



Published in final edited form as:

Nat Commun. ; 5: 3729. doi:10.1038/ncomms4729.

Oncogenic Ras induces inflammatory cytokine production by up-regulating the squamous cell carcinoma antigens SerpinB3/B4

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Abstract

Mounting evidence indicates that oncogenic Ras can modulate cell autonomous inflammatory cytokine production, although the underlying mechanism remains unclear. Here we show that squamous cell carcinoma antigens 1 and 2 (SCCA1/2), members of the Serpin family of serine/cysteine protease inhibitors, are transcriptionally up-regulated by oncogenic Ras via MAPK and the ETS family transcription factor PEA3. Increased SCCA expression leads to inhibition of protein turnover, unfolded protein response, activation of NF- κ B, and is essential for Ras-mediated cytokine production and tumor growth. Analysis of human colorectal and pancreatic tumor samples reveals a positive correlation between Ras mutation, enhanced SCCA expression, and IL-6 expression. These results indicate that SCCA is a Ras-responsive factor that has a role in Ras-associated cytokine production and tumorigenesis.

INTRODUCTION

Constitutively active mutants of Ras are found in a large number of human cancers^{1, 2}. Among the oncogenic activities of mutant Ras, its ability to alter the tumor microenvironment has been well appreciated with numerous studies implicating Ras in

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J.M.C., N.S., J.A.P., Y.S., and C.S. performed experiments. J.L., R.S.P., and H.C.C. aided with data analysis. J.M.C. and W.X.Z. wrote the paper.

Competing financial interests: The authors declare no competing financial interests.

various non-cell autonomous processes including basement membrane degradation, immune cell infiltration, and angiogenesis^{3, 4}. This tumor microenvironment altering function of Ras is at least partially caused by the ability of Ras to induce the production and secretion of proinflammatory and protumorigenic cytokines. As cellular senescence has been largely attributed to the cytokine secretory response termed senescence-associated secretion phenotype (SASP)^{5, 6}, the possibility remains that oncogene-induced secretory profile is a senescence-independent process upon Ras activation. Several reports have implicated Ras in the ability to modulate the tumor microenvironment at least due in part to Ras' ability to promote cytokine and chemokine production (i.e. IL-6, IL-8, GM-CSF) in a cell autonomous manner^{3, 4, 7, 8}. Therefore, it remains to be determined whether premature senescence is a prerequisite of oncogene-induced secretory phenotype, and how Ras mechanistically regulates the expression of the pro-inflammatory cytokines.

Squamous cell carcinoma antigens (SCCAs) are members of the serpin family of endogenous protease inhibitors. Approximately 98% and 92% homologous at their nucleotide and amino acid levels, respectively, SCCA1 (SerpinB3) and SCCA2 (SerpinB4) are "suicide-substrate" protease inhibitors with differing substrate specificities due to amino acid differences within their reactive site loop (RSL) domain⁹. Up-regulated in numerous cancers (cervical, lung, head and neck, liver, and breast)¹⁰⁻¹³, both SCCA1 and SCCA2 are thought to promote cell survival through the inhibition of cell death^{14, 15}. Moreover, the level of SCCA has been shown to predict pathological grade, disease stage, recurrence, and response to both radiotherapy and chemotherapy¹⁶⁻¹⁸. Despite SCCA's well-reported involvement in cancer, how SCCA is transcriptionally up-regulated during transformation and contributes to tumor development remains largely unknown. Here, we study the oncogenic regulation of SCCA and uncover a novel proinflammatory role for SCCA downstream of mutant Ras. We demonstrate that through MAPK signaling and the ETS transcription factor PEA3, oncogenic Ras up-regulates the expression of SCCA1/2 (SerpinB3/B4) to promote inflammatory cytokine production and xenograft tumor growth. Moreover, SCCA up-regulation is observed in human colorectal and pancreatic cancer. Our findings suggest an important role of serpins in Ras-driven tumorigenesis.

RESULTS

Oncogenic Ras up-regulates SCCA expression

Elevated expression of SCCA has been found in numerous human cancers. However, the underlying molecular mechanism of its up-regulation remains unclear. We began to study this by expressing several oncoproteins (HRas^{V12}, myristolated-Akt (myr-Akt), and c-Myc) in IMR90 primary human lung fibroblasts. While myr-Akt and c-Myc failed to induce SCCA expression, HRas^{V12} led to a marked increase in SCCA protein levels (Fig. 1a). The SCCA antibody utilized was unable to distinguish between the SCCA isoforms¹⁰, though quantitative reverse-transcription PCR (qRT-PCR) analysis revealed an increase in the transcript levels of both SCCA1 and SCCA2 in response to Ras^{V12} expression (Fig. 1b). This is not surprising as the two SCCA isoforms are tandemly arranged on human chromosome 18q and their promoters are highly homologous¹⁹.

To determine whether persistent Ras signaling is required for SCCA expression, we used the estrogen receptor (ER):Ras^{V12} fusion protein that allows for Ras^{V12} stabilization upon the addition of 4-hydroxytamoxifen (4-OHT) to the culture medium²⁰. While 4-OHT induced the expression of Ras and SCCA (Fig. 1c), removal of 4-OHT led to a drastic reduction in Ras^{V12} protein levels and diminished downstream signaling indicated by decreased phospho-ERK, accompanied by a reduction of SCCA at both protein and transcript levels (Fig. 1c, d), indicating that sustained Ras signaling is required for maintaining SCCA expression.

In addition to HRas^{V12}, expression of KRas^{V12} or NRas^{Q61} also resulted in elevated SCCA expression in IMR90 cells (Fig. 1e). Similar to IMR90 cells, increased SCCA expression was observed by expressing HRas^{V12} in the BJ primary human foreskin fibroblast cells, and by expression of KRas^{V12} in a number of cancer cell lines with wild-type Ras, including the colon cancer cell lines HT-29 and Caco-2, and HeLa cells (Fig. 1f). Taken together, these results indicate that up-regulation of SCCA expression is a general feature of oncogenic Ras proteins in various human cell lines.

It is important to note that in IMR90 and BJ fibroblasts expression of Ras^{V12} elicits oncogene-induced senescence²¹. The up-regulation of SCCA in response to Ras^{V12} in Caco-2, HT-29, and HeLa cells, which do not undergo senescence^{3, 22}, suggests that up-regulation of SCCA by Ras^{V12} is independent of cellular senescence. To further elucidate that the increased SCCA expression is not a consequence of cellular senescence, we induced premature senescence by treating IMR90 cells with etoposide or H₂O₂, and replicative senescence by continually passaging the cells. Indeed, unlike Ras^{V12}, these senescence-inducing conditions failed to up-regulate SCCA expression (Fig. 2). These results indicate that expression of oncogenic Ras stimulates expression of SCCA in a manner that is independent of oncogene-induced senescence.

