confirmed that SLU7 is a caspase substrate as it is included as a proteolytic event enriched in apoptotic cells. Moreover, in a quantitative MS-based enzymology study of caspases,[35] SLU7 is also listed as a direct substrate of different caspases. Accordingly, using a GFP-SLU7 construct we found that the caspase-dependent cleavage of SLU7 occurs very near the N-terminus (Fig. 5C) and using a mutated SLU7 construct we demonstrate that it occurs at the putative caspase target site D7 (Fig. 5D).

These results seem to rule out the possibility that SLU7 protects UPF1 from caspase-mediated degradation because SLU7 itself is a target of these proteases. However, they uncover the relevant role that induction of apoptosis can play in promoting hepatocarcinogenesis through the degradation of SLU7 and UPF1, and the subsequent inhibition of NMD, and the

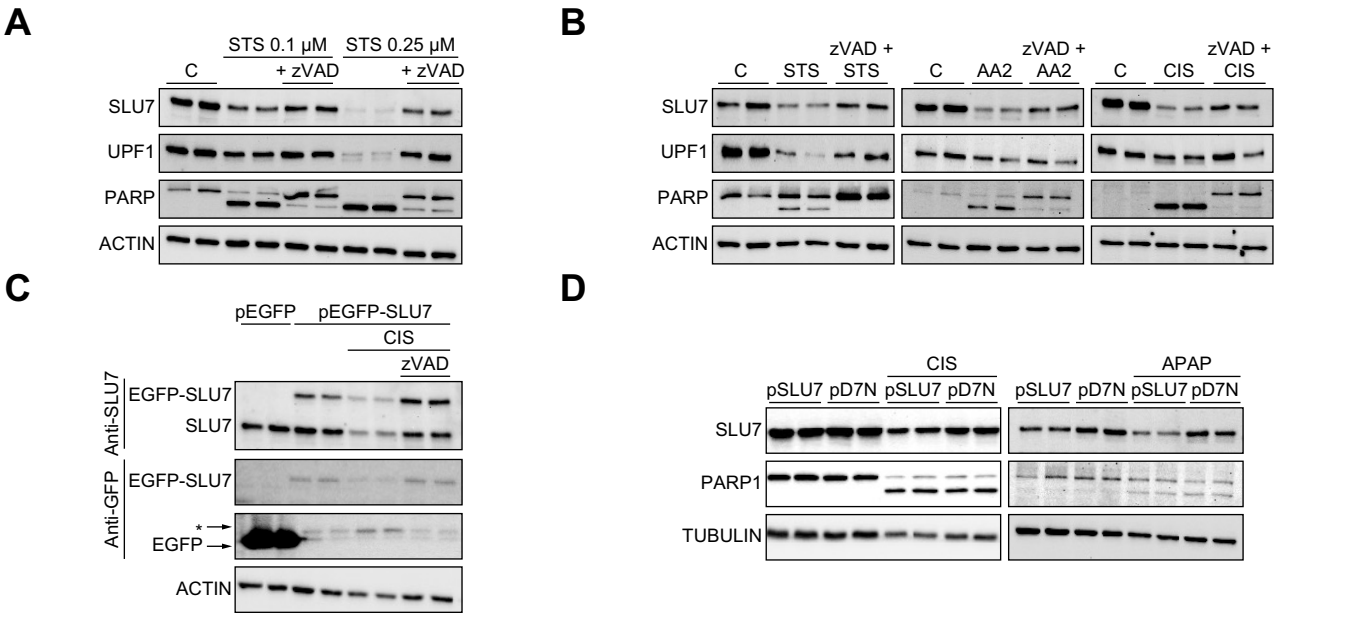

Fig. 5. UPF1 and SLU7 are substrates of caspases. (A) UPF1, SLU7, PARP and Actin (loading control) western blots in HeLa cells treated overnight with two different concentrations of STS in the absence or presence of the pan-caspase inhibitor zVAD-fmk. (B) SLU7, UPF1, PARP and Actin (loading control) Western blots in PLC/PRF/5 cells treated with STS, AA2 or CIS in the absence or presence of zVAD-fmk. (C) Western blot of GFP-SLU7 and SLU7 in HeLa transfected cells after treatment with cisplatin in the absence or presence of zVAD-fmk. (D) SLU7, PARP and Tubulin (loading control) Western blots in HeLa or HepaRG cells transfected with a plasmid over-expressing SLU7 (pSLU7) or a D7N mutant isoform and treatment with cisplatin or APAP respectively. AA2, apoptosis activator; APAP, acetaminophen; CIS, cisplatin; STS, staurosporin.

activation of relevant events associated with SLU7 downregulation, including cell dedifferentiation and genome instability.

