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USP39 Deubiquitinase Is Essential for *KRAS* Oncogene-driven Cancer*[S]

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***KRAS* is the most frequently mutated oncogene in human cancer, but its therapeutic targeting remains challenging. Here, we report a synthetic lethal screen with a library of deubiquitinases and identify *USP39*, which encodes an essential splicing factor, as a critical gene for the viability of KRAS-dependent cells. We show that splicing fidelity inhibitors decrease preferentially the proliferation rate of KRAS-active cells. Moreover, depletion of *DHX38*, encoding an USP39-interacting splicing factor, also reduces the viability of these cells. In agreement with these results, USP39 depletion caused a significant reduction in pre-mRNA splicing efficiency, as demonstrated through RNA-seq experiments. Furthermore, we show that *USP39* is up-regulated in lung and colon carcinomas and its expression correlates with *KRAS* levels and poor clinical outcome. Accordingly, our work provides critical information for the development of splicing-directed antitumor treatments and supports the potential of USP39-targeting strategies as the basis of new anticancer therapies.**

Ras family members are the most frequently activated oncogenes associated with human malignancies (1). Although genetic alterations have also been found in *HRAS* and *NRAS*, *KRAS* is the most frequently mutated member of this oncogene family. Remarkably, and in accordance with the oncogene addiction concept, mutated KRAS proteins are not only involved in the process of neoplastic transformation but also required for maintaining the viability of cancer cells with mutations in these genes (2–4). The dependence on KRAS of several human cancers, such as lung and colorectal carcinomas, has been clearly established over the last decades. However, the therapeutic targeting of this oncogene has been challenging so far and there is a clear need of effective treatment for cancers harboring oncogenic mutations in *KRAS* (5, 6).

Cancer cells are exposed to several types of stress, including DNA damage, protein misfolding, and metabolic or oxidative stress, which do not affect normal cells at the same extent. As a consequence, the normal function of multiple proteins is critical for tumor development and maintenance, even without undergoing activating mutations. In this regard, non-oncogene addiction was originally defined as the exacerbated dependence of tumor cells on the activity of genes and pathways that are not required at the same degree for the viability of normal cells (7–9). Non-oncogene addiction has provided the conceptual framework for the exploration of a wide spectrum of potential targets for anti-cancer therapies, whose inhibition could selectively reverse the oncogenic phenotype. To exploit this concept for the development of anti-cancer approaches, several groups have carried out synthetic lethal RNA interference screens designed to identify genes whose silencing is preferentially deleterious for cells carrying particular oncogenic mutations (10). The use of this experimental approach has led to the identification of numerous proteins whose normal function is especially required for the survival of *KRAS*-mutant cells (11). Thus, Luo *et al.* (12) demonstrated that silencing of Polo-kinase 1 (*PLK1*), a gene with mitotic function, is selectively lethal for KRAS-active cells. Using the same technology, additional targets have been identified, including integrin β6, *RON*, and *SYK* tyrosine kinases, *CDC6*, *GATA2*, TGF-β activated kinase 1 (*TAK1*), TANK-binding kinase 1 (*TBK1*), *MED23*, *CDK1*, *SNAIL2*, and Wilms tumor 1 (*WT1*) (13–21). Furthermore, synthetic lethal interactions between *KRAS* mutations and MEK inhibition combined with suppression of BCL-XL or RAF1 have also been uncovered (22, 23). Finally, using different experimental approaches, the combined inhibition of DDR1 and Notch signaling has been recently proposed as another therapeutic strategy for KRAS-driven lung adenocarcinoma (24).

Among all proteins potentially involved in non-oncogene addiction of cancer cells, proteases represent a large and diverse group of enzymes that participate in virtually all the physiological mechanisms related to cancer cell growth and survival (25).

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[S] This article contains supplemental Figs. S1–S5 and Tables S1 and S2.

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On this basis, we have recently proposed that protease addiction would be a common phenomenon in human cancer (7). The sensitivity of *KRAS*-mutant cells to proteasome inhibitors such as bortezomib has provided pharmacological evidence to this proposal (9). To further explore these concepts, we have focused on DeUbiquitinases (DUBs),[3] due to their wide functional diversity and profound impact on the regulation of multiple biological processes that are frequently altered in human malignancies (26). DUBs constitute a large group of proteases with the ability to hydrolyze the peptide and isopeptide bonds that link ubiquitin chains to target proteins. DUBs are classified into six families according to sequence and structural similarity: ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases, ovarian-tumor proteases, Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases, and monocyte chemotactic protein-induced protein family (26–28). The cysteine proteases of the USP family represent the largest group of DUBs, with more than 50 members in humans (29). Notably, the number of human malignancies in which DUBs show changes in their expression levels or are mutated behaving as oncogenes or tumor suppressors has substantially grown over the last few years (26, 30, 31).

In this work, we have performed an RNAi-based synthetic lethal screen aimed at identifying DUBs required for the survival of KRAS-dependent cancer cells. This approach has revealed that down-regulation of *USP39* selectively abrogates the growth and tumorigenic potential of lung and colorectal cancer cells whose viability depends on KRAS. Furthermore, we have proved that pharmacological inhibition of splicing fidelity selectively decreases the proliferation rate of KRAS-dependent cancer cells. We have also demonstrated that down-regulation of the splicing factor gene *DHX38* affects the viability of KRAS-active cancer cells. In agreement with these results, we have validated USP39 function in pre-mRNA splicing efficiency through RNA-sequencing (RNA-seq) experiments. Finally, we have found that *USP39* expression significantly correlates with *KRAS* levels and is associated with poor prognosis in both lung and colon cancers. Consequently, we propose USP39 as a new promising target for the treatment of KRAS-addicted human malignancies.

Results

USP39 Is an Indispensable Gene for the Survival of KRAS-dependent Lung Cancer Cells—To identify DUBs that are essential for the viability of KRAS-dependent tumors, we performed a synthetic lethal screen using RNAi-based technology. For this purpose, we designed a custom library of 360 small hairpin RNAs (shRNAs) targeting most DUBs, with up to six shRNAs per gene. The library was next cloned into the TRMPV-Neo backbone (Fig. 1*A*), a vector optimized for negative-selection screening (32, 33), and the representation of each shRNA was analyzed by deep sequencing. This analysis revealed an optimal distribution with only 1.7% of the shRNAs under-represented (Fig. 1*B*).

To allow the inducible expression of the DUB shRNAs, we retrovirally transduced the library as one pool into Tet-On cell lines. We selected two lung cancer cell lines whose dependence on *KRAS* oncogene had been previously described: H358 as KRAS-dependent and A549 as KRAS-independent cells (13). To ensure single-cell transduction, we used a low multiplicity of infection (less than 20% of transduced cells) and maintained a large enough number of cells during all the experiment to keep a 1000-fold representation of the library (at least 1000 cells per shRNA). After neomycin selection, shRNA expression was induced by addition of doxycycline and genomic DNA was extracted after approximately 10 cell population doublings (Fig. 1*C*).