Ras-induced SCCA is dependent on the MAPK/PEA3 pathway

To elucidate the mechanism by which Ras^{V12} stimulates SCCA expression, we used pharmacological inhibitors to selectively inhibit MAPK and Akt pathways that are well-characterized to be activated by Ras^{2, 23}. While inhibition of Akt signaling had little to no effect on SCCA expression (Fig. 3a, b), the MEK inhibitor U0126 caused a marked inhibition of both SCCA1 and SCCA2 transcription in both IMR90 (Fig. 3c, d) and HeLa cells (Supplementary Fig. 1a, b). Similarly, expression of the dominant-negative ERK2 blocked Ras-induced SCCA expression (Supplementary Fig. 1c, d). Along the Ras/Raf/MAPK/ERK pathway, expression of oncogenic BRAF^{V600E} also induced SCCA expression (Supplementary Fig. 1e). We next sought to identify what transcription factor may be mediating Ras-induced expression of SCCA. The ETS transcription factor family member PEA3 has been shown to be modulated by MAPK via sumoylation²⁴ and can activate SCCA transcription²⁵. Indeed, silencing of PEA3 in Ras^{V12}-expressing cells (Fig. 3e) resulted in a drastic decrease of both SCCA1 and SCCA2 at protein and transcript levels (Fig. 3f, g), without affecting MAPK signaling indicated by similar levels of phospho-ERK in Ras^{V12}-expressing cells (Fig. 3f). This PEA3-dependent SCCA expression was also observed in Ras^{V12}-expressing HeLa cells (Supplementary Fig. 2a-c) and was rescued by expression of

wild-type murine PEA3, but not by a PEA3 sumoylation-defective mutant²⁴ (Supplementary Fig. 2d-f). These results indicate that mutant Ras leads to SCCA up-regulation via the MAPK/PEA3 pathway.

SCCA is essential for Ras-induced cytokine production

Next we examined the biological role of elevated SCCA expression. We chose to focus on using the IMR90 cell line that is widely used for studying the effect of Ras activation during the early phase of tumorigenesis. We began by examining the downstream signaling pathways activated by oncogenic Ras (MAPK, Akt, NF- κ B). To this end, SCCA expression was silenced in Ras^{V12}-expressing cells using two independent shRNA constructs that effectively silenced both SCCA1 and SCCA2 (Fig. 4a), and the signaling pathways examined by respective phosphorylation antibodies. While loss of SCCA had little to no effect on the ability of mutant Ras to activate Akt and MAPK phosphorylation (Fig. 4b), loss of SCCA significantly diminished Ras^{V12}-induced RelA/p65 phosphorylation that is indicative of NF- κ B activation (Fig. 4b). This effect was further confirmed by utilizing a NF- κ B luciferase reporter construct (Fig. 4c).

Activation of NF- κ B signaling has been attributed to cytokine production downstream of Ras activation²⁶. Indeed, inhibition of NF- κ B signaling using the pharmacological inhibitor BAY11-7082 abrogated Ras-induced cytokine production in IMR90 and HeLa cells (Supplementary Fig. 3). Therefore, we examined whether SCCA plays a role in Ras-induced cytokine production and performed a quantitative cytokine array analysis using cell culture media. In agreement with literature^{5, 6}, Ras^{V12}-expressing cells displayed a marked increase in a spectrum of cytokines including IL-6, IL-8, CXCL1, G-CSF, and GM-CSF (Fig. 4d). Consistent with the observed decrease in NF- κ B signaling (Fig. 4b, c), this increased cytokine expression was significantly abrogated upon SCCA silencing (Fig. 4d, e). Quantitative RT-PCR analysis revealed that the suppression of cytokine production was due to transcriptional down-regulation (Fig. 4f) and was rescued by ectopically activating NF- κ B signaling with TNF α treatment (Fig. 4g). The SCCA-dependent NF- κ B activation and cytokine production downstream of oncogenic Ras were also observed in HeLa and Caco-2 cells (Supplementary Fig. 4). The introduction of oncogenic Ras in IMR90 elicits a DNA damage response that has been reported to mediate cytokine production^{6, 27}. However, silencing of SCCA had virtually no effect on Ras-induced DNA damage indicated by the phosphorylation of H2A.X (Fig. 5a, b) or the senescence-associated cell cycle arrest (Fig. 5c). Taken together, these results indicate that SCCA plays an essential role in Ras-mediated NF- κ B activation and inflammatory cytokine production that is independent of the DNA damage response.

SCCA promotes cytokine production by inducing the UPR

We next sought to understand how SCCA promotes Ras-mediated NF- κ B activation and cytokine production. A possible mechanism is through up-regulation of the unfolded protein response (UPR), which has been implicated in mediating NF- κ B activation and inflammatory transcriptional programs²⁸. Our previous work showed that SCCA leads to decreased lysosomal and proteasomal protein turnover, two major protein degradation pathways, hence increases the steady-state level of ER stress²⁹. Thus, we speculated that

mutant Ras induces ER stress through up-regulation of SCCA. Indeed, the ability of Ras^{V12} to promote an ER stress response has been reported³⁰. Silencing of SCCA relieved the Ras-induced inhibition of protein turnover as measured through total ubiquitinated proteins (Fig. 6a). Furthermore, while Ras^{V12} cells showed a robust increase in UPR indicated by the appearance of proteolytically cleaved ATF6 as well as both unspliced and spliced XBP1, this response was significantly reduced in SCCA silenced cells (Fig. 6b), indicating that SCCA mediates the UPR induced by Ras^{V12}. Silencing of ATF6 or XBP1 in Ras^{V12}-expressing cells (Fig. 6c) drastically diminished Ras^{V12}-induced cytokine production (Fig. 6d-f; Supplementary Fig. 5) that was accompanied by decreased RelA/p65 phosphorylation (Fig. 6g; Supplementary Fig. 5). Conversely, ectopic expression of the transcriptionally active spliced variant of XBP1 (XBP1s) restored cytokine production in Ras^{V12} cells when SCCA was silenced (Fig. 6h, i). These results indicate that SCCA modulates cytokine production downstream of mutant Ras by stimulating UPR.

SCCA is up-regulated in colorectal and pancreatic cancers

Our results thus far show that SCCA is a Ras-responsive gene that plays an essential role in Ras-induced cytokine production. We next sought to identify an *in vivo* connection between Ras and SCCA expression. Using TCGA (The Cancer Genome Atlas) data of human colorectal cancer, which was available with sizable samples with KRas mutation, we identified a positive correlation between the presence of mutant Ras and SCCA up-regulation (Fig. 7a). We also chose to examine pancreatic ductal adenocarcinoma (PDAC), where mutant KRas functions as the primary driver³¹. It also offers a unique model where the precursor lesions to PDAC have a defined pathological progression³¹. To this end, we analyzed the pancreatic cancer gene expression datasets for SCCA expression through Oncomine. In the 7 available patient datasets, SCCA1 mRNA was up-regulated in 6 and SCCA2 mRNA in 3 independent datasets³²⁻³⁷ (Fig. 7b-d). Of note, the Logsdon dataset³⁷ identified both SCCA1 and SCCA2 to be up-regulated in pancreatic cancer when compared to chronic pancreatitis (Fig. 7d). To further examine the association of SCCA expression in human pancreatic cancer, we performed IHC for SCCA using a pancreatic cancer tissue microarray. While all non-neoplastic/normal pancreatic samples were negative for SCCA expression, positive staining for SCCA was seen throughout all stages of pancreatic cancer progression. The incidence of SCCA positivity increased in relation to the progression of pancreatic cancer: 2 out of 17 (11.8%) PanIN-1 lesions, 5 out of 19 (26.3%) PanIN-2 lesions, 8 out of 15 (53.3%) PanIN-3 lesions, and 20 out of 30 (66.7%) of PDAC (Fig. 7e; $p < 0.001$). Moreover, consistent with the result that SCCA promotes inflammatory cytokine production in cultured cells, IHC of IL-6 on the same panel of pancreatic tissues showed a positive correlation between IL-6 and SCCA in both PDAC (Fig. 7f, g) and PanIN (Supplementary Fig. 6) samples.

