Targeted Degradation of KRAS by an Engineered Ubiquitin Ligase Suppresses Pancreatic Cancer Cell Growth In Vitro and In Vivo

Yihui Ma1,3, Yumei Gu1, Qiang Zhang1,2,4, Yongqing Han2, Shuangni Yu1, Zhaohui Lu1, and Jie Chen1

Abstract
KRAS is an attractive pancreatic ductal adenocarcinoma (PDAC) therapeutic target. E3 ligase is thought to be the component of the ubiquitin conjugation system that is directly responsible for substrate recognition. In this study, an engineered E3 ubiquitin ligase (RC-U) was generated to target the KRAS oncoprotein for ubiquitination and degradation. The engineered E3 ubiquitin ligases (RC-U) were constructed (pRC-U and lentivirus-expressing RC-U). After transfecting the pRC-U plasmid into human pancreatic cancer cells, KRAS expression levels were determined. KRAS expression was also evaluated in cells transfected with pRC-U and treated with MG-132 or cycloheximide. Interactions between RC-U and KRAS as well as whether RC-U could ubiquitinate KRAS were investigated. Extracellular signal–regulated kinase 1/2 (ERK1/2) and phosphorylated ERK 1/2 (pERK1/2) levels were examined in pancreatic cancer cells transfected with pRC-U. The effects of RC-U on pancreatic cancer cell growth were assessed. RC-U decreased KRAS protein levels. After pRC-U transfection, KRAS stability was increased in the presence of MG-132. HEK 293T cells were transfected with a mutant KRAS construct together with pRC-U and incubated with cycloheximide to inhibit new protein synthesis. The exogenous mutant KRAS oncoprotein was degraded more quickly. RC-U can bind KRAS and KRAS can be ubiquitinated by RC-U. pERK1/2 protein levels were decreased. RC-U resulted in reduced cell proliferation in vitro and in vivo. KRAS destruction by RC-U occurred through a ubiquitin-dependent, proteasome-mediated degradation pathway. RC-U inhibited pancreatic cancer cell growth in vitro and in vivo. Mol Cancer Ther; 12(3); 1–9. ©2012 AACR.

Introduction
Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States, with a median survival of 6 months (1) and a dismal 5-year survival rate of 6% (2). Although surgical resection provides a potential cure, approximately 70% of patients still develop early recurrence within 6 to 12 months after surgery (3). Systemic chemotherapy is only palliative because most patients have advanced or metastatic disease at the time of diagnosis (4). Novel effective therapeutic strategies are urgently needed for the treatment of this disease.
Activating mutation of the KRAS oncogene is one of the earliest genetic events observed in 30% of human pancreatic intraepithelial neoplasias (PanIN), a frequency that increases to nearly 100% in advanced pancreatic ductal adenocarcinoma (PDAC; ref. 5). Such findings make KRAS an attractive therapeutic target. Many approaches for KRAS inhibition are in preclinical trials. For example, a farnesyl transferase inhibitor, which inhibits lipid modification of the C terminus of Ras proteins, showed excellent experimental anticancer effects (6, 7). Ras silencing at the gene level by RNA interference (RNAi) is considered a promising gene therapy method and has been used successfully in experimental pancreatic cancer models (8, 9).
Targeted ubiquitination and degradation of oncoproteins via the ubiquitin–proteasome pathway (UPP) represents an alternative therapeutic strategy (10). Ubiquitin conjugation is achieved by several enzymes that act in concert, including the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3; refs. 11–13). The E3 ligase is thought to be the component of the ubiquitin conjugation system that is directly responsible for substrate recognition. The E3 ligase is a protein or protein complex that generally serves as a scaffold that brings together the E2 and the substrate.
The E3s are a large, diverse group of proteins characterized by one of several defining motifs, for example, a HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene), or U-box (a modified RING motif without the full complement of Zn$^{2+}$-binding ligands) domain (15). While HECT E3s have a direct role in catalysis during ubiquitination, RING and U-box E3s facilitate protein ubiquitination and act as adaptor-like molecules (16, 17). Indeed, engineered E3 enzymes have been shown to eliminate oncoproteins.