Changes in library representation were analyzed by performing deep sequencing of PCR-amplified shRNAs (Fig. 1*D*). The library included two positive-control shRNAs that targeted an essential gene, *RPA1*, and were strongly depleted in both cell lines. By contrast, the integration of two neutral-control shRNAs (Renilla.713 and Luc.1309) did not cause any apparent effect on cell viability (Fig. 1, *E* and *F*). We assessed the dependence on KRAS of each cell line by comparing the number of reads of three independent *KRAS* shRNAs at the beginning and at the end of the experiment. As expected, *KRAS* shRNAs were depleted only in H358 cells (Fig. 1, *E* and *F*), confirming the addiction of the cell line to this oncogene (13). Interestingly, A549 cells present *KRAS* mutations but do not depend on this oncogene for their viability. In this regard, cancer cell lines harboring mutations in *KRAS* can be classified as KRAS-dependent or KRAS-independent cells, based on whether they are addicted or not to this oncogene, respectively. Therefore, although both A549 and H358 cell lines harbor mutations in *KRAS*, only H358 cells are addicted to this oncogene (13). Of note, two shRNAs targeting *USP39* were depleted more than 2-fold in each of the three independent replicates of H358 cell line, whereas the same shRNAs showed little change in A549 cells. After analyzing the mean of all replicates, we found that four of six *USP39* shRNAs and all the *KRAS* shRNAs were depleted more than 2-fold in H358 cells (Fig. 1*E*), with no depletion in A549 cells (Fig. 1*F*), thereby identifying *USP39* as the top scorer in our screen (supplemental Table S1). In conclusion, by screening a library of DUB shRNAs, we have selectively identified *USP39* as an essential gene for the viability of a KRAS-dependent lung cancer cell line.

USP39 Depletion Inhibits Proliferation and Tumorigenicity of KRAS-dependent Cells—To validate the results obtained in the synthetic lethal screen, we stably transduced A549 and H358 lung cancer cells with an independent shRNA targeting a different region of *USP39*. Down-regulation of *USP39* expression was verified by Western blot (Fig. 2*A*) and quantitative (q) RT-PCR in both cell lines (supplemental Fig. S1*A*). As expected, *USP39* silencing significantly decreased the proliferation rate of H358 without affecting the viability of A549 cells (Fig. 2, *B* and *C*). To analyze *in vivo* the impact of *USP39* down-regulation on the tumorigenic potential of these lung cancer cells, we next used a mouse xenograft model based on the subcutaneous inoculation of control or *USP39*-silenced A549 and H358 cells. In

[3] The abbreviations used are: DUB, deubiquitinases; RNA-seq, RNA sequencing; PARP, poly(ADP-ribose) polymerase; qRT-PCR, quantitative RT-PCR; USP, ubiquitin-specific protease; SpS, splicing score; RQ, relative quantification.

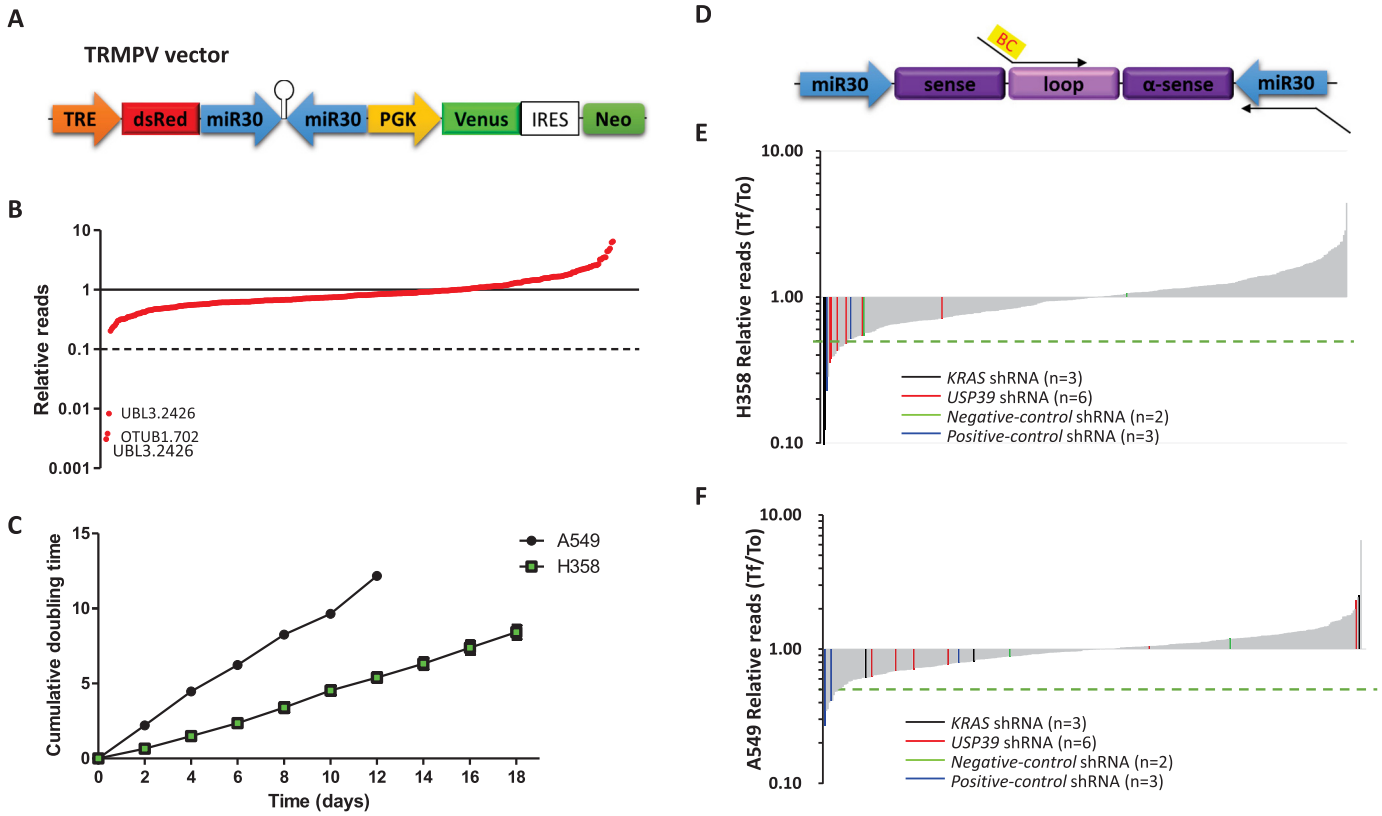

FIGURE 1. **RNAi screen identifies *USP39* as an essential gene for KRAS-dependent lung cancer cell viability.** *A,* schematic representation of the retroviral TRMPV-Neo vector in which the DUB shRNA library was cloned. *B,* DUB library representation. The number of reads of each shRNA was divided by the total number of reads and multiplied by total number of shRNAs to calculate DUB library representation. *C,* Tet-On A549 and Tet-On H358 cell population doublings. *D,* PCR strategy to amplify template libraries from genomic DNA (T_0 and T_f) for deep sequencing. *BC,* barcode. *E* and *F,* pooled negative-selection screen in H358 (*E*) and A549 (*F*) cell lines. shRNA abundance ratios were calculated as the number of reads at T_f divided by the number of reads at T_0 and plotted as the mean of three replicates in ascending order.

agreement with the above results, silencing of *USP39* in H358 cells significantly decreased their tumorigenicity when injected into nude mice. By contrast, tumor growth of KRAS-independent A549 cells was not affected by the depletion of USP39 (Fig. 2*D*).

