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Role of squamous cell carcinoma antigen (SCCA) in oncogenic Ras-mediated tumorigenesis

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Abstract of the Dissertation

Role of squamous cell carcinoma antigen (SCCA) in oncogenic Ras-mediated tumorigenesis

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Squamous cell carcinoma antigens (SCCAs) are members of the serpin family of endogenous serine/cysteine protease inhibitors. First identified in the serum of a patient with squamous cell carcinoma of the uterine cervix, both SCCA1 and SCCA2 have now been found to be elevated in cancers of the lung, head and neck, and liver. Despite SCCA's long known involvement with human cancer, little is known about SCCA's functional role within tumor development and progression.

I began my study examining SCCA expression in numerous epithelial cancers. In assessing 1,300 breast cancer samples, SCCA expression was found to correlate with high-grade, advanced-stage cancers. Furthermore, SCCA-positivity predicted both a worse overall survival and recurrence-free survival. My study then turned to dissecting SCCA's oncogenic regulation within human cancers. After an initial screen, I identified oncogenic Ras-mediated up-regulation of SCCA that is dependent on MAPK signaling and the Ets family transcription factor PEA3. Downstream of oncogenic Ras, SCCA expression leads to inhibition of protein turnover, unfolded protein response (UPR), activation of NF-kB transcription factors, and production of

proinflammatory cytokines. Analysis of human colorectal and pancreatic cancer patient samples revealed a positive correlation between Ras mutation, enhanced SCCA expression, and proinflammatory cytokine production. Lastly, silencing of SCCA impairs proinflammatory cytokine production and xenograft tumor growth of pancreatic cancer cells. These findings uncover SCCA as a Ras-responsive factor that plays an essential role in Ras-mediated secretory response, and offers new insight on the regulation of oncogene-associated cytokine production in human malignancy.

Table of Contents

List of Figures and Tables

List of Abbreviations

ATF-6:	activating transcription factor 6
CAF:	cancer associated fibroblast
CDK:	cyclin dependent kinase
CIN:	cervical intraepithelial neoplasia
CXCL1/GROα:	chemokine ligand 1
DDR:	DNA damage response
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor
ETS:	E26 transformation-specific
ER:	estrogen receptor
ERK:	extracellular signal-regulated kinase
GAP:	GTPase activating protein
GEF:	guanine nucleotide exchange factor
G-CSF:	granulocyte colony-stimulating factor
GM-CSF:	granulocyte-macrophage colony-stimulating factor
IL-6:	interleukin-6
IL-8/CXCL8:	interleukin-8
MAPK:	mitogen-activated protein kinase
MEK/MAP2K:	mitogen-activate protein kinase kinase
NF-κB:	nuclear factor κB
OIS:	oncogene-induced senescence
OS:	overall survival
PanIN:	pancreatic intraepithelial neoplasia
PDAC:	pancreatic ductal adenocarcinoma
PEA3:	Polyomavirus enhancer activator 3
PI3K:	phosphoinositide 3-kinase
PR:	progesterone receptor
RFS:	recurrence-free survival
RSL:	reactive site loop
SAHF:	senescence-associated heterochromatic foci
SASP:	senescence-associated secretory phenotype
Serpin:	serine protease inhibitor
TAM:	tumor-associated macrophage
TCGA:	The Cancer Genome Atlas
TMA:	tissue microarray
UPR:	unfolded protein response
XBP-1:	x-box binding protein 1

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Chapter 1: The principles of cancer and SCCA

(A) The basics of cancer

The progression from normal, healthy cell to a fully malignant one is a multi-step process that involves the acquisition and loss of several phenotypes all contributing to the development of a cancerous cell. In a normal cell, evolution has selected for a fine-tuned circuitry that decides when a cell should proliferate and when it should enter a non-replicative quiescent state. Only when the proper cues have been received from both within and outside the cell will it enter the cell cycle and divide. A cancerous cell has hijacked these processes and rewired the circuits such that it proliferates even if it has not received the appropriate signals. Once a cell has successfully achieved this state of chronic proliferation it is considered transformed and fully malignant. It is this feature that is shared universally among all cancers.