β-TrCP is an E3 ubiquitin ligase in the F-box protein complex, which mediates β-catenin degradation. β-TrCP induces β-catenin degradation in other cell types (18, 19). In another line of experiments, the functional domain of the CHIP (carboxyl terminus of Hsc70 interacting protein) E3 ligase, the U-Box, was ligated with the bHLH/LZ protein Max to construct a chimeric molecule called Max-U, which can interact with the oncopgenic c-Myc protein and limit its activity (20). In addition, a small molecule named Protac was synthesized to target the estrogen receptor and the androgen receptor to the UPP (21).

Ras-binding domains/Ras-associating domains (RBD/RAD) are the primary Ras-binding sites in Ras effector proteins. The modules, which facilitate interactions with Ras/Rap1-GTP, are conserved from yeast to mammals (22). Raf-1 is a pivotal effector of Ras. Among the Ras family members, it is preferentially activated by KRAS (23). However, the Raf-1 RBD alone is not sufficient to support a stable interaction with Ras in living cells, the KRAS (23). However, the Raf-1 RBD alone is not sufficient to support a stable interaction with Ras in living cells, the KRAS family members, it is preferentially activated by KRAS (22). Raf-1 is a pivotal effector of Ras. Among the Ras family members, it is preferentially activated by KRAS (23). However, the Raf-1 RBD alone is not sufficient to support a stable interaction with Ras in living cells, the addition of the cysteine-rich domain (CRD) is required for the RBD to remain bound to active Ras and form a more stable complex in the plasma membrane (24–26).

In this study, the U-Box-based chimeric E3 ligase (RBD+CRD)$^{\text{Raf-1, U-Box (RC-U)}}$ was generated to target the KRAS oncoprotein for ubiquitination and degradation. Our results showed that this chimeric molecule (RC-U) can decrease KRAS protein expression by UPP and suppress pancreatic cancer cell growth in vitro and in vivo.

Materials and Methods

Cell lines and cell culture

The human pancreatic cancer cell lines PANC-1, Mia PaCa-2, AsPC-1, and BxPC-3 and human HEK 293T cells were obtained from the American Type Culture Collection. Cells were reauthenticated using short tandem repeat (STR) profiling and maintained in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich) or RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Tian Jin Hao Yang Biological Manufacture) and cultured at 37°C in a 5% CO$_2$ atmosphere.

cDNA cloning and expression vector construction

The cDNA fragments encoding the U-box and (RBD+CRD)$^{\text{Raf-1, (RC)}}$ were amplified by PCR amplification of cDNA derived from normal human lung tissue. The 2 cDNA fragments were inserted into the pcDNA3.1 vector (Invitrogen) by cloning the RC fragment into the BamHI/EcoRI sites and the U-box into the EcoRI/XhoI sites. Four glycine-serine repeats (GSGSGSGS) were used to connect RC with the U-box to avoid interference of these 2 regions. DNA encoding Flag-tagged mutant or wild-type KRAS was amplified by PCR amplification of cDNA derived from PANC-1 and BxPC-3 cells, respectively, and cloned into the BamHI/XhoI sites of the pcDNA3.1 vector. The fusion cDNA (RC-U) was subcloned into the pLenti6.3-MSC-IRE5-EGFP (Invitrogen) plasmid to generate pLenti6.3-RC-U-IRE5-EGFP. Lentiviruses were packaged in HEK 293T cells. Viruses were harvested 48 hours after transfection and the lentiviral titers were determined.

Cell transfection and infection

For transient transfection, cells were transfected with the RC-U, RC, and U-box expression vectors and pcDNA3.1 in 6-well plates using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. For stable expression, cells ($7 \times 10^6$) were infected with $1 \times 10^8$ recombinant lentivirus-transduction units in the presence of 8 μg/mL polybrene (Sigma-Aldrich).