Endogenous SCCA promotes IL-6 production and tumorigenesis

To further explore the physiological relevance of SCCA, we tested the expression and function of SCCA in a setting when mutant Ras is expressed at endogenous levels. Ras^{V12} failed to induce the expression of the SCCA paralogs Serpinb3a or Serpinb3b in immortalized MEF NIH3T3 (Supplementary Fig. 7a) or in primary MEFs (Supplementary Fig. 7b). Since we were unable to detect up-regulation of SCCA paralogs in the murine

system, we instead examined a panel of human pancreatic cancer cell lines that harbor mutant KRas. We found 5 out of 9 cell lines to be positive for SCCA expression (Fig. 8a). In the cell lines that displayed detectable levels of SCCA and can tolerate loss of KRas (CFPAC-1, L3.6, and Capan-1), silencing of mutant KRas significantly reduced SCCA expression (Fig. 8b). Consistent with the essential role of SCCA in Ras-mediated cytokine production, silencing of SCCA in three SCCA-positive cell lines resulted in decreased expression of IL-6, whereas the SCCA shRNAs did not affect IL-6 expression in the SCCA-low PANC-1 cells (Fig. 8c). Moreover, while silencing of SCCA in CFPAC-1, L3.6, and HPAF-II cells did not compromise cell proliferation in cell culture (Supplementary Fig. 8), it led to impaired xenograft tumor growth in all three cell lines (Fig. 8d). These results, taken together with the above finding that SCCA positivity correlates with progression of pancreatic cancer development (Fig. 7e), indicate that SCCA expression is under the control of mutant Ras expressed at endogenous level, and that the SCCA-mediated inflammatory response plays a pro-tumorigenic role in Ras-driven cancer.

DISCUSSION

In this study, we show that the endogenous serine/cysteine proteinase inhibitor SCCA is up-regulated upon activation of oncogenic Ras, in a manner that is dependent on hyper-active MAPK signaling and the ETS transcription factor PEA3. Downstream of oncogenic Ras, SCCA promotes activation of NF- κ B signaling and cytokine production by blocking protein turnover and inducing the UPR.

Our study implicates SCCA as a direct link between oncogenic Ras mutation, NF- κ B activation, and cytokine production. While it has been clearly demonstrated that mutant Ras can activate NF- κ B and induce inflammatory cytokine production in various settings, the underlying molecular mechanism has remained elusive. In numerous settings, inflammatory cytokine production has been attributed to oncogene-induced senescence that is associated with excess production of reactive oxygen species (ROS) and subsequent DNA damage or loss of p53^{6, 27}. We found that oncogenic Ras up-regulates SCCA to promote cytokine production, whereas SCCA is not up-regulated in the setting of drug-induced and replicative senescence. These results suggest that the secretory response seen in cells with oncogenic Ras mutations may be distinct from SASP induced by DNA damage or loss of p53. Although the physiological significance of Ras-induced cellular senescence remains an open topic, our results show that Ras can induce SCCA expression in cells where Ras does not induce senescence (Fig. 1f). Furthermore, we found that SCCA-dependent cytokine production occurs in human cells expressing endogenous level of mutant Ras (Fig. 8) and this provides a mechanism that uncouples cellular senescence from inflammatory cytokine production upon Ras activation. Importantly, our study shows that the incidence of SCCA positivity within PanIN lesions and PDAC increases with disease progression (Fig. 7e). Therefore, monitoring SCCA levels may become an attractive diagnostic tool in pancreatic cancer patients, as the levels of SCCA have been used in numerous studies to successfully predict disease stage and response to therapy¹⁶⁻¹⁸.

Our work uncovers SCCA as a critical mediator of Ras-induced UPR. Until now, SCCA's involvement in cancer was largely attributed to its protective role against lysosomal

permeability that leads to unscheduled activation of lysosomal proteases^{29, 39}. We have previously reported that through its protease inhibitory activity, SCCA leads to a decrease in both lysosomal and proteasomal protein turnover and elicits a non-lethal level of ER stress response²⁹. It remains to be determined how SCCA mechanistically suppresses protein turnover, and whether other functions of SCCA such as its possible role in the nucleus contribute to increased UPR. Nevertheless, here we show that SCCA-mediated UPR plays a critical role in the context of oncogenic Ras mutation. Enhanced UPR has been well implicated in cancer, largely owing to the highly proliferative status of cancer cells and their encounter of growth limiting conditions such as nutrient deprivation and hypoxia^{40, 41}. Targeting the enhanced UPR of cancer cells remains an attractive therapeutic option, though the precise prediction of sensitivity has been difficult. Consistent with this notion, tumors driven by mutant Ras have been shown to be susceptible to proteotoxic therapies⁴². Our current work showing that SCCA expression is stimulated by oncogenic Ras and plays an essential role in Ras-induced ER stress response, together with our previous report demonstrating SCCA sensitizes cells to proteotoxic stress²⁹, suggest that SCCA may be a valuable predictor of response to proteotoxic agents in tumors harboring oncogenic Ras.

The contribution of the tumor stroma and inflammation to tumor development and progression, especially in pancreatic and colorectal cancer, is well-accepted^{43, 44}. While NF- κ B signaling has been shown to mediate the SASP response²⁶, it is critical for the development of Ras-driven lung adenocarcinoma⁴⁵. The pro-tumorigenic role of SCCA mediating NF- κ B activation and inflammatory cytokine production is consistent with these reports. This adds a new pro-tumor mechanism of SCCA in addition to its well-documented anti-cell death function^{15, 46-49}. In a parallel study, we find that ectopic expression of SCCA can independently promote the transformation of the non-tumorigenic mammary epithelial cell line MCF10A (Sheshadri et al., in preparation). As numerous studies utilizing anti-inflammatories to target the tumor microenvironment have shown great promise in the treatment of malignancies^{50, 51}, the ability of SCCA to promote cytokine production and tumorigenesis suggests a therapeutic value of targeting its protease inhibitory activity.

METHODS

Cell lines and culture

293T, IMR90, BJ, HT-29, Caco-2, HeLa, AsPC-1, Capan-1, Capan-2, CFPAC-1, HPAF-II, L3.6, PANC-1, PL45, and S2-013 cells were cultured according to ATCC recommendations. 293T, HeLa, PANC-1, PL45, and S2-013 cells were cultured in DMEM supplemented with 10% FBS (HyClone). IMR90, BJ, and HPAF-II cells were cultured in MEM supplemented with 10% FBS. HT-29 and Capan-2 cells were cultured in McCoy's 5a supplemented with 10% FBS. Caco-2 cells were cultured in MEM supplemented with 20% FBS. AsPC-1 and L3.6 cells were cultured in RPMI supplemented with 10% FBS. Capan-1 cells were cultured in Iscove's supplemented with 20% FBS. CFPAC-1 cells were cultured in Iscove's supplemented with 10% FBS. All media was supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen).

