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RASSF6 promotes p21$^{Cip1/Waf1}$-dependent cell cycle arrest and apoptosis through activation of the JNK/SAPK pathway in clear cell renal cell carcinoma

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Abbreviations: ccRCC, clear cell renal cell carcinoma; *VHL*, von Hippel-Lindau; HIFs, hypoxia-inducible factors; *RASSF*, Ras-association domain family; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction

Clear cell renal cell carcinoma (ccRCC) is a highly aggressive and common pathological subtype of renal cancer. This cancer is characterized by biallelic inactivation of the von Hippel–Lindau (*VHL*) tumor suppressor gene, which leads to the accumulation of hypoxia-inducible factors (HIFs). Although therapies targeted at HIFs can significantly improve survival, nearly all patients with advanced ccRCC eventually succumb to the disease. Thus, additional oncogenic events are thought to be involved in the development of ccRCC tumors. In this study, we investigated the role of *RASSF6* in ccRCC. Downregulation of *RASSF6* was commonly observed in primary tumors relative to matched adjacent normal tissues. Moreover, functional studies established that ectopic re-expression of *RASSF6* in ccRCC cells inhibited cell proliferation, clonogenicity, and tumor growth in mice, whereas silencing of *RASSF6* dramatically enhanced cell proliferation in vitro and in vivo. Mechanistic investigation suggested that *RASSF6* triggers p21$^{Cip1/Waf1}$ accumulation to induce G_1 cell cycle arrest and promote apoptosis upon exposure to pro-apoptotic agents, and both of these mechanisms appear to be mediated by activated JNK signaling. Together, these findings suggest that *RASSF6* may play a tumor suppressor role in the progression of ccRCC.

Introduction

Renal cell carcinoma (RCC) is the most common malignant renal tumor, and the occurrence of RCC has increased worldwide in recent years. Clear cell RCC (ccRCC), which accounts for approximately 80% of all RCCs, is characterized by inactivation of the von Hippel–Lindau (*VHL*) tumor suppressor gene. Loss of this gene leads to the stabilization of hypoxia-inducible factors (HIFs), which mediate the effects of hypoxia and play a central role in tumorigenesis.[1] Drugs targeting the HIF axis (including mTOR inhibitors) have been approved for advanced RCC.[2,3] However, the efficacy is thought to be limited, and treatment response is not long-standing.[4] Therefore the overall survival of ccRCC remains poor.[5-7] The pathogenesis of ccRCC is quite complicated, and it is unrealistic to expect that any single mechanism will uncover the truth. Additional tumorigenic events was supposed to contribute to the genesis and development of ccRCCs.[8-10]

In recent years, the aberrant activation of Ras-MAPK signaling has been reported in renal cell carcinoma.[11,12] Serve as RAS effector, Ras-association domain family (*RASSF*) genes 6 harbor Ras-association (RA) domains in their C-terminal region and have the ability to bind to RAS.[13] Ras superfamily proteins are activated by various extracellular stimuli and play important roles in regulating intracellular MAPK signaling pathways that control gene expression, mitosis, motility, and cell survival and differentiation. Recently, *RASSF6* was reported to be frequently lost in primary tumors (including in a relatively small number of kidney cancers) and to play an important role in regulating several physiological processes, including cell proliferation, apoptosis, and tumor development.[14,15] These findings suggest that *RASSF6* may function as a tumor suppressor gene in cancers. However, the role of *RASSF6* in ccRCC has not been previously investigated. In the present study, we investigated *RASSF6* expression status in ccRCC samples, and found that

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it was significantly downregulated in renal cancer tissues and cultured cells. Both in vitro and in vivo functional studies were also performed to characterize the growth-inhibiting effects of *RASSF6* in ccRCC. Moreover, the biological role of *RASSF6* in cell cycle arrest and the promotion of apoptosis was mechanistically associated with the activation of JNK/SAPK signaling. These results collectively indicate a suppressive role for *RASSF6* in ccRCC tumorigenesis.

Results

***RASSF6* is frequently downregulated in archived ccRCC tissues and cell lines**

RASSF6 mRNA expression levels were initially measured in 20 pairs of primary ccRCC samples and their corresponding non-tumor tissues by real-time quantitative PCR (qPCR). The relative expression level of *RASSF6* was significantly lower in tumor tissues compared with the non-tumor counterparts (**Fig. 1A**, $P < 0.01$, paired t test). Western blotting further showed that downregulation of *RASSF6* protein occurred in 5/8 randomly selected pairs of ccRCC and normal tissues (**Fig. 1B**). Downregulation of *RASSF6* was also observed in all tested ccRCC cell lines compared with HK-2 immortalized human renal proximal epithelial tubular cells (**Fig. 1C and D**). These findings indicate that a reduction in the *RASSF6* expression level is associated with the development of ccRCC.

***RASSF6* demonstrates tumor suppressive ability in vitro and in vivo**

To evaluate the function of *RASSF6* in ccRCC development, *RASSF6* was stably overexpressed in 2 ccRCC cell lines, 786-O and SKRC-39 (786-O-RF6 and SKRC39-RF6). Empty vector-transfected 786-O and SKRC-39 (786-O-Vec and SKRC-39-Vec) cells were used as controls. The expression of *RASSF6* in these cells was confirmed by western blot analysis (**Fig. 2A**). In vitro assays revealed that ectopic expression of *RASSF6* effectively inhibited cell proliferation, resulting in a significant inhibition of the cell growth rate (**Fig. 2B**, $P < 0.01$, Student t test) and a reduction in colony formation ability (**Fig. 2C**, $P < 0.01$, Student t test). To further explore the tumor suppressive role of *RASSF6* in vivo, 786-O-RF6 and 786-O-Vec cells were subcutaneously injected into nude mice, and their capacity for tumorigenesis was evaluated. Tumor growth was significantly suppressed in mice injected with *RASSF6*-expressing 786-O cells compared with controls (**Fig. 2D**, $P < 0.05$, Student t test). We next stably suppressed *RASSF6* expression in ACHN cells using 2 different shRNAs (ACHN-KD1 and ACHN-KD3, **Fig. 3A**). Suppression of *RASSF6* led to a significant increase in cell viability, as analyzed by MTS and colony-formation assays (**Fig. 3B and C**). In vivo study further revealed that tumors formed from *RASSF6* deplection ACHN cells presented significantly increased growth and weight compared with tumors formed from vector-transfected ACHN cells. These results strongly suggest that *RASSF6* plays a tumor suppressor role in the development of ccRCC.