Immunoblotting and immunoprecipitation

Cells transfected with the different expression vectors were cultured in media with or without MG-132 (100 nmol/L) or cycloheximide (50 μg/mL; Sigma-Aldrich). The vector of hemagglutinin (HA)-tagged ubiquitin was kindly provided by Dr. Dahai Zhu (National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). For immunoblotting, 60 or 40 μg of total protein extract was separated on a 12% SDS-PAGE gel. For immunoprecipitation, cell lysates containing 1 to 1.5 mg total protein were incubated with anti-Flag M2 (1:2,000, F1804, Sigma-Aldrich) for 4 hours at 4°C followed by protein A/G Sepharose beads (Santa Cruz Biotechnology) for 2 hours at 4°C and then were transferred to polyvinylidene difluoride membrane (Millipore). The membranes were incubated with antibodies against Flag (1:2,000, F1804), Myc (1:2,000, sc-40, Santa Cruz Biotechnology), KRAS (1:1,000, sc-30, Santa Cruz Biotechnology), HA (1:2,000, sc-57592, Santa Cruz Biotechnology), extracellular signal–regulated kinase 1/2 (ERK1/2; 1:2,000, 8544, Cell Signaling), phosphorylated ERK 1/2 (pERK1/2; 1:2,000, 4348, Cell Signaling), and β-actin (1:2,000, sc-47787, Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with their corresponding secondary antibodies (1:2,000, Zhongshan Goldenbridge Biotechnology) at room temperature for 2 hours. The membrane-bound proteins were detected using ECL Western blotting substrate (Pierce).

Quantitative real-time reverse transcription PCR

One microgram of total extracted RNA was reverse-transcribed into cDNA with oligo(dT)15 using M-MLV
reverse transcriptase (Promega). The cDNAs were used to amplify KRAS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by PCR. The PCR primers were as follows:

KRAS-F: 5'-GACTCTGAAGATGTACCTATGGTCCT-3', KRAS-R: 5'-CATCATAACACCCCGTCTGGTGC-3' (27), GAPDH-F: 5'-GCACCGTCAAGGCTGAGAAC-3', and GAPDH-R: 5'-GCTCTCTCATGGTGTTGA-3'.

Real-time PCR was conducted using SYBR Green PCR Mix (ABI) according to the manufacturer's protocol. The relative mRNA expression level was calculated using the 2^(-ΔΔCt) method (27).

**Immunohistochemistry**

Four-micrometer thick sections were prepared from the paraffin-embedded tissues. Immunostaining was carried out by PV-6000 polymer detection system. The sections were deparaffinized in xylene, rehydrated with graded alcohol, and then boiled in EDTA buffer (pH 9.0) for 2.5 minutes with an autoclave. They were incubated at 4°C overnight with anti-KRAS polyclonal antibody. The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB). PV-6000 system, anti-KRAS polyclonal antibody, and DAB were obtained from Zhongshan Goldenbridge Biotechnology. Slides were also stained in the absence of primary antibody, and these served as negative controls. The staining intensity was assessed by experienced pathologists.

**Immunofluorescence and confocal microscopy**

The cells, which were grown on coverslips, were fixed with 4% paraformaldehyde, followed by washing with 0.2% Triton X-100. The coverslips were incubated at 4°C overnight with anti-Flag (2.5 μg/mL, F1804) or Myc (1:100, sc-40) antibodies. The cells were incubated with the appropriate fluorochrome-conjugated secondary antibody (1:200, Zhongshan Goldenbridge Biotechnology). Nuclei counterstaining was conducted using 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and detected using a microscope. Colonies were visualized by adding 5 mg/mL MTT (Sigma-Aldrich) and detected using a microscope. Colonies were counted. Each assay was conducted in triplicate.

**CCK-8 assay**

Transfected pancreatic cancer cells were seeded at 1,000 cells per well in a 96-well plate. In brief, 10 μL of CCK-8 solution (Dojindo Laboratories) was added to 90 μL of culture media in each well. The plate was incubated for 1.5 hours at 37°C, and the absorbance at 450 nm was measured using a Vmax microplate spectrophotometer (Molecular Devices). Each sample was assayed in triplicate.

**Soft agar colony formation assay**

A 1.5 mL volume of culture medium containing 10% FBS with 0.7% agar was added to the bottom of a 24-well plate. A total of 3,000 cells per well were mixed with culture medium containing 10% FBS and 0.35% agar and plated into the prepared wells. After 14 days, the colonies were visualized by adding 5 mg/mL MTT (Sigma-Aldrich) and detected using a microscope. Colonies were counted. Each assay was conducted in triplicate.