Next, we decided to extend our validation of USP39 addiction to colorectal cancer, a different tumor type whose dependence on KRAS has also been demonstrated (34). Thus, we silenced *USP39* in a pair of isogenic DLD-1 cell lines that only differ in the presence or absence of a *KRAS* mutation (35), representing an optimal model to analyze the synthetic lethal interaction between *USP39* and *KRAS*. Depletion of USP39 was verified by Western blot (Fig. 2*E*). As observed in lung cancer cells, *USP39* down-regulation selectively decreased the proliferation rate of *KRAS*-mutant DLD-1 cells without affecting the viability of cells in which the mutant *KRAS* had been deleted (Fig. 2*F*). We then analyzed USP39 addiction in a second pair of isogenic colorectal cancer cell lines, derived from HCT116. Down-regulation of *USP39* was verified in both cell lines by Western blot (Fig. 2*G*) and qRT-PCR (supplemental Fig. S1*B*). As expected, only the proliferation rate of KRAS-dependent cells was significantly reduced upon depletion of USP39 with the same two shRNAs (Fig. 2*H*). Interestingly, USP39-depleted cells showed no increase in PARP cleavage when compared with control cells, ruling out apoptosis as the cause of the reduced cell proliferation in shRNA-treated cells (supplemental Fig. S1*C*). Then, we analyzed the effect of *USP39* silencing on the tumorigenic potential of isogenic HCT116 cells. In agreement with the results obtained with lung cancer cell lines, *USP39* down-regulation selectively decreased the tumor growth of *KRAS*-mutated HCT116 cells when injected into nude mice, without affecting in the same way to *KRAS*-wild type HCT116 cells (Fig. 2*I*).

So far, one of the main problems with RNAi-based screens has been the undesired off-target effects due to unspecific binding to other nontargeted mRNAs. To rule out this possibility, we developed a retroviral vector encoding a shRNA-resistant *USP39* cDNA and we transduced it into HCT116 and DLD-1 cells. As shown in Fig. 3, *A* and *B*, ectopic expression of this cDNA completely abrogated the effects of the shRNA-mediated *USP39* silencing in both cell lines, demonstrating the absence of noticeable off-target effects. Rescue of USP39 protein levels by the transduced construct was verified by Western blot analysis (Fig. 3, *C* and *D*). Taken together, these results indicate that *USP39* expression is critical for the survival of KRAS-dependent tumors, in both lung and colorectal carcinomas.

To further explore the association between *KRAS* and *USP39* dependence in different tumor types, we examined the data derived from a genome-wide shRNA screen in 216 cancer cell lines from multiple tumor types (Project Achilles) (36). As shown in supplemental Fig. S2*A*, there is a significant positive correlation between the antiproliferative effects of silencing

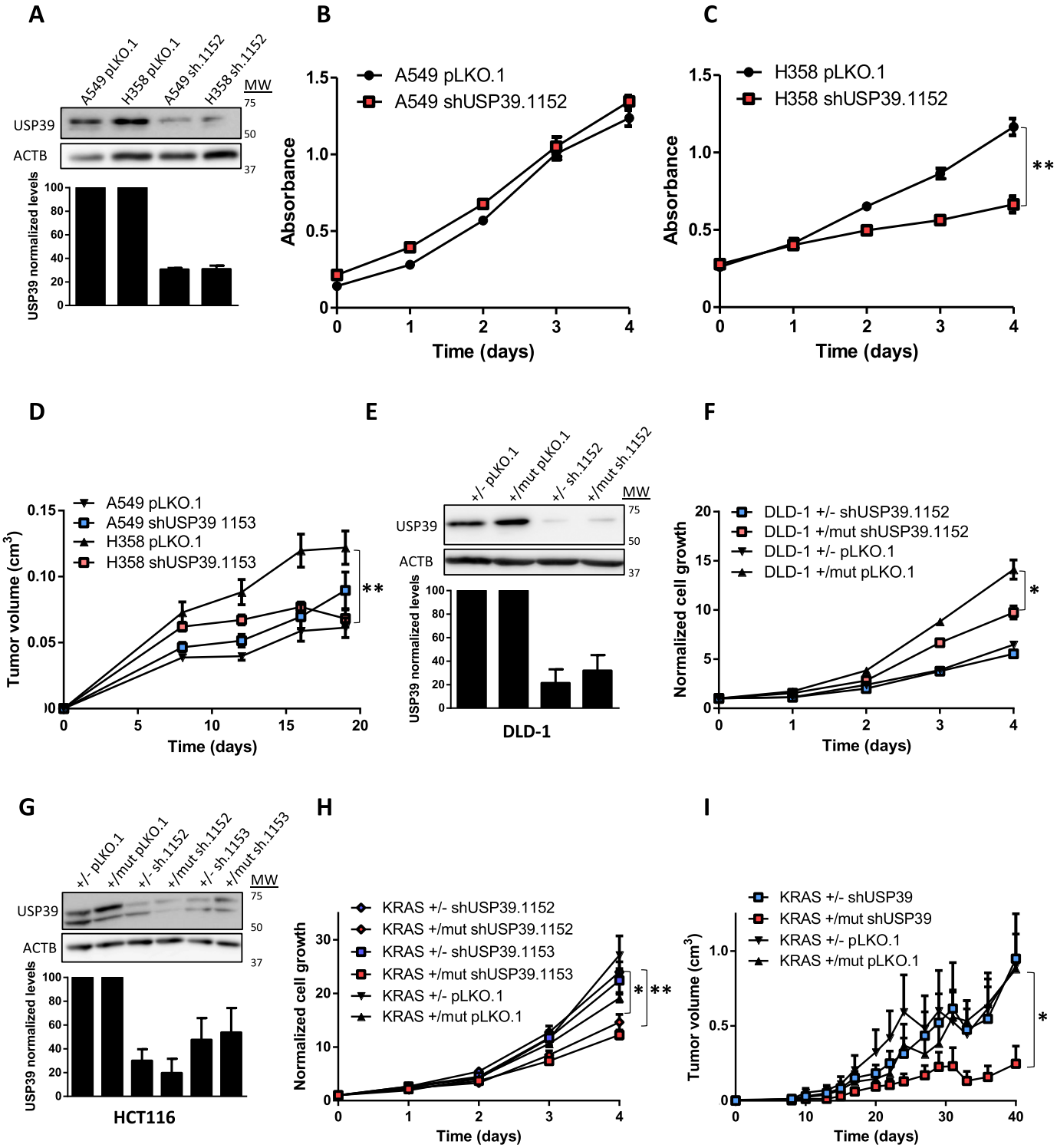

FIGURE 2. **USP39 depletion decreases the proliferation and tumorigenicity of KRAS-dependent lung and colon cancer cell lines.** *A,* Western blot and densitometry quantification of USP39 in A549 and H358 cell lines transduced with the *USP39* shRNA vector (shUSP39.1152) or with the empty vector as a control (pLKO.1). *B* and *C,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis showed decreased proliferation when *USP39* was down-regulated only in H358 cells, being unaltered in the case of A549 cell line. *D,* tumor xenograft experiment. Nude mice were injected subcutaneously with A549 or H358 cells transduced with either control or *USP39*-specific shRNA vectors. *E,* USP39 levels were analyzed in isogenic DLD-1 by Western blot and densitometry quantification. *F, USP39* depletion selectively reduced the proliferation of *KRAS*-mutant DLD-1 cells. *G,* knockdown of *USP39* was verified by Western blot in isogenic HCT116 cell lines. *H,* MTT analysis demonstrated a reduction in the proliferation rate of *KRAS*-mutant HCT116 transduced with shUSP39.1152 or shUSP39.1153. *I,* tumor xenograft experiment. Nude mice were injected subcutaneously with either control or *KRAS*-mutant HCT116 cells transduced with either control or *USP39*-specific shRNA vectors. A representative blot and average of densitometry quantification of three independent experiments are represented for all Western blot analyses. A representative graph of at least two independent experiments is shown for each MTT proliferation assay, in which *error bars* represent technical replicates ($n = 5–6$). All data are presented as mean ± S.E. and statistical significance was assessed by using a non-parametric Mann Whitney-Wilcoxon test (*, $p < 0.05$; **, $p < 0.01$).