***RASSF6* regulates the G_1/S phase transition of the cell cycle through $p21^{Cip1/Waf1}$**

Ectopic expression of *RASSF6* in 786-O and SKRC39 cells significantly increased the proportion of cells in G_0/G_1 phase and decreased the proportion in S phase (**Fig. 4A**). Conversely, *RASSF6* depletion in ACHN cells decreased the proportion of cells in G_0/G_1 phase and increased the proportion in S phase (**Fig. 4B**). Cell cycle progression from G_1/S phase is orchestrated by a tightly regulated interplay between cyclins D, E and A and their inhibitors. To confirm the role of *RASSF6* in the G_1/S-phase transition, these regulators were further evaluated. As shown in **Figure 4C**, *RASSF6*-expressing 786-O and SKRC39 cells

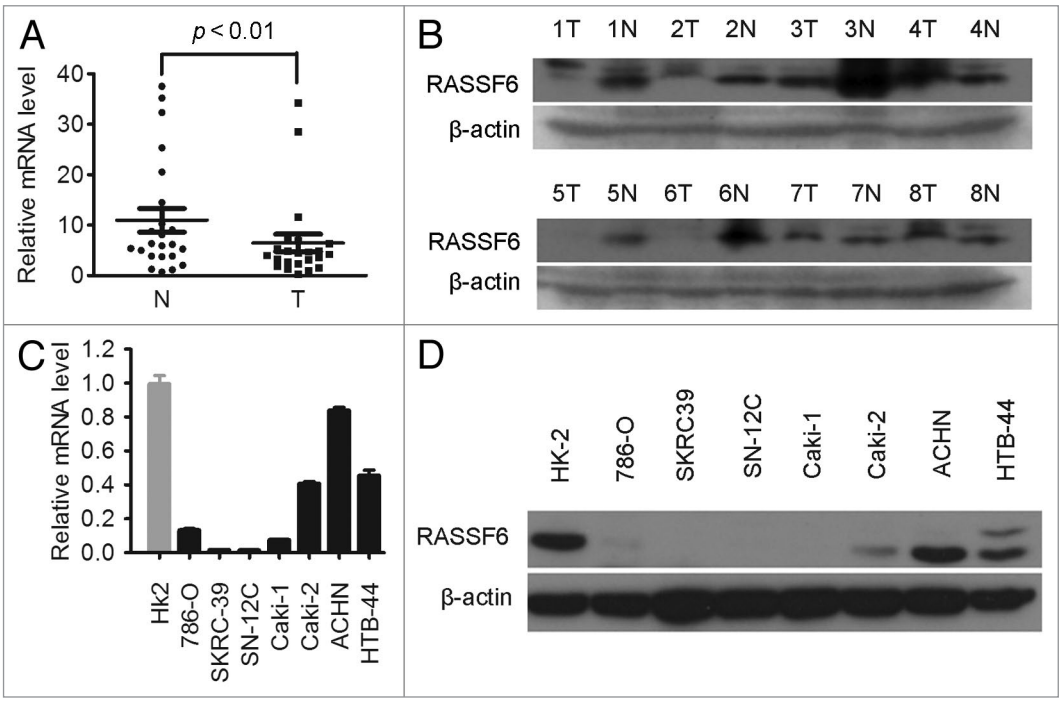

Figure 1. Downregulation of RASSF6 expression in ccRCC tissues and cell lines. (**A**) RASSF6 mRNA expression levels in 20 matched primary ccRCC tissues (T) and adjacent noncancerous tissues (N) were determined by qPCR assays. GAPDH and 18S were used as reference genes. $P < 0.01$, paired t test. (**B**) Western blotting analysis of RASSF6 protein levels in another randomly selected 8 pairs of matched ccRCC tissues (T) and adjacent noncancerous tissues (N). (**C and D**) qPCR (**C**) and western blotting (**D**) analysis of RASSF6 expression in ccRCC cell lines and HK-2 immortalized renal proximal epithelial tubular cells.

displayed a reduction in cyclin D1 levels and an accumulation of $p21^{Cip1/Waf1}$. In contrast, *RASSF6* depletion in ACHN cells resulted in cyclin D1 upregulation and $p21^{Cip1/Waf1}$ downregulation. Transient silencing of $p21^{Cip1/Waf1}$ in *RASSF6*-expressing 786-O cells partially prevented the G_0/G_1 phase accumulation caused by *RASSF6* (**Fig. 4D**, upper panel, and **4E**; **Fig. S1**). Restoration of $p21^{Cip1/Waf1}$ expression in ACHN-KDs and ACHN-NC cells (**Fig. 4D**, lower panel) restored the G_0/G_1-phase reduction mediated by *RASSF6* depletion (**Fig. 4F**; **Fig. S1**). To further confirm the relationship between *RASSF6* and $p21^{Cip1/Waf1}$, we measured expression of both of them in ccRCC samples by using quantitative PCR, and found out $p21^{Cip1/Waf1}$ expression was positively associated with *RASSF6* expression in clinical samples (**Fig. 4E**). These results indicate the *RASSF6*-mediated cell cycle arrest is dependent on $p21^{Cip1/Waf1}$.

***RASSF6* promotes apoptosis in renal cancer cells**

The potential role of *RASSF6* in apoptosis was studied by treating *RASSF6*-expressing or empty vector control 786-O and SKRC39 cells with cisplatin, a strong and widely used chemotherapy drug that can induce apoptosis in a wide variety of cancer cells. As measured by Annexin-V/7-AAD staining, the apoptotic index (total number of Annexin-V-positive/PI-negative and Annexin-V-positive/PI-positive cells) was similar between *RASSF6*-expressing cells and vector-transfected cells without cisplatin treatment. After cisplatin exposure, the apoptotic index was significantly increased in *RASSF6*-expressing 786-O and SKRC39 cells (**Fig. 5A**). Conversely, *RASSF6* silencing in ACHN cells resulted in a reduced amount of apoptosis upon cisplatin treatment (**Fig. 5B**). The apoptotic markers cleaved-PARP and Bax were dramatically increased in *RASSF6*-expressing cells after cisplatin treatment compared with control cells, whereas the converse results were observed in ACHN cells with *RASSF6* depletion. However, no obvious alterations in p53 or Bcl-2 levels were detected in the ccRCC cells with altered *RASSF6* expression (**Fig. 5C**).

***RASSF6* mediates $p21^{Cip1/Waf1}$-dependent cell cycle arrest and apoptosis by modulating the JNK/SAPK pathway**

To elucidate the molecular basis of the cell cycle arrest and apoptosis promoted by *RASSF6*, we analyzed the effects of *RASSF6* on Ras-MAPK signaling. Ectopically expressed *RASSF6* specifically enhanced the phosphorylation of JNK (except for SKRC39 cells, indicating that phosphorylation of C-jun was independent of JNK phosphorylation in SKRC39 cells) and c-Jun, but not that of p38 kinase or ERK. The phosphorylation of JNK and c-Jun was conversely inhibited in *RASSF6*-depleted ACHN cells.