**Xenograft studies in nude mice**

BALB/c female nude mice (nu/nu; 4-6-weeks old) were obtained from Vital River. Animal handling and experimental procedures were approved by the Peking Union Medical College animal experiments committee. PANC-1 cells were subcutaneously inoculated into the dorsal region of a single mouse to generate several primary tumor masses. Once the tumors reached 1 cm^3 in size, they were removed, cut into masses of 3 mm in diameter, and transplanted into the right dorsal region of other mice. In vivo-jetPEI (Polyplus transfection), was used to conduct the in vivo transfection. The mice treated earlier were randomly divided into 3 groups of 6 mice each. Once the tumors reached 50 mm^3, they were injected with the vector+in vivo-jetPEI, pRC-U+in vivo-jetPEI, or in vivo-jetPEI reagent only (mock). The mice were injected twice (day 1 and 7). After the first injection, tumor size was measured every 3 days, and tumor volume was calculated as (length) × (width)^2/2. When the tumor diameter reached 15 mm, the mice were euthanized. Finally, the tumors were fixed in 10% formalin and embedded in paraffin.

**Statistical analysis**

Statistical analysis was conducted using SPSS 16.0 software. Unless otherwise indicated, the level of significance for differences between datasets was assessed using t-test and one-way ANOVA. Data are expressed as the mean ± SD. P < 0.05 was considered statistically significant.

**Results**

**Construction of a chimeric ubiquitin ligase to target the KRAS protein**

To generate a U-Box-based chimeric ubiquitin ligase that specifically targets the KRAS oncoprotein for degradation, the RBD and CRD of Raf-1, which have been reported to interact with KRAS in vivo, were chosen as the "interaction domain" of the chimeric protein. As shown in Fig. 1A, the chimeric protein was designated Myc-tagged (RBD+CRD)^Raf-1.U-Box (RC-U). We also used constructs that expressed Myc-tagged RC (RC) and Myc-tagged U-Box (U-Box) as negative controls. The Flag-tagged mutant KRAS and RC-U constructs were cotransfected into human HEK 293T cells. Cotransfection of RC and mutant KRAS served as a positive control, whereas transfection alone was the negative control. We measured the potential interaction between KRAS and RC-U by immunoprecipitation. The results showed that the RC-U chimeric protein communoprecipitated with Flag-tagged mutant KRAS as efficiently as RC (Fig. 1B), and the U-Box and charged domain at the C-terminus did not interfere with the interaction between RC and KRAS.
KRAS destruction by the RC-U chimeric protein through a ubiquitin-dependent, proteasome-mediated degradation pathway

Four pancreatic cancer cell lines were examined by immunoblotting to determine endogenous KRAS protein expression levels. As shown in Fig. 2A, PANC-1 cells carrying the mutant KRAS gene showed the highest level of KRAS protein. To evaluate the effect of RC-U on KRAS protein expression, endogenous KRAS protein levels were determined in PANC-1 cells 48 hours posttransfection. Ectopically expressed RC-U dramatically decreased the KRAS protein level compared with the controls (Fig. 2B). We also cotransfected Myc-tagged RC-U and Flag-tagged mutant KRAS expression plasmids or the empty vector into HEK 293T cells. By immunofluorescence staining, we found that mutant KRAS was decreased in the presence of RC-U (Fig. 2C). To investigate whether the lower KRAS protein level is independent of KRAS gene transcription, we used real-time reverse transcription PCR (RT-PCR) to analyze KRAS mRNA levels after RC-
U transfection. No significant changes were observed among the groups, suggesting that RC-U’s regulation of mutant KRAS occurs at the posttranslational level (P > 0.05; Fig. 2D). The ubiquitin ligase activity of RC-U was assessed using a ubiquitination assay. We transfected HEK 293T cells with Flag-tagged mutant KRAS and HA-tagged ubiquitin in combination with RC-U, RC, or U-Box. As shown in Fig. 3A, polyubiquitylation of the mutant KRAS oncoprotein was markedly enhanced by RC-U as compared with RC and U-Box. The result of the ubiquitination assay indicated that KRAS could be ubiquitinated by RC-U. HEK 293T cells were transfected with the Flag-tagged mutant KRAS construct together with either the Myc-tagged RC-U or pcDNA3.1 and then incubated with cycloheximide for various times to inhibit new protein synthesis. As a result, the exogenous KRAS oncoprotein was degraded more quickly in RC-U-transfected cells than in empty vector-transfected cells (Fig. 3B). To further confirm the involvement of the UPP in RC-U-mediated KRAS protein degradation, we treated the RC-U-transfected PANC-1 cells with MG-132, a specific proteasome inhibitor. We found that KRAS stability was significantly increased in the presence of MG-132 (P < 0.05). Collectively, these results suggested that RC-U may function as an E3 ubiquitin ligase for the KRAS oncoprotein and that the polyubiquitylated KRAS is subject to proteasomal degradation. The structures of MG-132 and cycloheximide were shown in Fig. 3D and E.