In vivo apoptosis can compromise SLU7 and UPF1 stability and NMD activity

Apoptosis is a general event in liver diseases associated with almost any etiology.[21,33] Our previous results have demonstrated that SLU7 plays a very relevant role in preserving liver identity[20] and protecting the liver against damage,[21] and we have demonstrated that SLU7 downregulation results in hepatocyte dedifferentiation, loss of hepatic functions, genomic instability and DNA hypomethylation, all mechanisms implicated in the process of hepatocarcinogenesis.[19–22,24] Moreover, we have shown that SLU7 expression is significantly reduced in HCC and the damaged liver, including cirrhotic and acutely injured livers.[21,23] However, the mechanisms involved in the regulation of SLU7 expression are unknown. Based on our present results, we propose that caspase activation could be a general mechanism responsible for the inhibition of SLU7 expression and subsequently of UPF1 and NMD function, representing another mechanism contributing to liver disfunction and malignant transformation.

To obtain further evidence supporting this mechanism, we first studied whether SLU7 and UPF1 expression is reduced in the apoptotic liver when the death receptor Fas is activated *in vivo*. As shown in Fig. 6A and Fig. S6A, as fast as 5 h after the administration of the Fas agonistic antibody Jo2 to mice, and in a caspase-dependent manner, the hepatic expression of UPF1 and SLU7 proteins is reduced, without significant alterations in their mRNA levels, in parallel with the cleavage of PARP and caspase 3 and the accumulation of several endogenous NMD target mRNAs (Fig. 6B and Fig. S6B,C).

Moreover, present evidence suggests that the reduced expression of SLU7 detected by immunohistochemistry in the acutely APAP-damaged mouse liver[21] could be mediated by the activation of caspases and could also be accompanied by NMD inhibition. Accordingly, we found that APAP administration *in vivo* reduced the hepatic expression of SLU7 and UPF1 proteins in parallel to the induction of NMD targets (Fig. 6C and Fig. S6D), events exacerbated in *Slu7* haploinsufficient mice, highlighting the relevant role of SLU7 in controlling UPF1 and NMD activity *in vivo*. Finally, using *Mdr2*$^{-/-}$ mice where liver tumors spontaneously developed in association with progressive inflammation and fibrosis, we show that these mechanisms could play a role in the process of hepatocarcinogenesis as SLU7 and UPF1 proteins are reduced (Fig. 6D), and the accumulation of NMD targets is induced (Fig. S6E), in both the non-tumoral and tumoral liver of 17-month-old *Mdr2*$^{-/-}$ mice, while no changes were observed in 17-month-old wild-type mice.

Discussion

AS and NMD are coupled and regulated processes which dynamically control gene expression. Splicing factors are highly abundant among transcripts undergoing AS-NMD and they use this mechanism to maintain homeostatic levels.[36] On the other hand, multiple splicing factors have been shown to promote NMD.[37,38] Therefore, AS homeostasis is affected by NMD and vice versa and an intact splicing and NMD machinery