As mentioned above, *RASSF6*-induced G_0/G_1 arrest depended on increased expression of $p21^{Cip1/Waf1}$ (**Fig. 4**), which indicated either a direct effect of *RASSF6* on $p21^{Cip1/Waf1}$ or an indirect mode of action via another unknown modulatory event. In fact, JNK activation has been reported to contribute to cell cycle arrest through $p21^{Cip1/Waf1}$.[16-18] We therefore explored whether the *RASSF6*-mediated upregulation of $p21^{Cip1/Waf1}$ proceeds via activation of the JNK pathway. *RASSF6*-expressing 786-O or SKRC39 cells were treated with SP600125, a selective inhibitor of JNK, and the efficacy of the treatment was confirmed by reduced phosphorylation of c-Jun. This treatment effectively blocked the significant increase in $p21^{Cip1/Waf1}$ expression induced by *RASSF6* (**Fig. 6B**, upper panel).

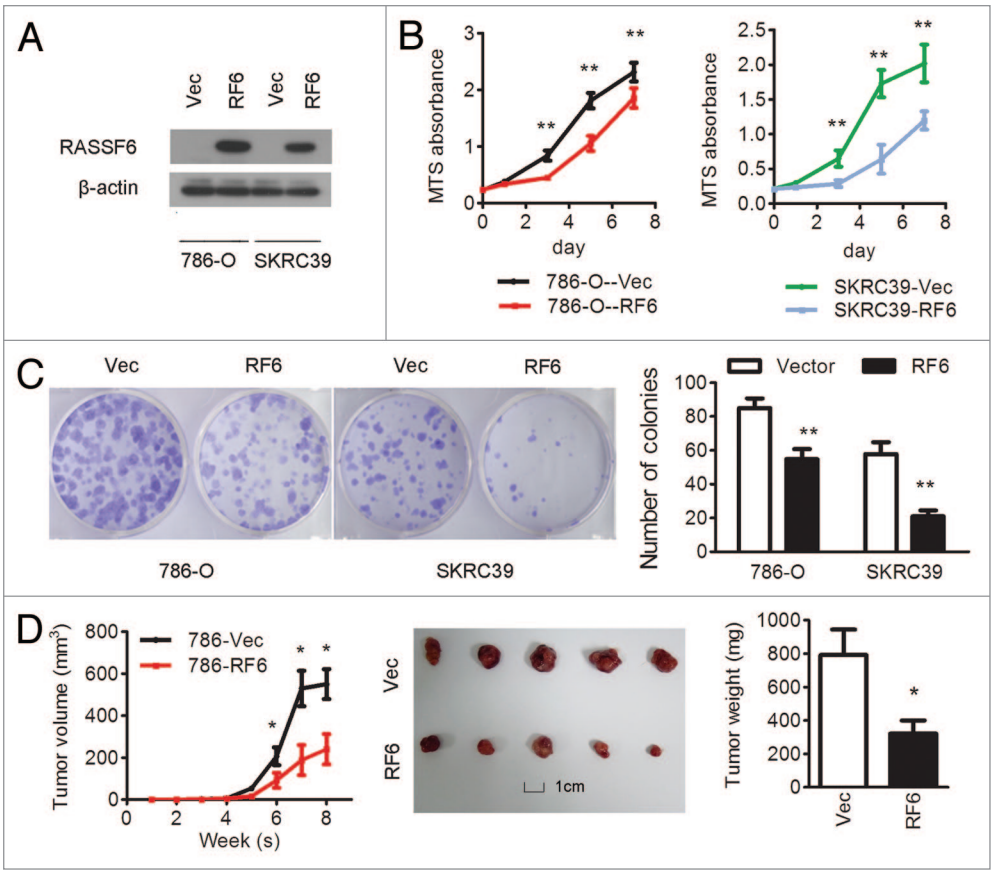

Figure 2. Overexpression of RASSF6 inhibits the proliferation of ccRCC cells in vitro and in vivo. (**A–C**) 786-O and SKRC39 cells stably overexpressing RASSF6 (RF6) or transfected with empty vector (Vec) were analyzed as follows. (**A**) RASSF6 protein expression levels were determined by western blot analysis; β-actin was used as a loading control. (**B**) Cell proliferation was determined by the MTS assay; $*P < 0.05$, $**P < 0.01$, Student *t* test. (**C**) Colony formation ability; representative micrographs (left) and quantification (right) of crystal violet-stained cells from 3 independent experiments; $*P < 0.05$, $**P < 0.01$, Student *t* test. (**D**) Control or RASSF6-overexpressing 786-O cells were inoculated subcutaneously into nude mice (n = 5/group). Tumor volumes were measured (left) and weighed (right) on the last day of the experiment. Representative images of isolated tumors (middle) are presented; $*P < 0.05$, Student *t* test; scale bar in picture: 1 cm.

Moreover, SP600125 also reduced the *RASSF6*-induced G_0/G_1 cell cycle arrest (**Fig. 6C**; **Fig. S2**), which mimicked the effect of transient silencing of p21 in *RASSF6*-expressing cells. These data support a model wherein *RASSF6* mediates $p21^{Cip1/Waf1}$-dependent G_0/G_1 accumulation through JNK signaling in ccRCC.

We next sought to clarify whether *RASSF6*-induced apoptosis depended on the JNK signaling pathway. We co-treated RASSF6-expressing and vector-transfected cells with cisplatin in the absence or presence of the specific JNK inhibitor SP600125. This experiment showed that treatment with SP600125 resulted in an inhibition of *RASSF6*-induced PARP activation (**Fig. 6B**, lower panel) and a significant increase in cell viability after cisplatin treatment compared with controls (**Fig. 6D**; **Fig. S3**). These results indicate that activation of JNK is required for the pro-apoptotic function of *RASSF6*.