Plasmids and reagents

Retroviral expression vectors for WZL-hygro and WZL-HRas^{V12} were a kind gift of Dr. Alea Mills at Cold Spring Harbor Laboratory²¹. pBABE-puro and pBABEKRas^{V12} were a kind gift of Dr. Scott Lowe at Memorial Sloan-Kettering Cancer Institute. pBABE-NRas^{Q61} was purchased from Addgene (Dr. Channing Der; Plasmid 12543)⁵². pLNCXER:HRas^{V12} was a kind gift of Dr. Masashi Narita at University of Cambridge²⁰. pcDNA3-FLAG-ERK2-WT and pcDNA3-FLAG-ERK2-T138A (DN) were a kind gift of Dr. Scott Eblen at the Medical University of South Carolina⁵³. pcDNA3-PEA3-WT and pcDNA3-PEA3-K123R (MUT) were a kind gift of Dr. Andrew Sharrocks at the University of Manchester²⁴. Human XBP1s was cloned by RT-PCR from total RNA from IMR90 cells. Primers used were as follows, forward with HindIII restriction and FLAG tag:

5' AAGCTTATGGATTACAAGGATGACGATGACAAGTGGTGGT
GGCAGCCGCGCCGAACCC-3' and reverse primer with HindIII restriction site: 5'-
AAGCTT TTAGACACTAATCAGCTGGGGAAAG-3'. The RT-PCR product was digested with HindIII and ligated into the pLPC retroviral expression vector. All shRNA lentiviral constructs were in the pLKO (Sigma) backbone. shRNA targeting sequences used are: shGFP: 5'-TACAACAGCC ACAACGTCTAT-3'; shScramble: 5'-
CAACAAGATGAAGAGCACCAA-3'; shSCCA#1: 5'-
GCACAACAGATTAAGAAGGTT-3'; shSCCA#2: 5'-
CCGCTGTAGTAGGGATTCGGAT-3'; shPEA3: 5'-
GCTCCGATACTATTATGAGAA-3'; shATF6: 5'-GCAGCAACCAATTATCAGT TT-3';
shXBP1: 5'-GCCTGTCTGTACTTCCATTCAA-3'; shKRas: 5'-CAGTTGAGACCTTC
TAATTGG-3'. Bay-11-7082 (B5556), 4-hydroxytamoxifen (H7904), and crystal violet (C0775) were purchased from Sigma. U0126 (V1121) was purchased from Promega. AKT inhibitor VIII (124018) was purchased from Calbiochem. TNF α (210-TA) was purchased from R&D Systems. X-gal (15520-018) was purchased from Invitrogen. Etoposide (8154-1) was purchased from Clontech.

DNA transfection and viral infection

Both retrovirus and shRNA lentivirus were generated in 293T cells. Briefly, 293T were transfected by Lipofectamine 2000 (Invitrogen) with the plasmid of interest, packaging plasmid, and a plasmid encoding for the VSV-G envelope protein. 48 and 72 h after initial transfection, viral supernatant was harvested, filtered, supplemented with polybrene (10 μ g/ml) and used to infect target cells. 48 h after last infection, cells were selected with appropriate antibiotics. IMR90 cells were selected for 2 d with 100 μ g/ml of hygromycin, 2 μ g/ml of puromycin or 1.75 mg/ml of G418. HeLa and L3.6 cells were selected with 0.5 μ g/ml of puromycin for 2 d. Caco-2, Capan-1, CFPAC-1, and PANC-1 cells were selected with 1.0 μ g/ml of puromycin for 3 d.

Immunoblot analysis

Cell lysates were prepared in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 0.01M Tris pH 8.0, 0.14M NaCl). Protein expression was examined by western blotting using antibodies against SCCA1/2 (FL-390; Santa Cruz; 1:1000), Ras (Clone Ras10; Millipore; 1:10,000), p21 (C-19; Santa Cruz; 1:500), ERK1/2 (4695; Cell Signaling;

1:10,000), P-ERK1/2 (4370; Cell Signaling; 1:2,000), Akt (9272; Cell Signaling; 1:2,000), P-Akt (4058; Cell Signaling; 1:2,000), P-RelA/p65 (3031; Cell Signaling; 1:1,000), RelA/p65 (F-6; Santa Cruz; 1:2,000), ATF6 (F-7; Santa Cruz; 1:500), XBP1 (M-186; Santa Cruz; 1:500), Ubiquitin (P4D1; Covance; 1:1,000), and β -tubulin (Sigma; 1:10,000). All primary antibodies were incubated overnight at 4°C. Horseradish peroxidase or Alexafluorconjugated goat anti-rabbit (Rockland) or goat anti-mouse (Rockland) antibodies were used as secondary antibodies (1:2,000). Western blots were developed using an ECL detection kit (Thermo Scientific) or an Odyssey Imager (LI-COR).

Gene expression analysis and quantitative PCR (qPCR)

Total RNA was isolated and purified using the RNeasy kit (Qiagen). cDNA was obtained by reverse transcribing 1-2 μ g of total RNA using SuperscriptIII Reverse Transcriptase (Invitrogen) and used for qPCR. qPCR reactions were performed in triplicate using SYBR Green reagents (Quanta Biosciences) on a StepOnePlus (Life Technologies). GAPDH was used as an endogenous control. All results were normalized to GAPDH. Primers sets used are: GAPDH: 5'-AAGGTCGGAGTCAACGGATTT G-3' and 5'-CCATGGGTGGAATCATATTGGAA-3'; SCCA1: 5'-AGCCGCGGTCTCGTGC-3' and 5'-GGCAGCTGCAGCTTCTG-3'; SCCA2: 5'-AGCCACGGTCTCTCAG-3' and 5'-GCAGCTGCAGCTTCCA-3'; Serpinb3a: 5'-CATTTGTTTGCTGAAGCCACTAC-3' and 5'-CATGTTTCGAAATCCAGTGATTCC-3'; Serpinb3b: 5'-ATTCGTTTTTCATGCAGCTGATGT-3' and 5'-GAAAGCTGAAGTTAAATTTGTTTCG-3'; PEA3: 5'-GGACTTCGCCTACGACTCA G-3' and 5'-CGCAGAGGTTTCTCATAGCC-3'; IL-6: 5'-TCCACAAGCGCCTTCGGTCCA3' and 5'-AGGGCTGAGATGCCGTCGAGGA-3'; IL-8: 5'-AAGGAAACTGGGTGCAGAG-3' and 5'-ATTGCATCTGGCAACCCTAC-3'; CXCL1: 5'-CACCCCAAGAACATCCAAAG-3' and 5'-TAACTATGGGGGATGCAGGA-3'; G-CSF: 5'-ACTACAAGCAGCACTGCCCT-3' and 5'-AGCAGTCAAAGGGGATGACA-3'; GM-CSF: 5'-CAAGTGAGGAAGATCCAGGG-3' and 5'-AGAGAGTGTCCGAGCAGCAC-3'.

Cytokine array

Cells were washed once with PBS and incubated for 8 h with fresh media. Supernatant was collected, cleared by centrifugation, and used immediately. The amount of supernatant used was normalized to cell number and used with the human cytokine array kit (R&D Systems). IRDye 800CW Streptavidin (Rockland) was used as secondary (1:2,000) and arrays were imaged and quantified on an Odyssey Imager.