**RC-U inhibited the growth of pancreatic cancer cells in vitro**

The KRAS oncoprotein is involved in cell proliferation and KRAS knockdown can inhibit malignant tumor progression. In this study, CCK-8 and soft agar colony formation assays were conducted to assess the growth rate of transfected pancreatic cancer cells. As shown in Fig. 4A–C, PANC-1 and MIA PaCa-2 cells infected with lentiviruses expressing RC-U exhibited a decreased KRAS expression and low growth rate compared with the controls (P < 0.05; Fig. 4D and E). Furthermore, RC-U not only reduced PANC-1 and MIA PaCa-2 cells growth but also affected anchorage-independent growth in the colony formation assays (P < 0.05; Fig. 4F–H).

Because ERK1/2 is a major downstream KRAS effector in the Ras signaling pathway, we measured the level of ERK1/2 phosphorylation to further explore the possible mechanism by which RC-U suppresses PANC-1 cell growth. As shown in Fig. 5D, RC-U transfection significantly decreased the level of pERK1/2 compared with the controls.

---

**Figure 3.** The RC-U chimeric protein can degrade KRAS through a ubiquitin-dependent, proteasome-mediated degradation pathway. A, polyubiquitylation of the mutant KRAS oncoprotein was markedly enhanced by RC-U. B, in the presence of cycloheximide (50 μg/mL), the exogenous KRAS oncoprotein was degraded more rapidly in RC-U-transfected cells than controls. HEK 293T cells were transfected with the Flag-tagged mutant KRAS construct together with either Myc-tagged RC-U or vector and incubated with cycloheximide for various durations to inhibit new protein synthesis. C, KRAS stability was significantly increased in the presence of MG-132 (100 nmol/L), a specific proteasome inhibitor (*, P < 0.05). D, the structure of MG-132. E, the structure of cycloheximide.
The RC-U recombinant plasmid suppressed tumor xenograft growth in nude mice

To evaluate the effect of the RC-U recombinant plasmid on PANC-1 cell growth in vivo, we established a PANC-1 xenograft tumor model in nude mice. A significant difference in tumor volume was observed between the RC-U-treated and control groups on days 14 to 29 after the first injection (P < 0.05). However, there were no significant differences between the vector-treated and mock groups (P > 0.05; Fig. 5A). The gross morphology of the tumors was shown in Fig. 5B. RC-U inhibited KRAS expression in the mouse xenograft tumors. Using immunohistochemistry, the mock and vector groups showed relatively stronger cytoplasmic KRAS expression than the RC-U group did (Fig. 5C).

Discussion

Pancreatic cancer has a poor prognosis because of its early metastasis, rapid progression, and insensitivity to radio- and chemotherapy (28). Genetic changes in the KRAS, HER2, p53, p16, and Smad4 genes are early events in pancreatic carcinogenesis. These oncogenic or tumor suppressor genes play key roles in the progression and metastasis of pancreatic cancer (29). An increasing number of researchers and clinicians are focusing their attention on gene therapy methods for preventing and treating pancreatic cancer (30).