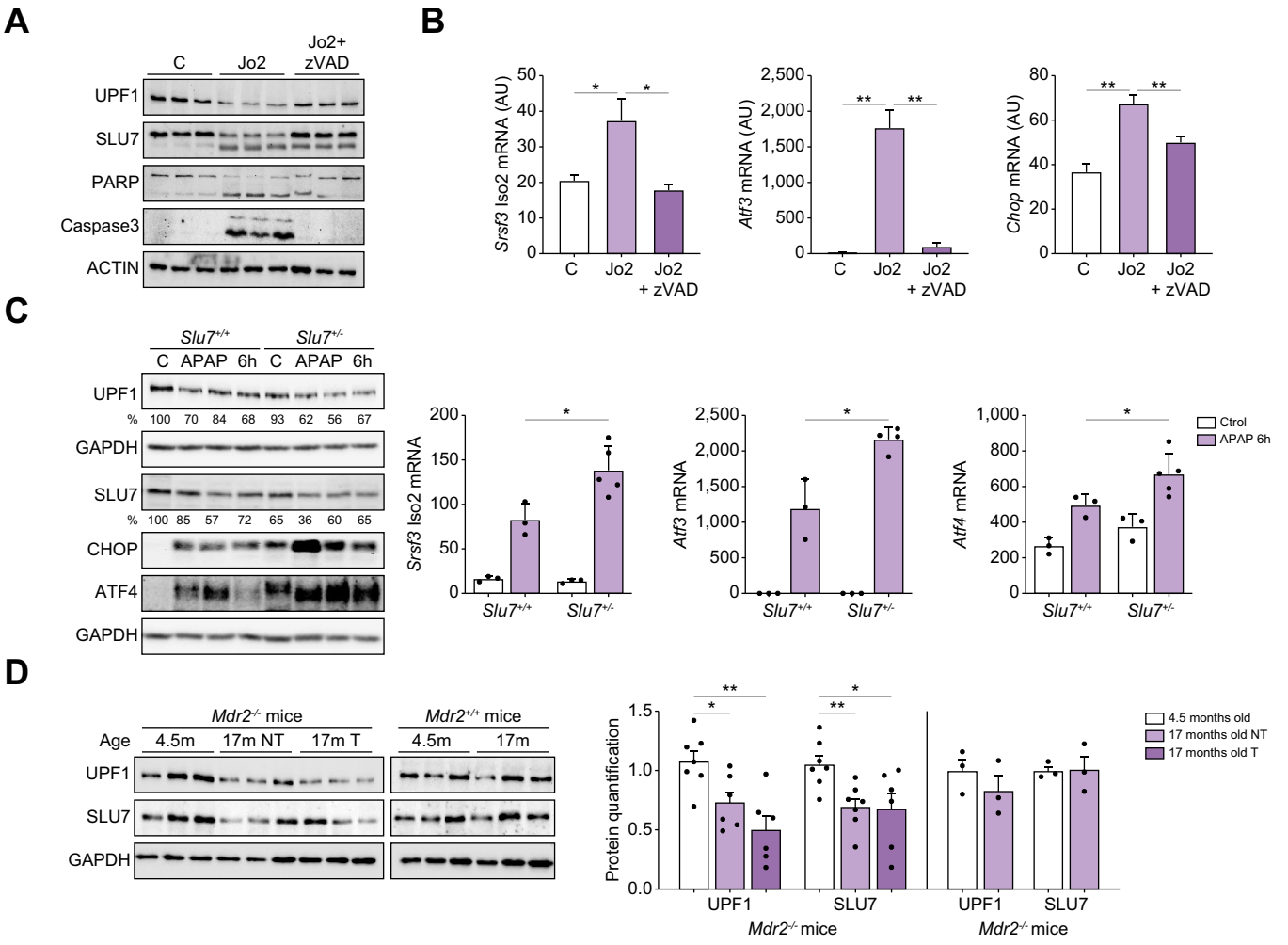

Fig. 6. *In vivo* hepatic UPF1 and SLU7 protein downregulation and NMD inhibition in mouse models of liver damage and hepatocarcinogenesis. (A) UPF1, SLU7, PARP, cleavage caspase 3 and Actin (loading control) western blots in control mouse liver and livers of mice 5 h after Fas agonistic Jo2 antibody intraperitoneal administration, without or with previous injection of zVAD. (B) Real-time PCR analysis of NMD targets in the livers described in (A). (C) UPF1, SLU7, CHOP, ATF4 and GAPDH (loading control) western blots in the liver of control (C) and 6-hour APAP-treated *Slu7*$^{+/+}$ and *Slu7*$^{+/-}$ mice. Graphs represent real-time PCR levels of mRNA NMD targets in the livers of APAP-treated mice. (D) UPF1, SLU7 and GAPDH (loading control) western blots in the liver of 4.5- and 17-month-old control mice (*Mdr2*$^{+/+}$) and Mdr2 knockout mice (*Mdr2*$^{-/-}$). Samples from tumors (T) detected in the 17-month-old *Mdr2*$^{-/-}$ mice were analyzed together with the NT liver. Graphs represents protein quantification data. Data are means ± SEM. **p* <0.05, ***p* <0.01. Mann-Whitney test. APAP, acetaminophen; m, month; NMD, nonsense-mediated RNA decay; NT, non-tumoral; T, tumor.

is required to secure the expression of the correct transcriptome responsible for cell identity and function. Indeed, aberrant splicing and changes in NMD efficiency are involved in reshaping the transcriptome in multiple diseases, including cancer.[2,4,7,39]

Regarding liver diseases, transcriptomic changes are considered tractable and clinically relevant markers of disease progression[40,41] and are associated with the complex and heterogeneous HCC phenotypes.[42] These changes include AS variants,[16,17,43–45] in many cases related with cancer hallmarks, such as genome instability and dedifferentiation,[16] and HCC-specific isoforms strikingly originating from aberrant intron retention.[18] The mechanisms implicated in these alterations are largely unknown. Small changes in the concentration of splicing factors have been shown to modify the selection of splice sites,[46] and accordingly, different RNA splicing factors have been found to be upregulated or downregulated in the diseased liver.[15,44] Moreover, we can hypothesize that the observed accumulation of PTC-containing NMD targets in HCC would be associated with the inhibition of NMD, according to the reduced expression of the core NMD effector UPF1 in HCC.[13,14]

Our previous results have demonstrated that alterations in the expression of the spliceosome component SLU7 can have very relevant effects in the process of hepatocarcinogenesis.[19] We have shown that SLU7 is required to preserved the mature and functional transcriptome[20] and unexpectedly the stability of relevant proteins such as the transcription factor HNF4α[21] and the DNA methyltransferase DNMT1.[24] Consequently, SLU7 downregulation impacts on the loss of hepatic functions, the induction of genomic instability and the increased sensitivity to liver damage,[20–22,24] therefore, hepatic SLU7 downregulation would play a relevant role in the process of hepatocarcinogenesis.