Senescence assays

Senescence was induced by oncogenic Ras, etoposide (100 μ M, 48 h), H₂O₂ (100 μ M, 1 h), or long term passaging (replicative senescence). All cells were analyzed 7 d post-selection or post-treatment. For SA- β -gal staining, cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 15 min and stained (150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 40 mM NaPi, pH 6.0, 1 μ g/ml X-Gal) overnight at 37°C. For BrdU staining, cells were cultured with BrdU (10 nM) for 6 h, fixed with acid ethanol (90% ethanol, 5% acetic acid, 5% H₂O) for 30 min at room temperature. Cells were then washed

once with PBS, incubated with 2 M HCl for 20 min, 0.1 M sodium borate, pH 8.5 for 2 min and washed once with PBS. Cells were blocked in 10% BSA in PBS for 1 h at room temperature and incubated with anti-BrdU (BD Pharmingen, 1:500 in 5% BSA in 0.1% PBS-tween) overnight at 4°C. Cells were washed, incubated with anti-mouse Alexa-594 (1:500) for 1 h at room temperature, washed, co-stained with DAPI and mounted.

NF- κ B luciferase activity assay

NF- κ B activity was determined by using an NF- κ B luciferase reporter construct where the luciferase gene is under control of the IL-6 promoter and internal control plasmid pCMV-RL using a dual-luciferase reporter system (Promega). Cells were plated 24 h prior to transfection at 5×10^4 cells/well of 24-well plate. 250 ng NF- κ B-luciferase vector and 100 ng pCMV-RL were used for transfection. 24 h post-transfection, cells were washed with PBS and lysed in 100 μ l passive lysis buffer for 10 min. Luciferase activity was determined following manufacturer's recommended protocol with SpectraMax M5 Microplate Reader. The ratios of firefly luciferase versus renilla luciferase is used as relative luciferase activities.

TCGA analysis

TCGA human colorectal cancer (study abbreviation: COADREAD) data were downloaded from Broad Institute's Genome Data Analysis Center (GDAC). Standard data of somatic mutations (Mutation_Packager_Calls_Level_3) and RNA expression (Merge_transcriptome_agilentg4502a_07_3_unc_edu_Level_3_unc_lowess_normalization_gene_level_data.Level_3) were used. There were 207 human colorectal tumors which have both somatic mutation and mRNA expression data available. KRas was mutated in 87 out of the 207 samples. SCCA mRNA expression level was compared between the groups with wild-type and mutant KRas.

Immunohistochemistry

Tissue microarrays came from the Vanderbilt GI SPORE Tissue Core. Distribution and the use of all human samples were approved by the Institutional Review Boards of Vanderbilt University Medical Center and Stony Brook University, and samples were obtained with informed consent. IHC was performed on a Ventana XT (Tucson, AZ, USA) autostainer, according to manufacturer's directions.

TMA analysis

Damaged core spots and those that did not contain cancerous tissue were eliminated from scoring. The sections were scored independently by two evaluators blinded to the clinical status of the patients. Samples were scored as previously described¹⁰. Briefly, samples were scored as percent of tumor cells with SCCA or IL-6 expression: 0, no expression; 1, <10%; 2, 10–50%; 3, >50%. For SCCA, a score 1 was considered positive. For IL-6, 0, Negative; 1, Weak; 2, Moderate; 3, Strong.

Xenograft tumor experiments

Male athymic nude mice, age 6–8 wk, were obtained from Taconic Farms. Mice were housed and monitored at the Division of Laboratory Animal Resources at Stony Brook University. All experimental procedures and protocols were approved by the Stony Brook University institutional animal care and use committee (IACUC). Tumors were established by resuspending 1×10^6 tumor cells in 100 μ l PBS and injecting the cells into the mid-flanks of mice using a 26-gauge needle. For each tumor, the tumor length (l) and width (w) was measured every 4–5 d with an electronic caliper. Tumor volume (v) was calculated using the formula $v = (l \times w^2)/2$ and plotted in mm^3 .

Statistical analysis

Statistical analyses were performed with GraphPad Prism (Graphpad Software Inc). For gene expression, luciferase activity and xenograft tumor growth significance was calculated with t -tests. Chi-squared tests were used to assess statistical significance of various categorical clinical features between SCCA-negative and SCCA-positive samples. For the TCGA colorectal cancer data analysis, Wilcoxon Rank Sum test was performed. For all tests, p values were considered statistically significant when <0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Drs. Alea Mills, Scott Lowe, Masashi Narita, Scott Eblen, Andrew Sharrocks, and Erich Mackow for reagents and Drs. Richard Lin and Dafna Bar-Sagi for critical reading. JMC was supported by the NCI T32 training grant (T32CA009176). This work was supported by grants from NIH (R01CA129536 and R01GM97355 to WXZ, U01CA168409 to RSP, and R01CA159222 and R01CA100126 to HCC), and the Carol Baldwin Breast Cancer Research Foundation (to WXZ). P50CA095103 for the Vanderbilt University Medical Center GI Spore Tissue Core.

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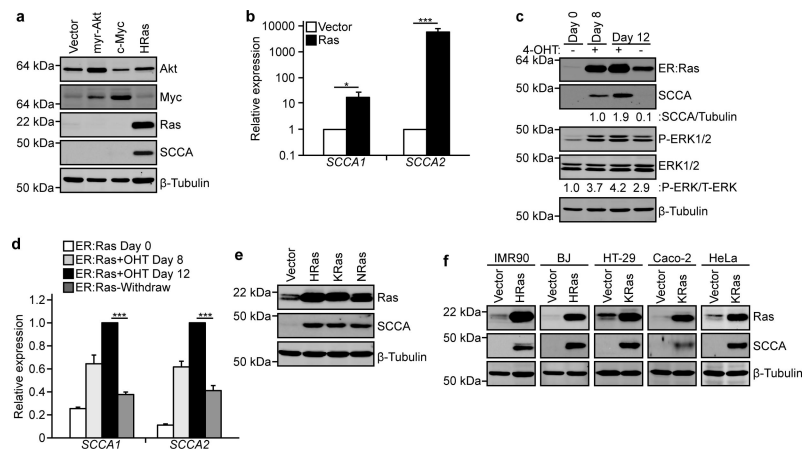


Figure 1. Oncogenic Ras up-regulates SCCA expression

(a) Indicated oncogenes were stably expressed in IMR90 cells. Whole cell lysates were obtained and analyzed by western blot with indicated antibodies. (b) Total RNA was extracted from vector-control or Ras^{V12}-expressing IMR90 cells. SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to that in vector control cells. Data shown are mean + SEM of three independent experiments performed in triplicate. (c, d) IMR90 cells expressing the ER:Ras^{V12} fusion protein were treated with 4-OHT for 8 d, split and either cultured in media containing 4-OHT or withdrew 4-OHT for additional 4 d. (e) Whole cell lysates were analyzed by western blot with indicated antibodies. (d) Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to that of Day 12 ER:Ras with 4-OHT cells. Data shown are mean + SEM of three independent experiments performed in triplicate. (e) Indicated oncogenic Ras proteins were stably expressed in IMR90 cells. Whole cell lysates were obtained and analyzed by western blot with indicated antibodies. (f) HRas^{V12} was stably expressed in IMR90 and BJ cell lines. KRas^{V12} was stably expressed in HT-29, Caco-2, and HeLa cells. Whole cell lysates were obtained and analyzed by western blot with indicated antibodies. *p<0.05; ***p<0.001 by t-test.