Ubiquitin-dependent protein degradation is a highly conserved method of protein degradation. Given the high specificity and rapidity of protein degradation by this system, the UPP has been considered a potentially effective approach to eliminate abnormal oncoproteins. In the
past 10 years, several chimeric E3 ligases have been shown to successfully degrade their specific target substrates and significantly suppress the malignant biologic behavior of cancer cells in vitro and in vivo. Several F-box–based chimeric E3 ligases were constructed in these studies; however, F-box–based E3 ligases require several other molecular components for full activity, which limits the efficiency with which they can induce the degradation of target proteins (31, 32). Like the CHIP E3 ligase (17, 33), the chimeric protein Max-U contains a catalytic U-box–based functional domain and a substrate-interacting domain in a single molecule, thus it can significantly degrade the c-Myc oncoprotein in cancer cells (20). In this study, we constructed a similar U-box–based chimeric ubiquitin ligase, RC-U, which can interact with the KRAS protein. As the results show, the RC-U chimeric protein interacts with, ubiquitinates and promotes KRAS degradation, which in turn, inhibits the growth of pancreatic cancer cells in vitro and in vivo, supplying an effective alternative therapeutic strategy for pancreatic cancers.

The ability of the chimeric E3 ligase to bind to the KRAS protein target was crucial. RBD/RAD are the primary Ras-binding sites of effector proteins. Raf-1 is a pivotal Ras effector that is preferentially activated by KRAS, suggesting that its RBD predominantly interacts with KRAS over other Ras proteins. However, the Raf-1 RBD alone is not sufficient to support a stable interaction with Ras in living cells, despite its reported ability to recognize active Ras in vitro. The addition of the CRD was required for the RBD to remain bound to active Ras and form a stable complex in the plasma membrane. We determined that the chimeric RC-U and mutant KRAS proteins interacted in a coimmunoprecipitation assay, suggesting that RC-U can target activated KRAS. As a control, we created another chimeric protein that included the RBD but not the CRD in the interaction domain. This protein failed to degrade the KRAS protein and did not seem to interact with KRAS in vitro (data not shown).

The decreased KRAS protein level was associated with a significant inhibition of proliferation in vitro together with tumor growth suppression in vivo. These results are consistent with reports that Ras and its downstream effectors alter the expression of many molecules that are dependent on the Raf/MEK/ERK pathway (34, 35). In addition, we observed decreased pERK1/2 expression, which is a pivotal downstream effector molecule of the Ras/Raf/MAPK signaling pathway.

RBD binds to the Ras protein effector domain. The Ras family has 3 members, HRAS, NRAS, and KRAS, which have identical effector binding domains. KRAS, HRAS, and NRAS mutations occur at varying frequencies in different human tumors and show a high degree of tumor-type specificity. HRAS is often mutated in papillary thyroid cancers (36), NRAS is often mutated in hematologic disorders (37), and KRAS mutations are predominant in pancreatic, colorectal (38), and non–small cell lung carcinomas (39). It has been suggested that the
expression of Ras gene family members is controlled in a tissue-specific manner, and therefore only the Ras gene with the appropriate expression characteristics will, when mutated, dominate the corresponding tumors (40). In our study, in addition to KRAS, RC-U downregulated the levels of HRAS and NRAS proteins (Supplementary Fig. S1). However, reductions in HRAS and NRAS did not influence the growth suppression caused by degradation of the mutant KRAS oncoprotein, which may indicate a predominant role of mutant KRAS and a preferred interaction between the mutant KRAS oncoprotein and the chimeric RC-U E3 ligase in pancreatic adenocarcinoma cells. Some of the previously reported chimeric E3 ligases can knockdown related family members. For example, CbIN-SHGrb2, which targets the Her2 protein in breast cancer, can also downregulate EGF receptor (EGFR), which belongs to the same family (41). Here, the ability to simultaneously degrade multiple protein family members by ubiquitin modification offers a simple method to provide greater therapeutic benefit. In Supplementary Fig. S1, although there is also a small decrease of KRAS, NRAS, and HRAS with the plasmid vector transfections, the quantification analysis results still showed there was no significant difference between plasmid vector transfection cells and mock group cells, whereas a significant downregulation of KRAS, HRAS, and NRAS of RC-U transfection cells were observed compared with other control groups (P < 0.05). We have repeated these experiments several times with similar results, and thus we consider that compared with untreated cells, plasmid vector transfections may affect the viability of cells.