Discussion

RASSF family proteins are potential mediators of the growth-inhibitory effects of Ras.[19-22] *RASSF6*, which demonstrates a different range of identity relative to other C-terminal RASSF proteins (RASSF1–5), is frequently lost in primary tumors, including breast, liver, colon, pancreas, stomach, and thyroid gland cancers. Therefore, a tumor suppressor role has been speculated for *RASSF6*.[14] Consistent with previous reports, we found, for the first time, that *RASSF6* was downregulated at both the mRNA and protein levels in ccRCC primary tumors and cell lines. CpG islands are predicted in the *RASSF6* promoter region, and studies have shown that heavy methylation of these *RASSF6* CpG islands is associated with expression silencing in leukemia cell lines[23] and neuroblastoma.[24] Based on these findings, we treated ccRCC cell lines exhibiting low levels of *RASSF6* (786-O, SKRC-39, Caki-1) with 5-Aza-dC, an inhibitor of DNA methylation. However, *RASSF6* expression was not significantly altered in the presence of 5-Aza-dC (**Fig. S4**), indicating that *RASSF6* is not epigenetically regulated in ccRCC. Allen et al.[14] also have reported nonmethylation-based mechanisms are involved in the downregulation of *RASSF6*, as 30–60% of primary tumor showed reduced levels of *RASSF6* mRNA but only 1/7 of the tumor cell lines examined demonstrated partial promoter methylation of the promoter of *RASSF6*. *RASSF6* is located at 4q13.3 in the genome, which has been reported to suffer from deletions during tumor development.[25] A high number of chromosomal imbalances and losses of 4q are involved in the progression of multiple myeloma,[26] hepatocellular carcinoma,[27] and pancreatic adenocarcinoma.[28] Thus, the association of *RASSF6* silencing with 4q loss warrants further investigation.

RASSF6 reduces cell viability in specific tumor cell lines via a poorly understood mode of action.[14,15] In the present study (the experimental design was presented in **Fig. S5**), we revealed the role of *RASSF6* in ccRCC to involve G_1/S-phase arrest as well as a pro-apoptotic effect that could be triggered by stimulation with apoptotic agents such as cisplatin. A model for the effect of *RASSF6* on cell cycle and apoptosis in ccRCC is shown in **Figure 7**. *RASSF6* inhibits the growth of both *VHL*-deficient (786-O) and *VHL* wild-type (SKRC39, ACHN)[29-31] cells, suggesting that RASSF6 may not depend on *VHL* status in ccRCC. The treatment strategy to enhance the inhibitory

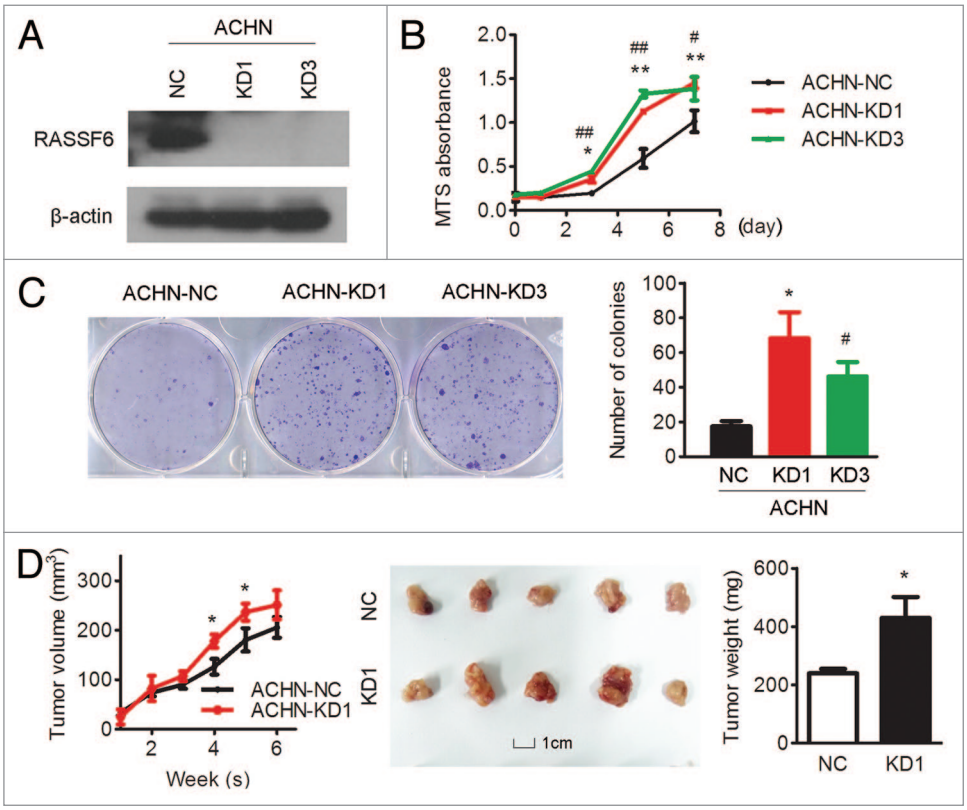

Figure 3. RASSF6 knockdown promotes cell growth in vitro and tumor growth in vivo. ACHN cells were stably transfected with one of 2 RASSF6 shRNAs (KD1, KD3) or negative control shRNA (NC). (**A**) Western blotting analysis of RASSF6 expression; β-actin was used as a loading control. (**B**) RASSF6 depletion enhanced ACHN cell proliferation compared with control cells as determined by an MTS assay; *$P < 0.05$, **$P < 0.01$ for KD1 cells compared with NC cells; #$P < 0.05$, ##$P < 0.01$ for KD3 cells compared with NC cells. (**C**) The colony-formation ability of RASSF6-depleted ACHN cells was increased compared with vector control cells; *$P < 0.05$ for KD1 cells compared with NC cells; #$P < 0.05$ for KD3 cells compared with NC cells. (**D**) ACHN cells transfected with RASSF6 shRNA (KD1) or empty vector (NC) were inoculated subcutaneously into nude mice (n = 5/group). Tumor volumes were measured (left) and weighed (right) on the last day of the experiment. Representative images of isolated tumors (middle) are presented; *$P < 0.05$, Student *t* test; scale bar: 1 cm.

function of *RASSF6* is promising in ccRCC, especially after treatment failure of targeting *VHL*.

Cell cycle regulation is a common characteristic of the *RASSF* family,[32-37] and *RASSF1* and *RASSF5* cause G_1/S arrest through p21, a regulator of cell cycle progression at G_1 and S phase.[38,39] We observed that reintroduction of *RASSF6* stimulated p21 protein expression, whereas inhibition of RASSF6 reduced p21 protein levels. This pattern was consistent with arrest in G_0/G_1 phase,