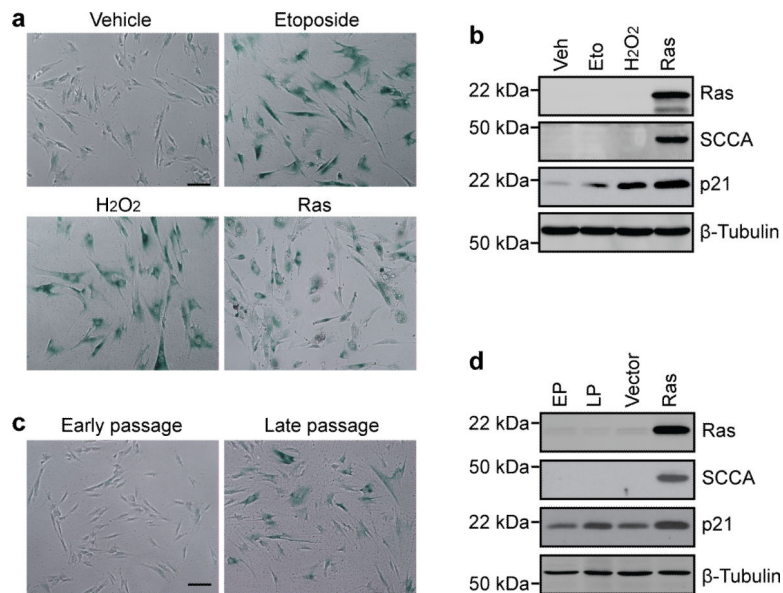


Figure 2. DNA damage-induced and replicative senescence fail to up-regulate SCCA
(a, b) IMR90 cells were treated with vehicle-control, etoposide (10 μ M) for 48 h, H₂O₂ (10 μ M) for 1 h, or stably transduced with HRas^{V12}, then analyzed 7 d post-treatment. **(a)** Cells were stained for β -Gal activity. Representative images are shown. **(b)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(c, d)** IMR90 cells were continuously passaged, and harvested at passage 15 as early passage (EP) or at passage 30 as late passage. **(c)** Cells were stained for β -galactosidase activity. Representative images are shown. **(d)** Whole cell lysates were analyzed by western blot with indicated antibodies. Note that while all the conditions induce cellular senescence, only Ras^{V12} led to SCCA expression. Scale bar = 100 μ M

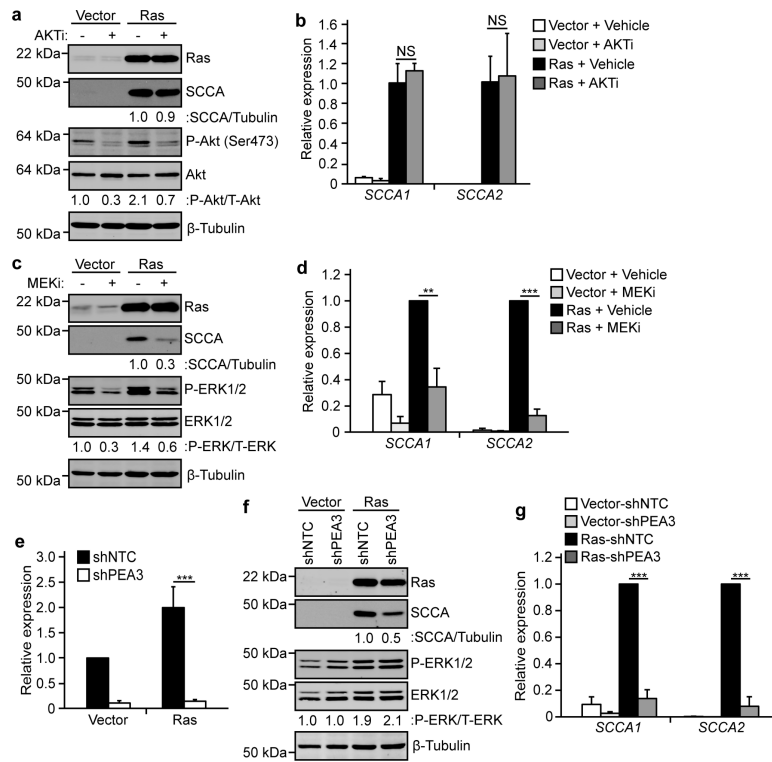


Figure 3. SCCA up-regulation is mediated by MAPK/PEA3

(a, b) Vector-control or HRas^{V12}-expressing IMR90 cells were treated with either vehicle control or AKTi (10 μ M) for 24 h. (a) Whole cell lysates were analyzed by western blot with indicated antibodies. (b) Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to Ras^{V12} cells treated with vehicle control. Data shown are mean + SEM of two independent experiments performed in triplicate. (c, d) Vector-control or HRas^{V12}-expressing IMR90 cells were treated with either vehicle control (DMSO) or U1026 (MEKi, 10 μ M) for 24 h. (e) Whole cell lysates were analyzed by western blot with indicated antibodies. (d) Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to that of Ras-expressing cells treated with vehicle. Data shown are mean + SEM of three independent experiments performed in triplicate. (e-g) Vector-control or HRas^{V12}-expressing IMR90 cells were stably infected with lentiviral shNTC (non-target control) or shPEA3. (e) Successful silencing of PEA3 was confirmed via qRT-PCR, and normalized to that in vector control cells with shNTC. Data shown are mean + SEM of three independent experiments performed in triplicate. (f) Whole cell lysates were analyzed through western blot with indicated antibodies. (g) Total RNA was analyzed for the transcript levels of SCCA1 and SCCA2 via qRT-PCR, and normalized to that in Ras-expressing cells with shNTC. Data shown are mean + SEM of three independent experiments performed in triplicate. **p<0.01; ***p<0.001; NS, non-significant by t-test.