Rap1A is another small GTPase protein that has an identical effector domain to that found in Ras (42); however, we did not observe Rap1A degradation by RC-U. It is possible that although the effector domains of these small GTPase proteins are identical, structures in addition to the effector domain may be required for interaction between the Ras family and Rap1A, and domains other than RBD or CRD may be necessary for interaction between Rap1A and Raf-1.

Although RBD can bind to KRAS and may compete with endogenous Raf-1 for KRAS binding to suppress oncogenic activity, no significant inhibitory effect on ERK1/2 phosphorylation was observed after RBD+CRD vector transfection. It is possible that mutant KRAS is sufficiently abundant to saturate the downstream signaling pathways.

Compared with RNAi, which silences genes at the transcriptional level (43), the targeted destruction of KRAS by the chimeric E3 ligase RC-U occurred at the posttranslational level and was more effective at achieving target protein degradation in a relatively short period of time. Both KRAS protein levels and cell proliferation were significantly reduced as early as 48 hours after transfection.

In our study, we used xenogeneic cell transplantation into nude mice to determine the therapeutic effects of our methods. This animal model is simple but powerful to get a rough evaluation of tumor growth capacity in an in vivo mammalian system, and had been successfully used by our laboratories (27, 44). It is better to confirm our results in other animal models of pancreatic cancer such as carcinogen-induced cancer or genetically engineered animals.

However, the techniques used in this study have limitations. Determining the specificity of the interaction between the targeted protein and the chimeric E3 ligase as well as the activity of the chimeric E3 ligase, which determines whether substrates can be ubiquitinated, are critical issues for the success of UPP-based gene therapies. Further work aimed at identifying specific interaction modules capable of distinguishing the wild-type KRAS protein from its mutant, oncogenic form, or distinguishing the 3 Ras family members would make this strategy more valuable, particularly in heterozygous samples.

Some of the difficulties associated with targeted ubiquitination and protein degradation approaches might be bypassed by exploiting a recently identified, naturally occurring alternative pathway for proteasome-dependent protein degradation that involves ornithine decarboxylase (ODC) and antizyme (AZ; refs. 45–47). ODC directly binds to substrates and is degraded by the 26S proteasome through a mechanism catalyzed by antizyme. This process is independent of ubiquitin. Our group is currently attempting to knockdown Ras proteins via the ODC–AZ system.

In summary, the U-Box–based chimeric E3 ligase generated in this study, RC-U, is able to promote the ubiquitination and degradation of KRAS, leading to inhibition of tumor cell growth in vitro and in vivo. This may represent an effective alternative strategy for targeted KRAS therapy in pancreatic cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Ma, Y. Gu, Q. Zhang, Z. Lu, J. Chen
Development of methodology: Y. Ma, Y. Gu, Y. Han, J. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ma, Y. Gu, Y. Han
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ma, Y. Gu
Writing, review, and/or revision of the manuscript: Y. Ma, Y. Gu, J. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Ma, Q. Zhang, S. Yu
Study supervision: Y. Ma, J. Chen

Grant Support
This work was supported by grants from National Natural Science Foundation of China, no. 30471970 and 30973470 (J. Chen); National Science and Technology Support Project (the 11th Five-Year Plan) of China, no. 2006BAI02A14 (J. Chen); Scientific Research Special Projects of Health Ministry of China, no. 200802011 (J. Chen); and the Youth Innovation Fund of the First Affiliated Hospital of Zhengzhou University (Y. Ma).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 28, 2012; revised November 27, 2012; accepted December 18, 2012; published OnlineFirst January 3, 2013.
References


Targeted Degradation of KRAS by an Engineered Ubiquitin Ligase Suppresses Pancreatic Cancer Cell Growth \textit{In Vitro} and \textit{In Vivo}

Yihui Ma, Yumei Gu, Qiang Zhang, et al.

\textit{Mol Cancer Ther} Published OnlineFirst January 3, 2013.

\textbf{Updated version} Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-0650

\textbf{Supplementary Material} Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/01/03/1535-7163.MCT-12-0650.DC1

\textbf{E-mail alerts} Sign up to receive free email-alerts related to this article or journal.

\textbf{Reprints and Subscriptions} To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

\textbf{Permissions} To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.