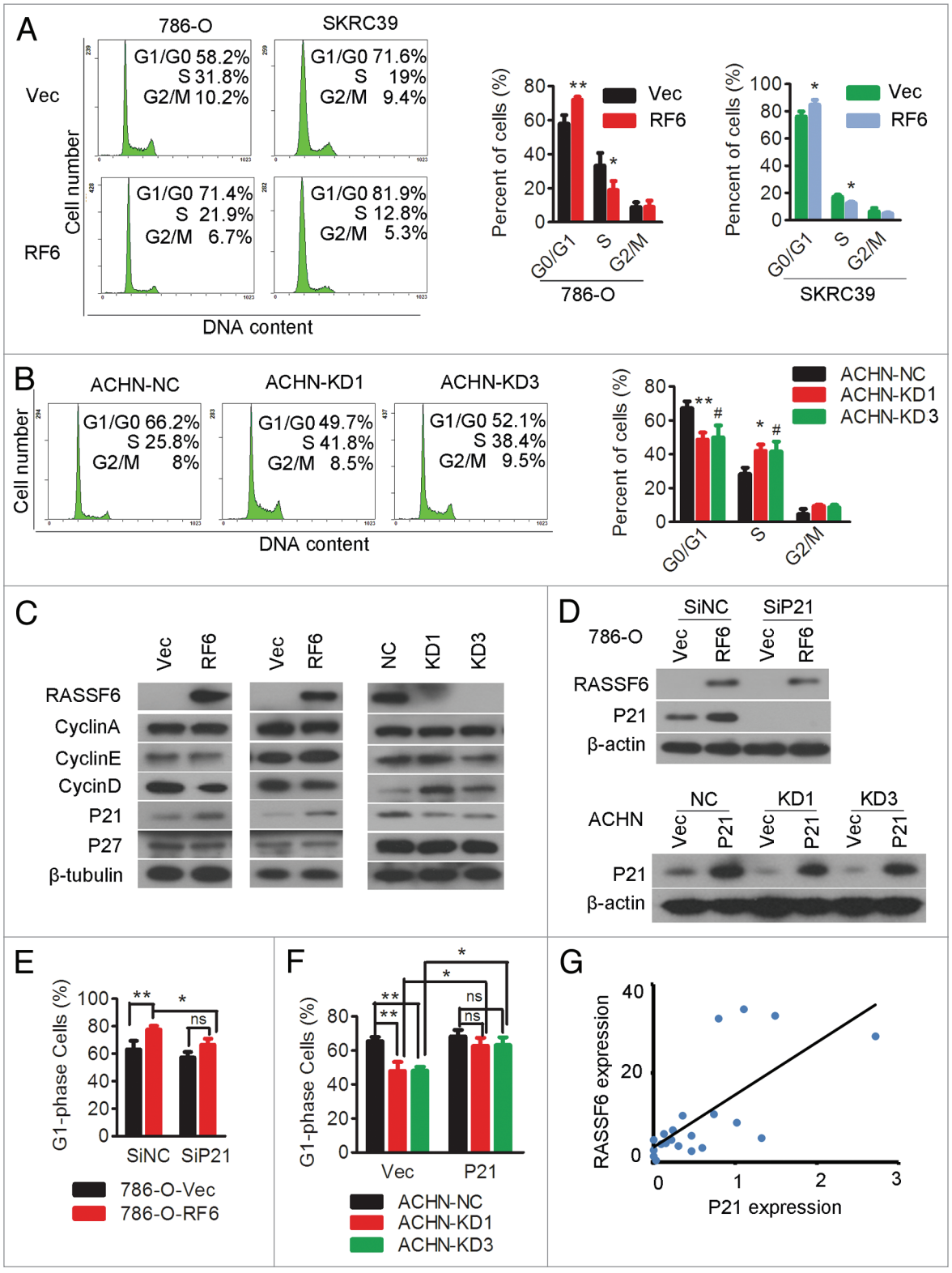

Figure 4. For figure legend, see page 1445.

Figure 4 (See previous page). RASSF6 promotes p21-dependent arrest at the G_1/S-phase transition. (**A**) 786-O or SKRC39 cells stably overexpressing RASSF6 (RF6) or transfected with empty vector (Vec) were subjected to cell cycle analysis by flow cytometry. Images and qualification of the cell cycle distribution in 3 experiments are shown; *$P < 0.05$, **$P < 0.01$, Student *t* test. (**B**) ACHN cells stably transfected with one of 2 RASSF6 shRNAs (KD1, KD3) or with negative control shRNA (NC) were subjected to cell cycle distribution analysis by flow cytometry; *$P < 0.05$, **$P < 0.01$ for KD1 cells compared with NC cells; #$P < 0.05$ for KD3 cells compared with NC cells. (**C**) Western blotting analysis of cyclin A, cyclin D, cyclin E, and their inhibitors p21 and p27 in the indicated cell lines. β-tubulin was used as a loading control. (**D and E**) RASSF6-expressing and control 786-O cells were transiently transfected with p21 siRNA (Sip21) or scrambled control siRNA (SiNC). p21 expression levels were evaluated by western blot analysis (**D,** upper panel), and cells were subjected to cell cycle analysis (**E**); *$P < 0.05$, **$P < 0.01$, ns, not significant. (**D and F**) ACHN cells stably transfected with RASSF6 shRNA (KD1, KD3) or negative control shRNA (NC) were transiently transfected with a p21 plasmid (p21) or empty vector (Vec). p21 expression levels were evaluated by western blot analysis (**D**, lower panel), and cells were subjected to cell cycle analysis (**F**); *$P < 0.05$, **$P < 0.01$, ns, not significant. (**G**) *RASSF6* mRNA levels were positively correlated with p21 expression in ccRCC tissues as determined by qPCR (*GAPDH* and *18S* were used as reference genes. Pearson analysis, $r = 0.712$; $P < 0.001$).

which can be mediated by the CDK inhibitor $p21^{Cip1/Waf1}$. The fact that transient silencing of $p21^{Cip1/Waf1}$ blocked the G_1 arrest caused by *RASSF6*, and that overexpression of $p21^{Cip1/Waf1}$ restored the G_1 phase further confirmed that RASSF6-mediated cell cycle arrest is closely associated with the activation of $p21^{Cip1/Waf1}$.

RASSF family proteins, including *RASSF6*, have also been shown to participate in pro-apoptotic programs.[14,20,22,40,41] For example, *RASSF1A* mediates some of its apoptotic functions by directly binding the pro-apoptotic Bax activator MOAP-1. *RASSF6* was also previously reported to activate Bax and interact with MOAP-1, although MOAP-1 knockdown partially suppressed RASSF6-induced Bax activation,[15,42] suggesting that other mechanisms may regulate the apoptosis mediated by *RASSF6*.[43-45]

p53 has also been reported to be involved in the cell cycle regulation and apoptosis mediated by *RASSF* proteins.[37,38,46] In the latest published finding, Iwasa showed that *RASSF6* regulates apoptosis and the cell cycle via stabilization of p53.[47] Interestingly, in our study, we did not observe any changes in p53 expression related to *RASSF6* in ccRCC, and we therefore suspect that *RASSF6*-induced cell cycle arrest and apoptosis in ccRCC is not dependent on p53 levels.