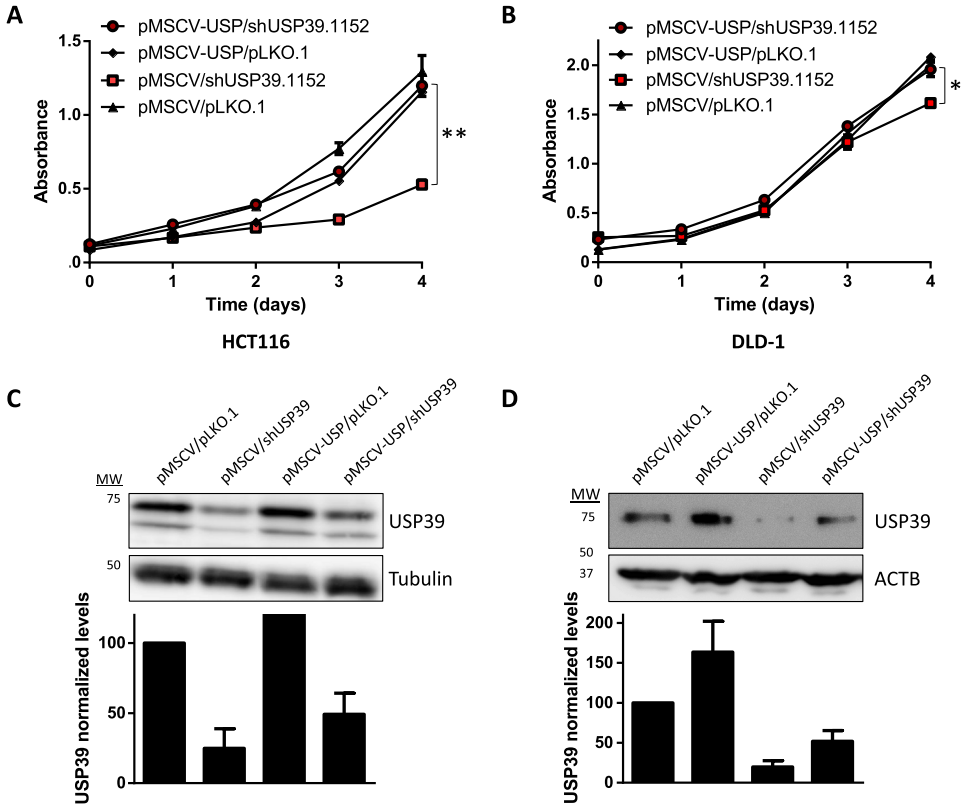

FIGURE 3. **Rescue experiments ruled out off-target effects of *USP39* down-regulation.** *A* and *B, USP39* shRNA effects on HCT116 (*A*) and DLD-1 (*B*) proliferation were rescued by the overexpression of a *USP39* shRNA-resistant clone (pMSCV-USP). *C* and *D,* Western blot analysis and densitometry quantification of USP39 expression in HCT116 (*C*) and DLD-1 cells (*D*) used in *A* and *B*. All data are presented as mean ± S.E. and statistical significance was assessed by using a non-parametric Mann Whitney-Wilcoxon test (*, $p < 0.05$; **, $p < 0.01$).

both genes. Consequently, we propose USP39 as a new example of non-oncogene addiction in cancer and a potential drug target for the clinical treatment of KRAS-active tumors of multiple histological origins.

Splicing Fidelity Inhibition Decreases Proliferation of KRAS-dependent Cancer Cells—USP39 is considered a non-protease homologue or pseudo-protease due to the lack of three residues critical for deubiquitinase activity (25, 26). However, it plays a role in the spindle assembly checkpoint and cytokinesis by regulating Aurora B kinase mRNA levels (37). Furthermore, USP39 is a component of the U4/U6-U5 tri-snRNP, one of the building blocks of the spliceosome, and its function is essential in pre-mRNA splicing (38–40). To determine whether the requirement of USP39 for proliferation of *KRAS*-mutant tumors could reflect a global dependence on the optimal function of the splicing machinery, we treated HCT116 cells with two well known splicing modulators, sudemycin D1 and sudemycin D6 (41–43). As shown in Fig. 4, *A* and *B*, after 72 h of treatment, the proliferation rate of *KRAS*-mutant cells was decreased to a higher degree than that of *KRAS*-wild type cells. Similarly, 24 h of treatment with another splicing modulator, FR901464, also preferentially diminished the viability of KRAS-dependent cells (Fig. 4*C*). In support of the exacerbated dependence on splicing efficiency of KRAS-dependent tumor cells, when we performed a Gene Set Enrichment Analysis (GSEA) (44) on the Project Achilles dataset comparing KRAS-dependent and -independent cells, we found a significant enrichment of genes involved in splicing (supplemental Fig. S2*B*). In summary, results from treatment with splicing inhibitors, together with data from the meta-analysis of a genome-scale shRNA screen of 216 cancer cell lines of multiple histological origins indicate that KRAS-dependent tumor cells exhibit addiction to both USP39 and the proper function of the splicing process.

To further evaluate the relevance of splicing efficiency for KRAS-dependent tumors, we transduced HCT116 cells with two different shRNAs against *DHX38*, which encodes PRP16, an USP39-interacting ATP-dependent RNA helicase involved in pre-mRNA splicing (38, 45, 46). *DHX38* down-regulation was verified by qRT-PCR (Fig. 4*D*). Remarkably, *DHX38* depletion decreased the proliferation rate of KRAS-dependent cells at a higher degree than that of *KRAS*-wild type cells (Fig. 4*E*). Furthermore, depletion of *DHX38* decreased USP39 protein levels (Fig. 4*F*). Altogether, these results allow us to hypothesize a concerted action of *USP39* and *DHX38* in the regulation of splicing and reinforce their essentiality for the viability of KRAS-dependent cells.

USP39 Regulates Global Pre-mRNA Splicing Efficiency—To further investigate the mechanism by which USP39 function becomes essential for the viability of KRAS-dependent tumors, we analyzed the pre-mRNA splicing efficiency of both KRAS-dependent and KRAS-independent HCT116 cells transduced with control or two *USP39*-specific shRNAs. Thus, after performing RNA-seq experiments, we calculated the splicing score (SpS) at exon-intron junctions across the genome (Fig. 5*A*;

