USP39 Deubiquitinase Is Essential for KRAS Oncogene-driven Cancer*§

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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, SNAI1L2, and Wilms tumor 1 (WT1) (13–21). Furthermore, synthetic lethal interactions between KRAS mutations and MEK inhibition combined with suppression of BCL-XL or RAFI 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).
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), 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. 1A), 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. 1B).

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. 1C).

Changes in library representation were analyzed by performing deep sequencing of PCR-amplified shRNAs (Fig. 1D). 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. 1E), with no depletion in A549 cells (Fig. 1F), 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-driven Cancer**—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. 2A) and quantitative (q) RT-PCR in both cell lines (supplemental Fig. S1A). 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
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. 2D).

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. 2E). 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. 2F). 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. 2G) and qRT-PCR (supplemental Fig. S1B). As expected, only the proliferation rate of KRAS-dependent cells was significantly reduced upon depletion of USP39 with the same two shRNAs (Fig. 2H). 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. S1C). 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. 2I).

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. S2A, 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).
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, 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. 4C). In support of the exacerbated dependence on 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).

**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. 5A;...
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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. 5D). 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, KIFI4, and ORC1) and validated by qRT-PCR their differences in splicing efficiency (supplemental Fig. S3A). As shown in Fig. 5E, 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. S3B). 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. 6A). Furthermore, cancer cell lines showed higher USP39 protein levels than control cells, corroborating the results obtained through qRT-PCR (Fig. 6B). 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. 6C, USP39 was consistently up-regulated in colorectal carcinoma samples, thus suggesting its pro-tumoral role. USP39 overexpression was verified by Western blot analysis (Fig. 6D). Moreover, a detailed analysis of USP39 and KRAS expression in these paired samples showed a positive correlation between both genes (Fig. 6E). 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 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).
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. 6F). Moreover, by analyzing expression data from an available microarray study of 293 lung tumors (49),
(GEO accession GSE30219), we found that USP39 and KRAS were significantly up-regulated in lung cancer compared with non-tumoral samples (Fig. 6G). 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. 6H). 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.
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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 KRASG12C 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 Técnico, Universidad de 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 $T_0$ 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: $n = \frac{\log N/N_0}{\log 2} + X$, where $n$ is the population doubling number at a given point, $N$ is the cell yield at that point, $N_0$ is the cell number used to begin that subculture, and $X$ is the doubling level of the inoculum used to initiate the subculture being quantified. After 10 cell population doublings, more than two million control or USP39-silenced HCT116 cells were transfected into NUDE mice (10 flanks per construction). Tumor size was measured twice per week using a caliper and mice were sacrificed 20 and 40 days post-injection.

RNA-seq—Total RNA from KRAS-dependent and KRAS-independent HCT116 cell lines transduced with control (pLKO.1) or USP39-specific shRNAs was isolated using RNAeasy 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 GRC37.82 genaset 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: $\text{SpS} = \log_2 \frac{[\text{E}+1]}{[\text{I}+1]}$. 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 <0.10 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.
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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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