Loss or altered expression of *RASSFs* has also been associated with altered activity of the Ras-MAPK signaling axis.[39,48,49] Like other *RASSFs*, *RASSF6* preferentially associates with activated K-Ras,[14] and Ras transduces extracellular information through a multitude of signaling cascades, primarily the ERK, JNK/SAPK, and p38 pathways. *RASSF1A* mediates cell cycle arrest by modulating the Raf-MEK-ERK pathway[39] or blocking the JNK pathway,[50] and *RASSF1C* participates in DNA damage through activation of JNK/SAPK.[49] In our study, we found that *RASSF6* overexpression resulted in activation of JNK signaling, but not ERK or p38 signaling. Therefore, the cell cycle arrest and apoptosis promotion induced by *RASSF6* most likely resulted from activation of the JNK pathway. Moreover, inhibition of the JNK pathway in *RASSF6*-expressing cells decreased *RASSF6*-mediated upregulation of p21 and reduced G_1 arrest, mimicking the effect of p21 silencing on cell cycle regulation. The relationship between JNK activation and $p21^{Cip1/Waf1}$ in the cell cycle was also reported by Densham and colleagues, who found that JNK activation contributed to cell cycle arrest through the stabilization of $p21^{Cip1/Waf1}$ via phosphorylation of Thr57.[16,17] All of these results support a model wherein *RASSF6*-mediated upregulation of $p21^{Cip1/Waf1}$ in ccRCC occurs through enhancement of JNK signaling. We confirmed that the JNK pathway was required for *RASSF6*-mediated apoptosis in ccRCC by showing that JNK inhibition partially abolished the apoptosis induced by *RASSF6*. Based on these findings, we conclude that the *RASSF6*-mediated pro-apoptosis effect is partially dependent on the JNK/SAPK pathway.

Taken together, our findings demonstrate that *RASSF6* is a novel tumor suppressor that plays an important role in the pathogenesis of ccRCC. In particular, *RASSF6* activates JNK signaling to promote $p21^{Cip1/Waf1}$-dependent inhibition of S-phase progression and apoptosis upon exposure to apoptotic agents. Thus, our findings reveal a central role for the *RASSF6* tumor suppressor in the inhibition of survival signals in ccRCC.

Materials and Methods

Cell line selection, cell culture, cellular growth curve, and colony-formation assays

To rule out the effect of *VHL*, we chose *VHL*-deficient cells lines (786-O), as well as wide-type *VHL* cell lines (SKRC39, ACHN). All of 3 cell lines are well-established lines and suitable for transfection. Moreover, their abilities of tumor formation in nude mice have been repeatedly reported.[29-31]

The ccRCC cell lines 786-O, SKRC39, and ACHN were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C and 5% CO_2. To plot the cellular growth curve, 3×10^3 cells suspended in 200 μl of medium were seeded into a 96-well plate (Corning) and cultured under normal conditions. At various time points after seeding, the cells in each well were stained with MTS (Promega, G5421), and the OD490 was determined with a microplate reader. For the colony-formation assays, cells were counted and plated at 500 cells per well in a 6-well plate (Corning). After 2 wk, the cells were washed with phosphate-buffered saline (PBS), fixed with methanol for 15 min at room temperature, and stained with crystal violet for 30 min. The colony was counted when it had more than 50 cells.

Paired tumor and tumor-adjacent tissues

Pairs of ccRCC tissues and matched tumor-adjacent morphologically normal tissues were obtained from Sun Yat-Sen University Cancer Center and frozen and stored in liquid nitrogen until used for comparing RASSF6 mRNA and protein expression levels.

Real-time quantitative PCR (qPCR)

Total RNA was extracted from cultured cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions

and then reverse-transcribed using a cDNA Synthesis Kit (Takara, 6111A). Real-time qPCR was performed using a SYBR Green PCR Kit (Bio-Rad, 172–5200). The expression of each target gene was normalized to the endogenous levels of GAPDH or 18S. The relative mRNA levels are shown as 2ΔCt values. The sequences of the PCR primers used for amplification were as follows: GAPDH forward, 5′-AAGGTCATCC CTGAGCTGAA-3′; GAPDH reverse, 5′- TGACAAAGTG GTCGTTGAGG-3′; 18S forward, 5′-CATGGCCGTT CTTAGTTGGT-3′; 18S reverse, 5′-CGCTGAGCCA GTCAGTGTAG-3′; RASSF6 forward, 5′-GGGGGAATTT GACGATCTCT-3′; RASSF6 reverse, 5′-TAGAGCACTG GGGAGTCTGG -3′; p21 forward, 5′-GGATGTCCGT CAGAACCCAT-3′; p21 reverse, 5′-GTGGGAAGGT AGAGCTTGGG-5′.

Plasmid transfection experiment

Plasmid construction and transfection were performed as previously described.[51] Briefly, full-length human p21 cDNA were cloned into pcDNA3.1. Growing ccRCC cells seeded at 2 × 10^5 cells per well of a 6-well tissue culture dish were transfected with 2 μg plasmid DNA or the corresponding empty control vector plasmid using Lipofectamine 2000 (Invitrogen, 11668–027) according to the manufacturer's instructions.

RNAi treatment

The sequence of the small-interfering RNA (siRNA) targeting p21 has been reported[52] and was synthesized by GenePharma. Growing cells were seeded at 2 × 10^5 cells per well in a 6-well tissue culture dish, and siRNAs were added 24 h later at a concentration of 80 nM using the RNAiMAX reagent (Invitrogen, 13778-075). The transfected cells were incubated for 6 h and then supplied with fresh medium containing serum.

Lentivirus transduction studies

A RASSF6 expression construct was generated by subcloning PCR-amplified full-length human RASSF6 cDNA into the pCDH-CMV-MCS-EF1-RFP plasmid. Cells stably expressing either RASSF6 short hairpin RNA (shRNAs) or a scrambled non-target shRNA were established using the LV3 plasmid