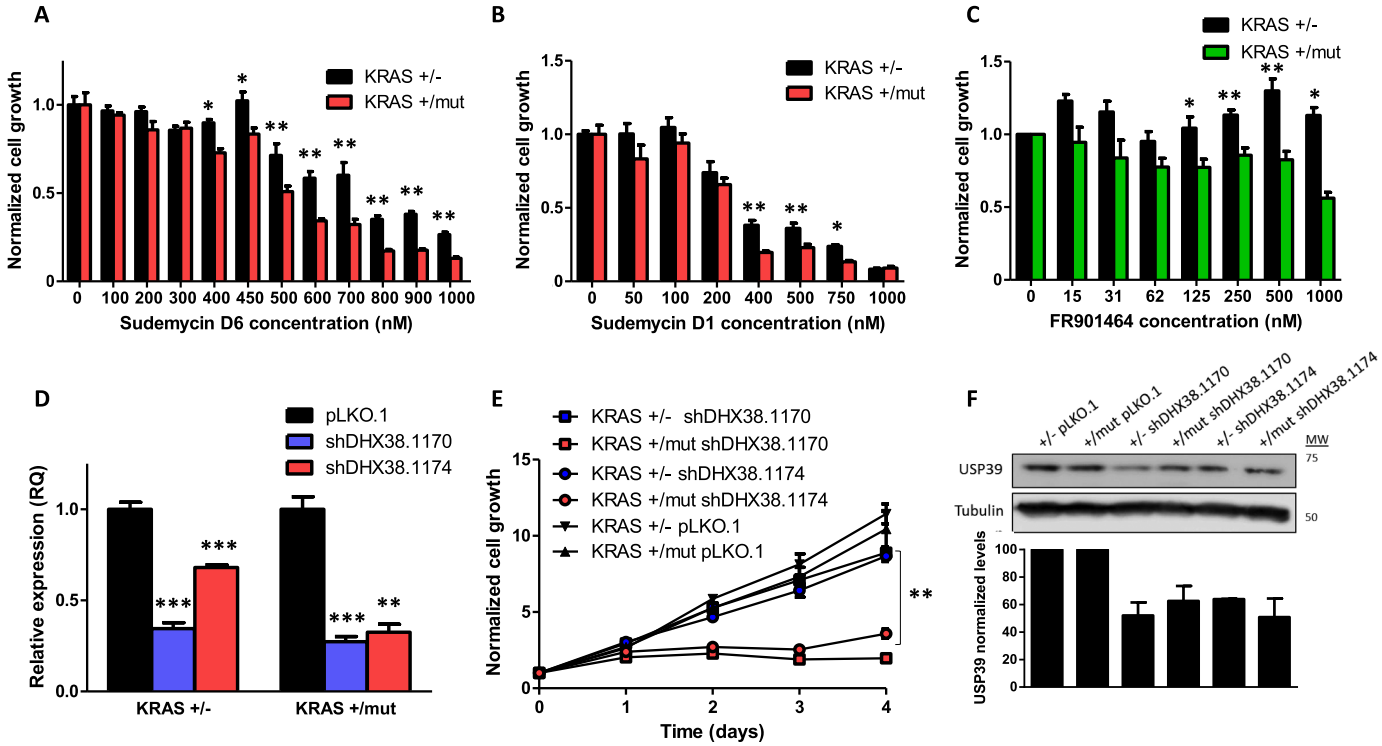

FIGURE 4. **Pharmacological modulation of splicing decreases the proliferation rate of KRAS-dependent cancer cells.** *A–C,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis of isogenic HCT116 cell lines after treatment with sudemycin D6 (*A*) or sudemycin D1 (*B*) during 72 h and FR901464 (*C*) for 24 h. *D,* SYBR Green-based qRT-PCR analysis of *DHX38* in HCT116 cells. Data are represented as RQ ± S.E., two-tailed Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). *E,* MTT analysis of isogenic HCT116 cell lines transduced with two different *DHX38* shRNAs (shDHX38.1170 and shDHX38.1174) or empty vector (pLKO.1). *F,* representative blot and densitometry quantification of two independent Western blot experiments of USP39 expression in *DHX38*-depleted HCT116 cells. Proliferation data are represented as mean ± S.E. and statistical significance was assessed by using a non-parametric Mann Whitney-Wilcoxon test (*, $p < 0.05$; **, $p < 0.01$).

supplemental Table S2). As it has been previously described, we restricted our analysis to reads directly spanning well established exon-intron and exon-exon junction sequences to avoid confounding effects (47). As expected, down-regulation of *USP39* affected the splicing of multiple genes in both KRAS-dependent and KRAS-independent cells. Thus, USP39-depleted cells exhibited a decreased SpS when compared with control cells (Fig. 5, *B* and *C*). Furthermore, using the GSEA algorithm we found a strong negative correlation between the decrease in SpS promoted by USP39 depletion and gene sets containing genes involved in cell cycle regulation, EGFR signaling, or DNA repair (Fig. 5*D*). Among all the exon-intron junctions whose splicing was affected by *USP39* down-regulation, we decided to focus on those from genes that could be more directly associated with KRAS-related processes, such as those selected in GSEA analysis. With this criterion, we selected several genes (*CASP8AP2*, *CDCA8*, *MED19*, *SERPINB6*, *TAF9B*, *BORA*, *KIF14*, and *ORC1*) and validated by qRT-PCR their differences in splicing efficiency (supplemental Fig. S3*A*). As shown in Fig. 5*E*, the splicing of these genes was affected by *USP39* depletion in both HCT116 isogenic cell lines. Interestingly, most of these splicing alterations were also present in *DHX38*-depleted cells (supplemental Fig. S3*B*). Altogether, these results corroborate the biological importance of USP39 function in RNA processing and suggest that USP39 addiction of KRAS-dependent tumor cells implies a more general phenomenon of dependence on an optimal function of the splicing machinery.

USP39 Expression Correlates with KRAS Levels and Is Associated with Poor Prognosis in Cancer—The requirement of USP39 function in KRAS-dependent cancer implies a possible oncogenic role of this protein. To further evaluate this hypothesis, we analyzed the expression of *USP39* in six human colorectal cancer cell lines (HCT116, HCT-15, HT29, RKO, SW620, and DLD-1) and in a colon epithelial cell line (FHC). In all cancer cells, *USP39* and *KRAS* expression was higher than in normal cells (Fig. 6*A*). Furthermore, cancer cell lines showed higher USP39 protein levels than control cells, corroborating the results obtained through qRT-PCR (Fig. 6*B*). Interestingly, those cancer cells with higher KRAS protein levels also exhibited the highest levels of USP39 protein. To further investigate *USP39* expression in colon cancer samples, we performed qRT-PCR analysis of this gene in a validation series of 29 patients, comprising pairs of tumor and matched normal mucosa (48). As shown in Fig. 6*C*, *USP39* was consistently up-regulated in colorectal carcinoma samples, thus suggesting its pro-tumoral role. *USP39* overexpression was verified by Western blot analysis (Fig. 6*D*). Moreover, a detailed analysis of *USP39* and *KRAS* expression in these paired samples showed a positive correlation between both genes (Fig. 6*E*). In this regard, it has been previously demonstrated that elevated *KRAS* expression is strongly correlated with KRAS dependence in KRAS-mutant cells (13). Therefore, the existence of a positive correlation between both genes in these samples supports the synthetic lethal interaction proposed herein.