Here we demonstrate that the aberrant accumulation of NMD targets, including intron retention isoforms,[18] that we detect in HCC may also be associated with SLU7 downregulation. Moreover, our results provide further evidence for the AS-NMD link, showing that SLU7 plays a role in NMD, not only in hepatic cells but in a general cellular context. Moreover, the fact that many of the NMD targets accumulated after SLU7 silencing are mRNA isoforms corresponding to genes coding for SRSF splicing factors, amplifies the impact of splicing alterations on the transcriptome in a vicious circle. Mechanistically we have demonstrated that SLU7 interacts with the essential effector of the NMD process the RNA helicase UPF1 in an RNA-dependent manner, and that SLU7 downregulation results in the proteolytic degradation of UPF1. Moreover, we found that this degradation is dependent on oxidative stress and the activation of caspases induced upon SLU7 silencing.

Relevantly, our present results also demonstrate that SLU7 is a target of caspases. Although further experiments are required to characterize its therapeutic implications, this finding is of particular relevance given the role that apoptosis plays in liver disease. Independently of the etiological agent, alcohol, fat, bile acids, viruses or drugs are able to induce the apoptosis of hepatocytes,[33,47] and apoptosis and caspase activation have been proposed as a mechanism for liver disease progression[33] and HCC development.[48,49] Thus, taking into account our present and past results, the apoptosis-mediated downregulation of SLU7 would promote the dedifferentiation of hepatocytes and the activation of oncogenic pathways,[20] together with genomic instability,[22] epigenetic modifications[24] and inhibition of NMD, all events associated with a complex reprogramming of the transcriptome. Moreover, this appears to occur through the modulation at the protein level of fundamental actors in the definition of the cellular phenotype, such as the epigenetic writer DNMT1,[24] the master transcription factor HNF4α[21] and, as uncovered here, the NMD key effector UPF1.

Therefore, the mechanism of caspase-dependent proteolytic cleavage of SLU7 described here in different cellular backgrounds, would explain the downregulation of SLU7 protein detected in different hepatic acute and chronic pathological situations.[21] Moreover, the fact that this newly identified proteolytic mechanism can occur concomitantly with SLU7 transcriptional downregulation,[23] underscores the relevant role that SLU7 reduction can play in the process of hepatocarcinogenesis.

Regarding the inhibition of NMD, it has been suggested to serve as an adaptive response to cellular stress, resulting in the accumulation of transcripts involved in the stress response, which are *bona fide* NMD targets.[8,9] However, as mentioned before, its protracted repression, which can be induced by a protumorigenic microenvironment, promotes cancer development.[8,9] Accordingly, the expression of UPF1 is downregulated in HCC and correlates with bad prognosis,[13,14] and UPF1 silencing fosters HCC growth and metastasis.[13] Here we provide a novel SLU7-dependent mechanism that, together with the described hypermethylation of the *UPF1* promoter,[13] would explain the marked downregulation of UPF1 protein observed in the damaged liver.

All in all, our results contribute to a better understanding of the molecular mechanisms implicated in hepatocarcinogenesis, highlighting the role of SLU7 as a relevant regulatory hub integrating epigenetic, transcriptional, splicing and surveillance mechanisms such as NMD. Moreover, we uncover caspase activation as responsible for SLU7 downregulation during the process of hepatocarcinogenesis, representing a link between apoptosis, transcriptome rewiring, hepatic dedifferentiation and malignant transformation.

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Abbreviations

APAP, acetaminophen; AS, alternative splicing; DNMT1, DNA methyltransferase 1; EJC, exon-exon junction complex; HCC, hepatocellular carcinoma; HNF4α, hepatocyte nuclear factor 4α; IRIs, intron-retaining isoforms; NAC, N-acetylcysteine; NMD, nonsense-mediated RNA decay; PTC, premature termination codon; RBPs, RNA-binding proteins; ROS, reactive oxygen species; SLU7, splicing factor SLU7; SRSFs, serine/arginine rich splicing factors.

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Conflict of interest

The authors of this study declare that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

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

Data availability statement

The RNA seq raw data performed in this study are available in GEO database with the accession number GSE246237. The mass spectrometry data (proteins that interacts with SLU7) were previously published (PMID: 34170569). All reagents, antibodies and protocols used in this research can be found in material and methods. Primers and siRNA sequences used are available from the corresponding author on reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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