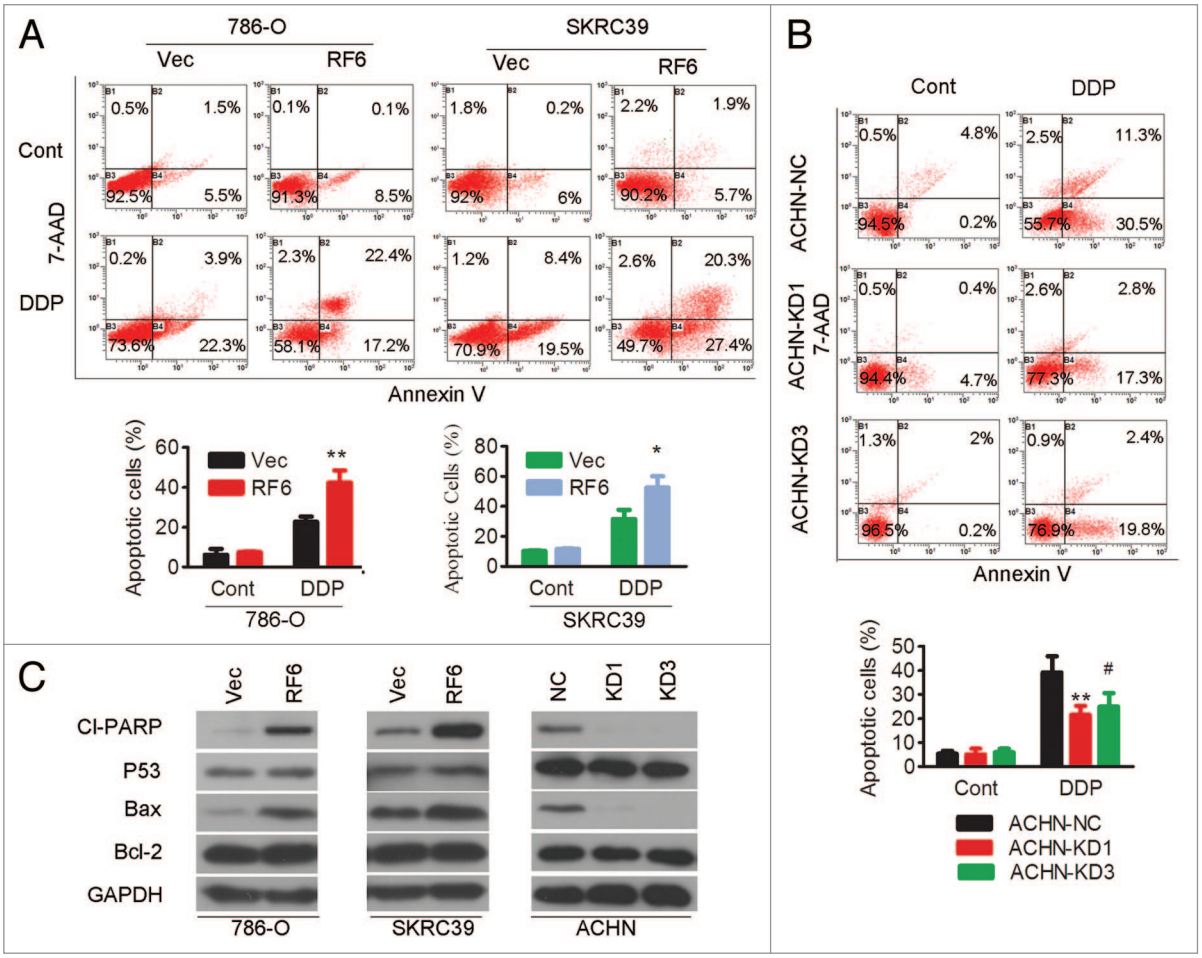

Figure 5. RASSF6 has a pro-apoptotic role in ccRCC cells. (**A**) 786-O and SKRC39 cells stably overexpressing RASSF6 (RF6) or transfected with empty vector (Vec) were treated with or without cisplatin (6 μM for 786-O cells and 10 μM for SKRC39 cells) and then subjected to apoptosis analysis by Annexin-V and 7-AAD double staining. The error bars represent the mean ± SD from 3 independent experiments; *P < 0.05, **P < 0.01. (**B**) ACHN cells stably transfected with RASSF6 shRNA (KD1, KD3) or negative control shRNA (NC) were treated with or without cisplatin and then subjected to apoptosis analysis by Annexin-V and 7-AAD double staining. The error bars represent the mean ± SD from 3 independent experiments; **P < 0.01 for KD1 cells compared with NC cells; #P < 0.05 for KD3 cells compared with NC cells. (**C**) Western blot analysis of cleaved PARP, p53, Bax, and Bcl-2 in the indicated cells; GAPDH was used as a loading control.

according to the manufacturer's instructions. The targets of RASSF6 shRNA-1 and shRNA-3 were 5′- GAACAAAGAC GACTAAAGA-3′ and 5′- GGAATTTGAC GATCTCTAT-3′, respectively. Retroviral production and infection were performed as previously described.[53] Stable cell lines expressing the shRNAs were selected with 5 μg/ml puromycin for 7 d.

Cell cycle analysis

Cells were harvested by trypsinization, washed in ice-cold PBS, and fixed in 75% ice-cold ethanol at –20 °C for at least 2 h. Before staining, cells were spun down and resuspended in PBS. RNase (Sigma, R3629) was added at a final concentration of 2 mg/ml, and the cells were incubated at 37 °C for 15 min, followed by incubation in 15 mg/ml propidium iodide (PI, Sigma, P4170) for 15 min at room temperature. At least 10 000 cells per sample were collected and analyzed on a flow cytometer (Beckman Coulter, Cytomics FC 500).

Apoptosis assays

After treating cells with or without cisplatin, both floating and adherent cells were harvested, washed with PBS, and stained with Annexin-V-Phycoerythrin/FITC (BD Biosciences, 559763/556547) and 7-AAD (BD Biosciences). The cells then were subjected to flow cytometry analysis.

Immunoblotting

Western blotting was performed according to standard methods as previously described.[54] The primary antibodies used included RASSF6 (Sigma, HPA037711), β-tubulin (Sigma, T3952), p53 (Santa Cruz, sc-126), and Bax (Proteintech, 50599-2), as well as a number of antibodies from Cell Signaling Technology, including human cleaved PARP (6525), caspase 3 (9662), SAPK/JNK (9258), phospho-SAPK/JNK (Thr183/Tyr185, 4668), c-Jun (60A8), phospho-c-Jun (Ser63, 9261), phospho-c-Jun (Ser73, 3270), ERK (4780), phospho-ERK1/2 (5726), p38 (8690), phospho-p38 (Thr180/Tyr182, 4511), β-actin (4970),