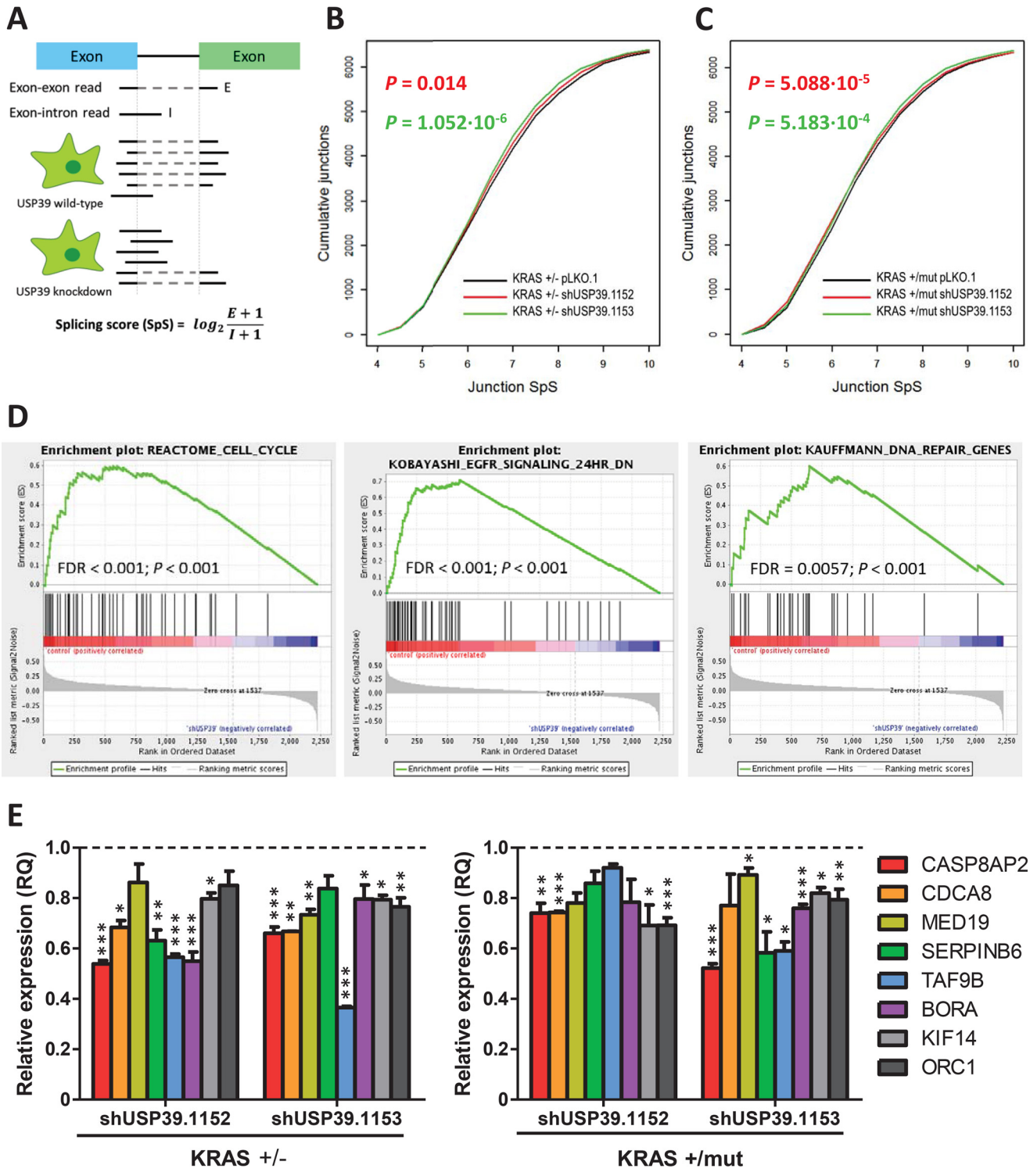

FIGURE 5. **USP39 regulates pre-mRNA splicing efficiency.** *A*, schematic representation of SpS calculation. *B* and *C*, empirical cumulative distribution of SpS coefficients for 6583 exon-intron junctions of KRAS-independent (*B*) and KRAS-dependent (*C*) cells transduced with either control or *USP39*-specific shRNAs. Distribution of SpS were compared using Wilcoxon test in R. *D*, GSEA analysis showed that SpS decreases upon USP39 depletion were correlated with gene sets composed of genes involved in cell cycle regulation, EGFR signaling, and DNA repair. Selected enriched pathways had a relaxed false discovery rate (*FDR*) < 0.01 and $p < 0.001$. *E*, selected exon-intron junctions were validated by SYBR Green-based qRT-PCR. The ratio of the expression values of the spliced and non-spliced forms for each of the selected exon-intron junctions between *USP39*-depleted cells *versus* controls was calculated as RQ $= (2^{-\Delta Ct\ \text{shRNA}})/(2^{-\Delta Ct\ \text{control}})$, where $\Delta C_t = C_t$ spliced $- C_t$ intron. These experiments were done in triplicate and the *p* values were calculated by comparing $2^{-\Delta Ct\ \text{shRNA}}$ with $2^{-\Delta Ct\ \text{control}}$, two-tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

To extend our results to a different cancer type, we analyzed the correlation between *USP39* and *KRAS* expression levels in a validation series of 14 lung cancer patients. Remarkably, both genes showed a strong positive correlation also in this cancer type (Fig. 6*F*). Moreover, by analyzing expression data from an available microarray study of 293 lung tumors (49)