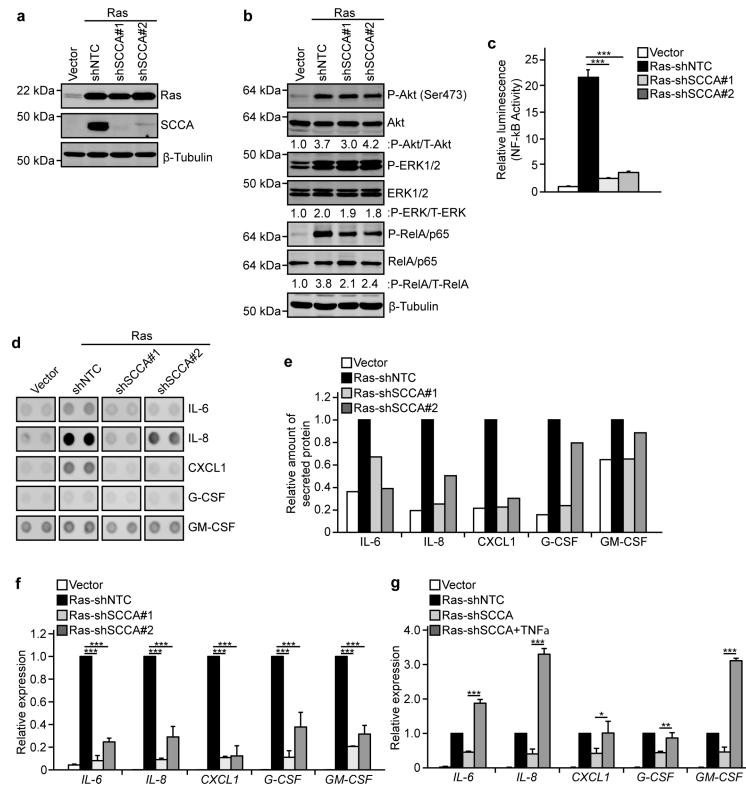


Figure 4. SCCA modulates Ras-induced cytokine production

(a-f) Vector-control or HRas^{V12}-expressing IMR90 cells were stably infected with lentiviral shRNA control (shNTC) or two independent hairpins targeting SCCA. (a, b) Whole cell lysates were analyzed by western blot with indicated antibodies. (c) Cells were transfected with an NF-κB luciferase reporter and a renilla luciferase construct. 24 h post-transfection, cells were lysed and luminescence was quantified. NF-κB luciferase activity was standardized based on renilla luciferase activity and normalized to that of vector-control cells. Data shown are mean + SEM of three independent experiments performed in triplicate. (d) Culture media were collected and subjected to a cytokine antibody array. The representative of two independent blots of indicated cytokines are shown. (e) The relative amount of cytokines was quantified and normalized to that of Ras^{V12}-shNTC cells. (f) Total RNA was extracted and cytokine transcript levels were analyzed via qRT-PCR, and normalized to that of Ras^{V12}-shNTC cells. Data shown are mean + SEM of three independent experiments performed in triplicate. (g) Ras-shSCCA cells were treated with TNFα (50 ng/μl) for 16 h. Total RNA was extracted and cytokine transcript levels were analyzed via qRT-PCR, and normalized to that of Ras^{V12}-shNTC cells. Data shown are mean + SEM of two independent experiments performed in triplicate. *p<0.05; **p<0.01; ***p < 0.001 by t-test.

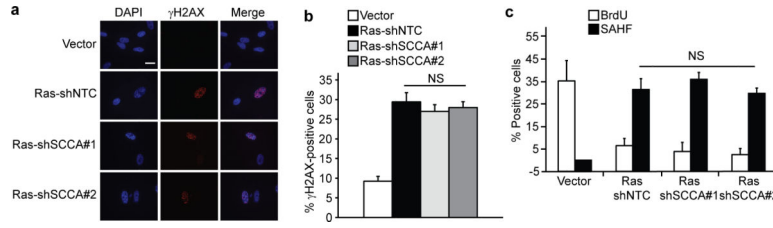


Figure 5. Silencing of SCCA does not interfere with the DNA damage response (a-c) Vector-control or HRas^{V12}-expressing IMR90 cells were stably infected with lentiviral shRNA control (shNTC) or two independent hairpins targeting SCCA (same cells as shown in Fig. 4). (a) Immunofluorescence against γ H2A.X was performed. Representative images are shown; scale bar = 20 μ M. (b) Quantification of percent γ H2A.X-positive cells is shown. Note that silencing of SCCA does not compromise Ras-induced DNA damage. Data shown are mean + SEM of three independent experiments. (c) Cells were cultured with BrdU (10 μ M) for 6 h and immunofluorescence against BrdU was performed. Quantification of BrdU-positive and senescence associated heterochromatin foci (SAHF)-positive cells are shown. Data shown are mean + SEM of three independent experiments. NS, non-significant by t-test.

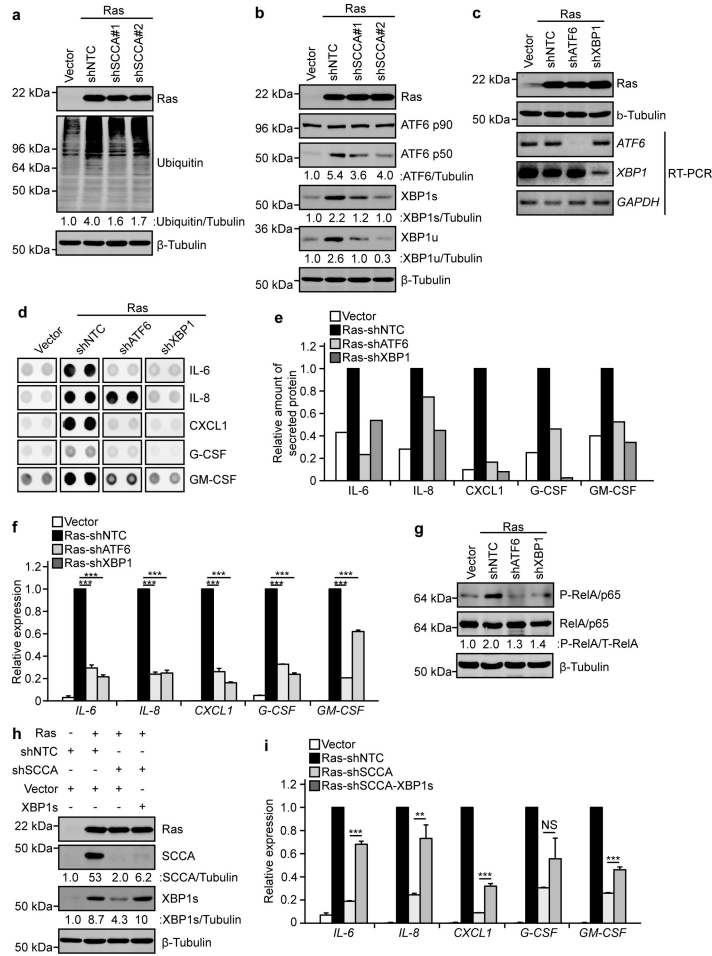


Figure 6. SCCA promotes cytokine production by inducing UPR
(a, b) Vector-control or HRas^{V12}-expressing IMR90 cells were stably infected with lentiviral shRNA control (shNTC) or two independent hairpins targeting SCCA. Whole cell lysates were analyzed by western blot with indicated antibodies. Quantification of the ubiquitin blot in (a) was performed by Image J using the full-length of each lane. **(c-g)** Vector control or HRas^{V12} IMR90 cells were stably infected with lentiviral shRNA control (shNTC) or shRNA hairpins targeting ATF6 or XBP1. **(c, g)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(d)** Culture media were collected and subjected to a cytokine antibody array. The blots of indicated cytokines are shown. **(e)** The relative amount of cytokines was quantified and normalized to that of Ras^{V12}-shNTC cells. **(f)** Total RNA was extracted and cytokine transcript levels were analyzed via qRT-PCR and normalized against that in Ras^{V12}-shNTC cells. Data shown are mean + SEM of three independent experiments performed in triplicate. **(h, i)** Vector-control or HRas^{V12} IMR90 cells were stably infected with lentiviral shRNA control (shNTC) or shSCCA, together with vector control or XBP1s-expressing construct. **(h)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(i)** Total RNA was extracted and cytokine transcript levels were analyzed via qRT-PCR and normalized against that in Ras^{V12}-shNTC cells. Data shown are mean + SEM of three independent experiments performed in triplicate. **p<0.01; ***p<0.001; NS, non-significant by t-test.