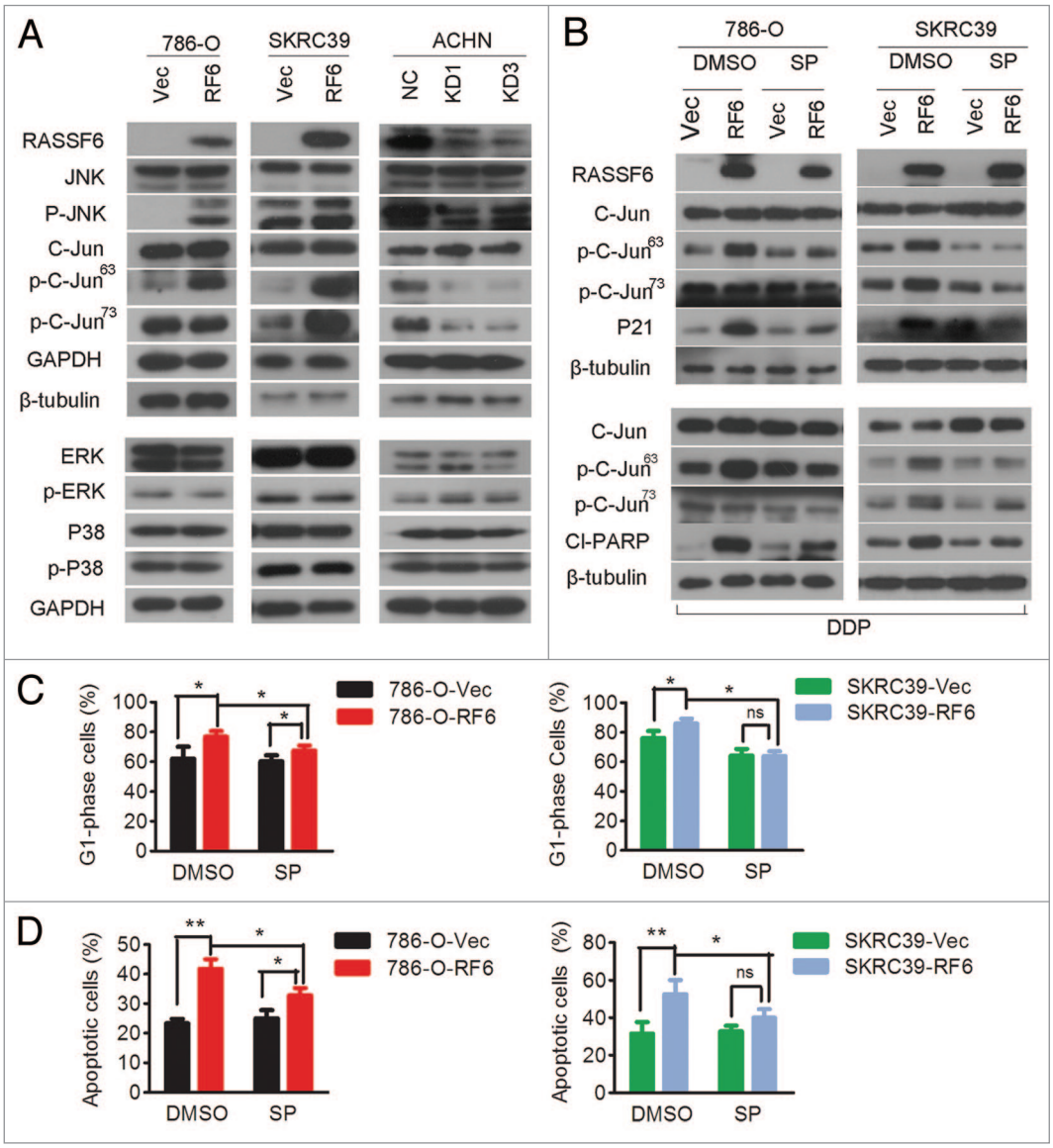

Figure 6. RASSF6 inhibits the G_1/S phase transition and promotes apoptosis though the JNK/SAPK pathway. (**A**) 786-O or SKRC39 cells stably overexpressing RASSF6 (RF6) or transfected with empty vector (Vec) and ACHN cells stably transfected with RASSF6 shRNAs (KD1, KD3) or negative control shRNA (NC) were analyzed for protein expression of phosphorylated JNK, total JNK, phosphorylated c-Jun (ser63), phosphorylated c-Jun (ser73), total c-Jun, phosphorylated ERK, total ERK, phosphorylated p38, and total p38. GAPDH or β-tubulin was used as the loading control. (**B**) RASSF6-expressing and control 786-O or SKRC39 cells were cultured in normal conditions (upper panel) or exposed to cisplatin treatment (lower panel) with or without SP600125 (5 μM for 786-O cells and 8 μM for SKRC39 cells). Levels of phosphorylated c-Jun, total c-Jun, p21, and cleaved-PARP (Cl^-PARP) were measured by western blot. (**C**) RASSF6-expressing and control 786-O or SKRC39 cells were incubated in the presence or absence of SP600125, and their cell cycle distribution was assessed by flow cytometry; $^*P < 0.05$; ns, not significant. (**D**) RASSF6-expressing and control 786-O or SKRC39 cells were incubated in the presence or absence of SP600125 (8 μM). Cisplatin was added as indicated, and the cells were incubated for an additional 48 h. Induction of apoptosis was determined by flow cytometry. The levels of apoptosis are presented as the mean ± SD from 3 independent experiments; $^*P < 0.05$, $^{**}P < 0.01$; ns, not significant.

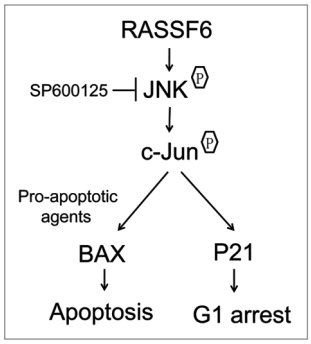

Figure 7. A model for the effect of *RASSF6* on cell cycle and apoptosis in ccRCC. When *RASSF6* is restored in ccRCC cells, *RASSF6* activated JNK/SAPK pathway. Activated JNK/SAPK pathway then promotes apoptosis when exposed to pre-apoptotic agents and regulates p21 accumulation that leads to G_1 arrest.

and GAPDH (2118). Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Promega.

Animal experiments

All animal experiments were approved by the Sun Yat-Sen University Cancer Center Institutional Animal Care and Usage Committee. Mice were housed under standard conditions and cared for according to institutional guidelines. BALB/c (nu/nu) nude mice (4 wk of age, 15–18 g) were randomly divided into groups of 5 mice each. Cells (1×10^6 for 786 cells with stable RASSF6 overexpression or transfected with an empty vector; 8×10^6 for ACHN cells transfected with RASSF6 shRNA (KD1) or negative control shRNA (NC) were suspended in 150 μl RPMI 1640 medium and injected subcutaneously into the flank of each mouse. Tumor diameters were measured, and the volume (length × width^2 × 0.5236) was calculated every week. Finally, the mice were euthanized, and the primary tumors were isolated and weighed.

Statistical analyses

All statistical analyses were performed using the SPSS 17.0 statistical software package. Data from 3 independent experiments are presented as the mean values with standard deviations. The differences between groups were evaluated using two-tailed Student *t* tests. The correlation between RASSF6 and $p21^{Cip1/Waf1}$ levels in tissues was evaluated using 2-tailed Pearson's correlation analysis. *P* values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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