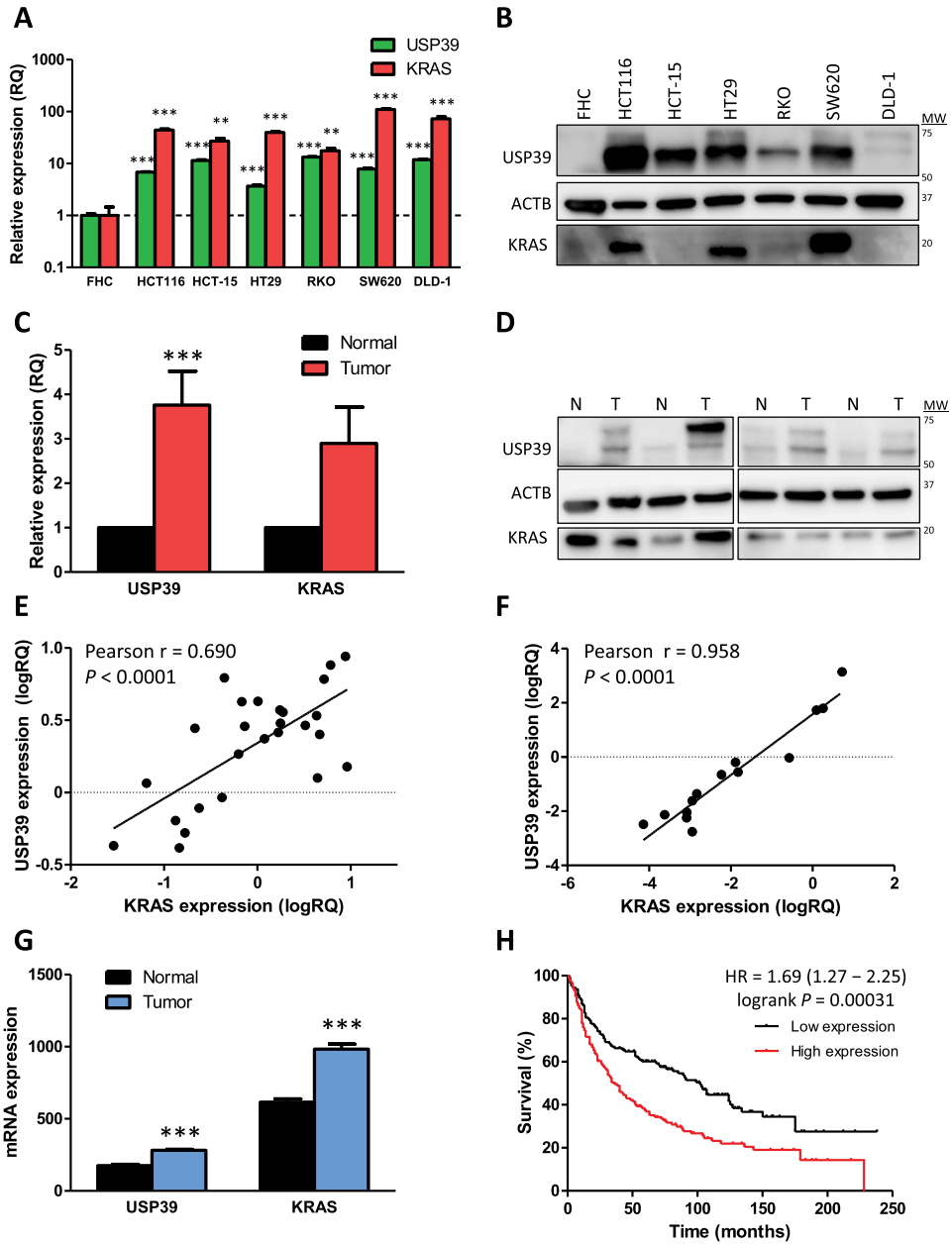

FIGURE 6. ***USP39* overexpression is associated with poor prognosis in colon and lung cancer samples.** *A,* qRT-PCR analysis of *USP39* expression in colon cancer cell lines (HCT116, HCT-15, HT29, RKO, SW620, and DLD-1) and FHC used as control. *B,* Western blot analyses of USP39 and KRAS expression in these cell lines. *C,* qRT-PCR analysis of *USP39* expression in 29 colon carcinoma-normal mucosa pairs. *D,* Western blot analyses of USP39 and KRAS expression in 4 colon carcinoma-normal mucosa pairs. *N*, normal; *T*, tumor. *E,* correlation of *USP39* and *KRAS* expression levels in colon samples. Pearson's *r* coefficient was calculated to analyze the parametric correlation between *KRAS* and *USP39* from the expression data shown in *C* transformed to logarithm ($p < 0.0001$). *F*, correlation of *USP39* and *KRAS* expression levels from lung cancer tumor samples. Pearson's *r* coefficient was calculated as in *E* ($p < 0.0001$). *G,* analysis of *USP39* expression in lung tumor samples compared with normal lung tissue from the GEO data set GSE30219. Statistical significance was assessed by a non-parametric Mann Whitney-Wilcoxon test (***, $p < 0.001$). *H*, Kaplan-Meier survival curve for the patient samples from the same dataset as in *E*, divided into two groups as a function of quantile expression of *USP39*. Relative expression is represented as relative quantification, RQ ± S.E., two-tailed Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$).

(GEO accession GSE30219), we found that *USP39* and *KRAS* were significantly up-regulated in lung cancer compared with non-tumoral samples (Fig. 6*G*). We further explored the clinical prognostic value of *USP39* by analyzing reported data on survival of patients of the same data set using the online application KM-Plotter (50). Interestingly, high expression levels of *USP39* were associated with short survival (Fig. 6*H*). Furthermore, the prognostic value of *DHX38* was also analyzed in lung cancer obtaining similar results ($p < 0.05$, supplemental Fig. S4). The inspection of a publicly available cancer genome database did not reveal frequent mutations in *USP39*, in neither lung nor colorectal cancers (http://cbioportal.org), although the amplification of *USP39* in breast cancer is noteworthy (supplemental Fig. S5). Altogether, these results demonstrate that USP39 is a candidate for non-oncogene addiction events in lung and colon cancers whose inhibition would become an appealing strategy for the development of anticancer therapies.

Discussion

Ras-family members, and especially KRAS, are major drivers in human tumors, and it is assumed that tumor cells harboring mutations in these genes are commonly dependent on the mutant Ras proteins for their proliferation and survival. Because of this, multiple efforts have been directed at targeting these proteins with therapeutic purposes. However, the therapeutic exploitation of *Ras* oncogene addiction has been hampered by a plethora of unfavorable factors, including incomplete understanding of signaling transduction, feedback loops, redundancy and tumor heterogeneity, as well as the difficulty in developing small-molecule inhibitors against Ras oncoproteins (51). Thus, a mutant-selective inhibitor of $KRAS^{G12C}$ has been recently described, although further work will be needed to determine its clinical efficacy in anticancer therapies (52, 53).

In this regard non-oncogene addiction offers new windows of therapeutic opportunities in which, according to our work, USPs represent potentially druggable candidates. Consistent with this hypothesis, several USPs have been identified as *bona fide* participants in non-oncogene addiction phenomena. Thus, USP2 inhibition has been described as an effective approach to induce growth suppression in cancer cells with addiction to cyclin D1 expression (54). Silencing of *USP11* caused spontaneous DNA damage repair activation and hypersensitivity to PARP inhibition, ionizing radiation, and other sources of genotoxic stress (55). Moreover, the disruption of USP9X and MCL1 interaction with gemcitabine sensitizes cells to ABT-737 treatment, inducing caspase-dependent apoptosis (56). Furthermore, depletion of USP4 mitigates TGF-β-induced epithelial-to-mesenchymal transition and metastasis (57) and *USP15* down-regulation leads to a decrease in TGF-β activity and oncogenesis (58). Pringle *et al.* (59) have demonstrated that USP6 acts as an oncogene by activating NF-κB, whose activity is essential for USP6-mediated tumorigenesis. Finally, inhibition of USP37 blocks the proliferation of lung cancer cells by reducing c-Myc levels (60) and depletion of USP1-UAF1 overcomes the resistance of cancer cells to cisplatin (61).

In this work we have identified *USP39* as an essential gene for the viability of KRAS-dependent cancer cells. Thus, by performing a pooled negative-selection RNAi screen we have demonstrated that down-regulation of *USP39* specifically decreases the proliferation rate of KRAS-dependent H358 lung cancer cells without affecting the viability of A549 KRAS-independent lung cancer cells. To rule out possible artifacts due to the cellular system used, we have tested this synthetic lethal interaction using a different experimental system, composed of two pairs of isogenic colorectal cancer cell lines (35). This approach revealed that *KRAS*-mutant DLD-1 and HCT116 colon cancer cells are also dependent on *USP39* expression, confirming the addiction to USP39 of KRAS-dependent tumor cells of different histological origins. Of note, rescue experiments, based on the ectopic expression of an *USP39* cDNA carrying silent mutations that render the encoded transcript resistant to the shRNA, have demonstrated the specificity of the *USP39*-silencing approach and ruled out noticeable off-target effects.

USP39 is a catalytically inactive member of the USP family of deubiquitinases with a reported essential role in pre-mRNA splicing (38–40). Accordingly, we hypothesized that the synthetic lethal interaction of *USP39* and *KRAS* in cancer could be based on the splicing-related function of USP39, and consequently KRAS-active tumors would be especially dependent on efficient pre-mRNA splicing. In agreement with this hypothesis, we show herein that tumor cells carrying a mutant *KRAS* allele are clearly more sensitive to the pharmacological inhibition of splicing fidelity (with three different drugs) than their isogenic counterparts that are not addicted to the mutated oncogene. Moreover, we have shown that down-regulation of *DHX38*, encoding an USP39-interacting splicing factor, also affects preferentially the viability of KRAS-dependent tumor cells, providing additional support to the importance of an optimal function of the splicing machinery in this phenomenon. Furthermore, by performing RNA-seq, we have confirmed that the depletion of USP39 affects the splicing of a large diversity of genes, including multiple genes involved in cell cycle regulation as well as DNA repair, which could explain the importance of the normal function of this protein for *KRAS*-mutant cancer cells. The results reported herein point to USP39 as a good candidate for drug targeting in anticancer therapies against KRAS-driven neoplasias. In agreement with this idea, we have found that *USP39* is up-regulated in colon and lung human cancer patients. Furthermore, *USP39* expression correlates with *KRAS* levels in both lung and colon cancers, supporting the synthetic lethality interaction identified in the present work. Remarkably, the clinical interest of *USP39* is also supported by the fact that its overexpression is associated with a poor prognosis in lung cancer patients.