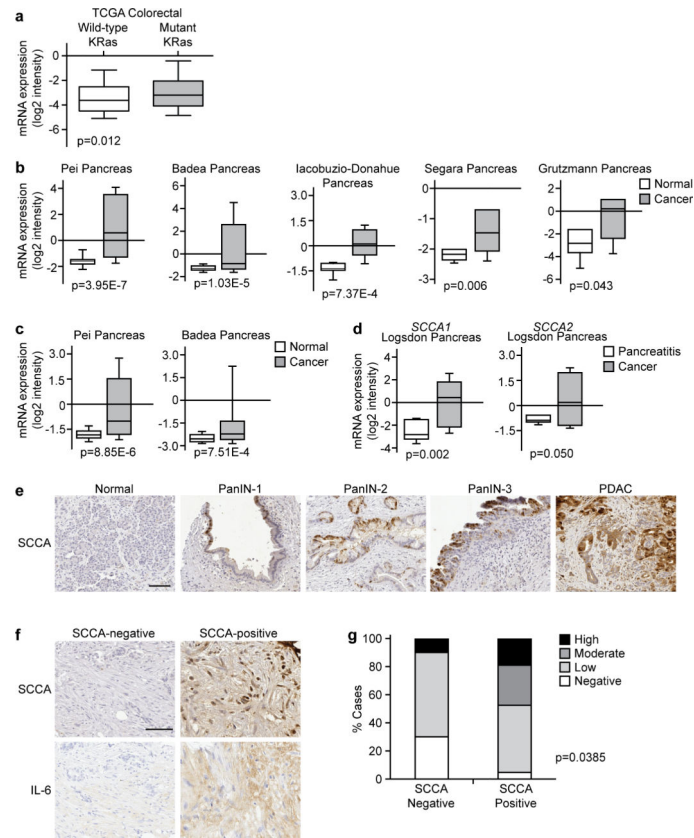


Figure 7. SCCA is up-regulated in human colorectal and pancreatic cancer

(a) TCGA human colorectal cancer data of somatic mutation and RNA expression from Broad Institute's Genome Data Analysis Center were analyzed. There were 207 human colorectal tumors that have both somatic mutation and mRNA expression data available. KRas was mutated in 87 out of the 207 samples. By comparing SCCA mRNA expression level of the groups with wild-type and mutated KRas, SCCA expression was found to be significantly higher in the group with KRas mutation. Boxplots with whisker from 10 to 90 percentile is shown. SCCA expression log₂ intensity values for wild-type (n=120) and mutant (n=87) KRas samples are shown. $p = 0.012$ by Wilcoxon Rank Sum test. (b-d) Oncomine (www.oncomine.org) datasets were analyzed for SCCA1 (b) or SCCA2 (c) mRNA expression levels in normal pancreatic tissue and pancreatic cancer, or for SCCA1 and SCCA2 mRNA expression levels in chronic pancreatitis and pancreatic cancer (d). The boxes represent the interquartile range. Whiskers represent the 10th–90th percentile range. Bars represent the median. p values were calculated by two-sample t-test. (e-g) IHC against SCCA and IL-6 was performed on the corresponding serial pancreatic tissue microarrays. (e) Representative images of normal pancreatic tissue and SCCA-positive PanIN1, PanIN2, PanIN3, and PDAC samples are shown; scale bar = 100 μ M. (f) Representative images of SCCA/IL-6-negative and SCCA/IL-6-positive grade III PDAC samples; scale bar = 50 μ M. (g) Quantification of IL-6 staining in SCCA-negative and SCCA-positive samples. Chi-squared test for trend was used to determine significance, $p = 0.0385$.

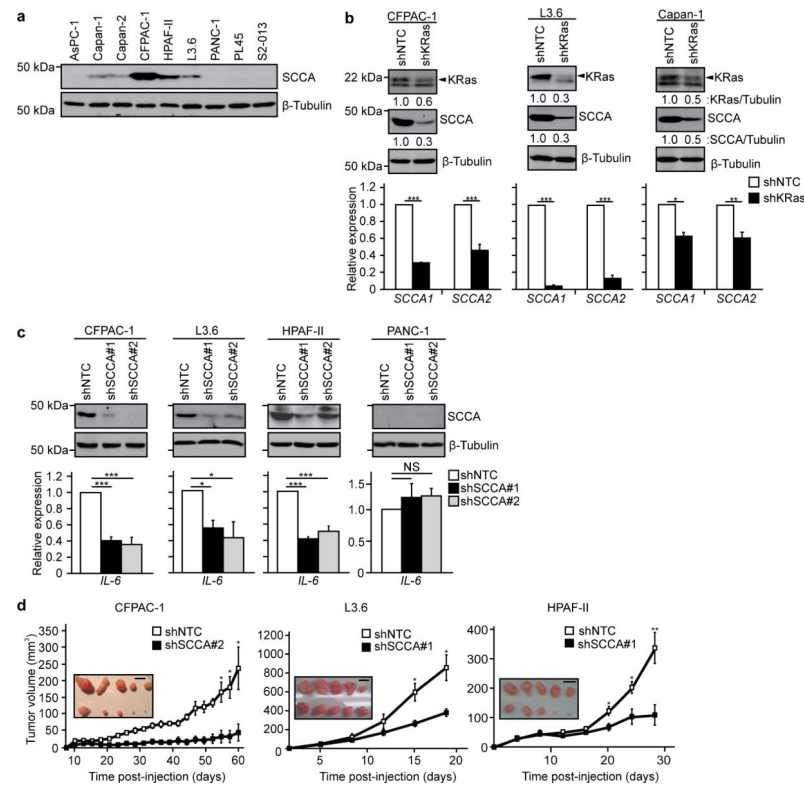


Figure 8. Ras-dependent SCCA expression promotes IL-6 production and tumorigenesis in human pancreatic cancer cell lines

(a) Whole cell lysates from a panel of pancreatic cancer cells were obtained and analyzed through western blot with indicated antibodies. (b) Indicated cell lines were stably infected with lentiviral shNTC or shKRas. Whole cell lysates were analyzed through western blot with indicated antibodies. Note that the Ras antibody utilized is a pan-Ras antibody and KRas is indicated by arrowhead. Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR and normalized to that of shNTC cells. Data shown are mean + SEM of three independent experiments performed in triplicate. (c) Indicated cell lines were stably infected with lentiviral shNTC or shSCCA. Whole cells lysates were analyzed through western blot with indicated antibodies. Total RNA was extracted and IL-6 transcript levels were analyzed via qRT-PCR. Data shown are mean + SEM of three independent experiments performed in triplicate. Relative level of transcript was normalized to that of shNTC cells. Note that silencing of SCCA in PANC-1 cells, which have undetectable SCCA expression, had virtually no effect on IL-6 production. (d) CFPAC-1, L3.6, and HPAF-II cells were injected into the flanks of athymic nude mice and monitored for tumor growth; $n = 5$. Representative images of tumor and the tumor growth curve \pm SEM are shown; scale bar = 1 cm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by t-test.