Several synthetic lethal screens have been carried out to identify proteins required for the maintenance of the tumorigenic phenotype of *KRAS*-mutant cells, yielding a remarkably heterogeneous array of results (10–23). The most recurrent finding in these screens corresponds to proteasome components, reflecting a requirement of efficient clearance of damaged or excess proteins resulting from elevated protein synthesis rates in *KRAS*-mutant cells. Similarly, these cells are especially sensitive to alterations in the mitotic machinery, reflecting mitotic stress as a hallmark of Ras transformation (12). By contrast, to our knowledge, this work is the first to describe an exacerbated dependence of KRAS-driven tumor cells on high-fidelity mRNA splicing, even though this phenomenon is consistent with the hyperactive protein biosynthesis associated with Ras oncogenesis. Our results from treatment with splicing fidelity inhibitors, *DHX38*-specific shRNAs, RNA-seq experiments, and the meta-analysis of a genome-scale shRNA screen of 216 cancer cell lines of multiple histological origins support this possibility. A variety of natural and synthetic small molecules with splicing modulatory activity are currently under investigation as potential antitumor drugs (42, 43, 62). In this regard, the results from the present work suggest that these compounds will be preferentially active against Ras-dependent cancer. Remarkably, it has been recently reported that Myc-associated tumors also present exacerbated susceptibility to spliceosome targeting (47). Together, these findings strongly support the development of splicing-directed antitumor therapies against neoplasias driven by these two major and previously undruggable oncogenes. Further characterization of

USP39 binding motifs and development of DHX38 inhibitors could facilitate the implementation of new strategies against KRAS-driven malignancies.

In summary, the use of an RNAi-based synthetic lethal screen has allowed us to demonstrate USP39 non-oncogene addiction in colon and lung cancers. Additionally, we have confirmed the importance of USP39 on splicing regulation and its requirement for the survival of KRAS-dependent tumors. Therefore, the results obtained in the present work support the introduction of DUB-targeting strategies as an essential component for the development of anticancer therapies.

Experimental Procedures

Cell Culture—HCT116, HCT-15, HT29, SW620, RKO, DLD-1, and FHC cells were purchased from the American Type Culture Collection (ATCC). Tet-On A549 and Tet-On H358 cell lines were derived from A549 and H358 (ATCC). DLD-1 and HCT116 isogenic pair cell lines were kindly provided by Dr. B. Vogelstein. Cells were authenticated by short tandem repeat profiling at Servicios Científico Técnicos, University of Oviedo.

Patient Samples—All subjects gave written consent to participate in the study, using a consent form approved by the Comité de Ética de la Investigación del Principado de Asturias. This study was also approved by Comité de Ética de la Investigación del Principado de Asturias, and research was carried out in compliance with the Helsinki Declaration.

Pooled Negative-selection RNAi Screening—A customized shRNA library was cloned into the retroviral TRMPV-Neo vector as described previously (32). Then, retroviral constructs were transfected into HEK293T packaging cells. Virus supernatant was collected at 24, 36, and 48 h and an optimized dilution was used to infect 20% of the cells. Transduced cells were selected for 10 days using 1 mg/ml of G418 (Invitrogen) and a large enough number of cells were maintained in every passage to preserve 1000-fold library representation. In triplicate, samples were collected at time = 0 (T_0) and cells were subsequently cultured with 0.5 mg/ml of G418 and 1 μg/ml of doxycycline to induce shRNA expression. The proliferation rate of Tet-On A549 and Tet-On H358 cell lines was analyzed to adjust the final point of the screen (T_f). To calculate population doublings, we used the following formula: $n = 3.32 (\log N/N_0) + X$, where n = population doubling number at a given point, N = the cell yield at that point, N_0 = the cell number used to begin that subculture, and X = the doubling level of the inoculum used to initiate the subculture being quantified. After 10 cell population doublings, more than two million shRNA-expressing cells ($dsRed^+/Venus^+$) were sorted for each triplicate using a FACSAriaIII (BD Biosciences). Genomic DNA extraction, deep sequencing, and sequence processing was performed as described previously (32).

shRNA Lentiviral Infection—The best two shRNAs of a set of 5 *USP39* and *DHX38*-specific shRNA vectors from Open Biosystems, Thermo Scientific, and empty vector (pLKO.1) were packaged in HEK293T cells using a VSVG-based package system. Viral supernatant was collected at 24 h and added in a 1:3 dilution to previously seeded cell lines, supplemented with 5 mg/ml of Polybrene (Millipore). Stably transduced cells were selected with puromycin at a final concentration of 1 μg/ml in HCT116 cells and 2 μg/ml in DLD-1, A549, and H358 cell lines.

Cell Proliferation Assay—To quantify cell proliferation, 5000 cells per well were seeded ($n = 6$) into 96-well plates and a Cell Titer 96 Nonradioactive cell proliferation kit was used following the manufacturer's instructions (Promega Corp.).

Real-time qRT-PCR Analysis—2 μg of total RNA was used to synthesize cDNA (Life Technologies) and qRT-PCR was performed using TaqMan® gene expression assay or Power SYBR® Green PCR Master Mix (Life Technologies), using an Applied Biosystems 7300HT Real-time PCR System. Relative expression was calculated as relative quantification (RQ) = $2^{-\Delta\Delta Ct}$.

Western Blot Analysis—Western blot analyses were performed as previously described (48) using anti-USP39 (Lifespan Biosciences) and anti-PARP (Cell Signaling) antibodies.

shRNA-resistant USP39 Cloning—Silent mutations were introduced into shUSP39.1152 target sequence using Q5® Site-directed Mutagenesis Kit (New England Biolabs) with primers: 5′-tattgtttagtcccacaatacccggcaggtaag and 5′-tcaaagctaacgattatgccaacgctgtcc.

Mouse Xenograft Model—Both flanks of 8-week-old athymic Nude-Foxn1nu/nu mice (Charles River) were injected subcutaneously with 2 million control or USP39-silenced HCT116 cells in 100 μl of PBS, resulting in eight flanks per construction. Regarding lung cancer cell lines, 2 million A549 cells and 4 million H358 cells transduced with empty vector or *USP39*-shRNA were mixed with Matrigel (1:1) (Corning) and injected into NUDE mice (10 flanks per construction). Tumor size was measured twice per week using a caliber and mice were sacrificed 20 and 40 days post-injection.

RNA-seq—TotalRNAfromKRAS-dependentandKRAS-independent HCT116 cell lines transduced with control (pLKO.1) or *USP39*-specific shRNAs was isolated using RNeasy kit (Qiagen). NGS libraries were constructed and sequenced as 101-bp paired-end reads by Illumina HiSeq4000 (Macrogen Inc., Seoul, Republic of Korea). Alignment of RNA-seq data were performed with TopHat version 2.0.14 (63). Transcripts were predicted and assigned to known transcripts with cufflinks version 2.2.1 and the GRCh37.82 geneset database from Ensembl, respectively. We considered constitutive junctions as junctions that are included within all isoforms of a given gene, do not overlap with any transcript annotated to a different gene symbol, do not coincide with the start or the stop of a transcript, and have more than 25 reads on average, as described elsewhere (47). We then calculated a SpS for each intron-exon junction. Briefly, reads overlapping this junction were classified as "intronic" (I) or "exonic" (E) if the read mapped to at least the first base of the intron or at least the first base of the subsequent exon, respectively. Then, we applied the following formula: $\mathrm{SpS} = \log_2[(E + 1)/(I + 1)]$. GSEA was performed as described (44), using GSEA release 2.06 and MSigDB release 2.5. Selected enriched pathways had a relaxed false discovery rate <0.100 and $p < 0.05$.

Survival Analysis—KM-plotter (50) was used to assess the effect of *USP39* or *DHX38* expression on survival of lung cancer patients.

Author Contributions—J. M. F. performed experimental work, data interpretation, and preparation of the manuscript. E. M., A. L., V. Q., and D. C.-I. performed experimental work. T. R. W. and S. W. L. provided critical materials and participated in the preparation of the manuscript. C. L.-O. and J. M. P. F. supervised research and project planning, data interpretation, and preparation of the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

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