At the heart of this transformation is the ability to replicate despite lack of proliferative signals, or achieve what is termed "growth factor independence". A cell can reach a state of growth factor independence in numerous ways. It can self-produce growth factors that are secreted and bind the cognate receptor (i.e. EGF binding EGFR) on the same cell resulting in an autocrine loop. It can over-express a growth factor receptor (i.e. EGFR) which competes for a growth factor that may be present in dilute amounts in the extracellular environment. It can also acquire a mutation that leads to the activation of an oncogene that promotes growth factor independence. For instance, mutations within EGFR render it constitutively active, constantly promoting growth signaling despite the lack of bound EGF. Reported mutations function either to enhance intrinsic tyrosine kinase activity or lead to loss of function of suppressor domains

(Pines et al. 2010). In both cases, autophosphorylation activity increases which results in increased signaling downstream of EGFR. The activation of the Ras oncogene will be discussed in detail in Chapter 3.

Another way to achieve growth factor independence is to acquire the ability to replicate in the presence of anti-proliferative signals coming from within the cell. The majority of these anti-proliferative programs are regulated by tumor suppressor networks that function to prevent cell division if upstream signals instruct them that the cell is not prepared for replication. For instance, under conditions of genotoxic stress the p16 tumor suppressor is up-regulated to prevent progression through the cell cycle. It functions in two ways, first it is a potent inhibitor of a cyclin-depedent kinase (CDK) that promotes entry into the S phase of the cell cycle and second, it stabilizes the p53 tumor suppressor by preventing its degradation (Sherr 2004). Not suprisingly, in many cancers the p16 locus is commonly lost through either a gene deletion event or epigenetic silencing.

While the presence of a single oncogene or the loss of a tumor suppressor can contribute to the development and progression of cancer, alone they are not sufficient to promote complete transformation. A healthy cell requires multiple insults for it to fully acquire the features of a cancerous cell. Along with the ability to sustain proliferative signals and evade growth suppressors, Hanahan and Weinberg established the six hallmarks of cancer that also includes resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). Through a process of natural selection

that involves the acquisition of oncogenes and the loss of tumor suppressors, a normal cell acquires these characteristics to become a fully transformed, malignant cell.

(B) The tumor microenvironment

Following Hanahan and Weinberg seminal "hallmarks of cancer" publication, 10 plus years of research expanded on these initial six hallmarks and it was realized that the contributing factors of transformation was much more expansive than these six hallmarks. Indeed, 11 years after their innovative six hallmarks were established, Hanahan and Weinberg added four additional characteristics to the hallmarks of cancer (Hanahan and Weinberg 2011). They included deregulating cellular energetics, genome instability and mutation, avoiding immune destruction, and tumor-promoting inflammation. All of which offered the possibility of developing targeted therapeutics in the treatment of cancer.

The connection between cancer and inflammation has long been appreciated. In fact, 150 years ago the renowned German physician Rudolph Virchow noted the presence of immune infiltrate in neoplastic tissues (Balkwill and Mantovani 2001). For a time, it was thought that this inflammatory response was the host's immune reaction in an attempt to fight off the malignant tissue. Recent research has now suggested that the inflammatory response, while possibly a futile attempt to eliminate cancer cells, is in fact contributing to both the development and progression of cancer. Along these lines, infectious agents that cause chronic infection have been causally linked to different cancer types. Highlighting the important connection between cancer and inflammation, it is estimated that 15% of all cancers are ascribed to infectious agents (Balkwill and Mantovani 2001). This idea is further supported by the notion that chronic inflammatory conditions such as Crohn's disease and pancreatitis, among many others, increase one's chance

of developing colorectal and pancreatic cancer respectively (Maitra and Hruban 2008; Terzic et al. 2010).

As markers have become more readily available, the different types of non-malignant cells that comprise a tumor have been dissected out. Endothelial cells, fibroblasts, and immune cells, both innate and adaptive, have now all been shown to be present within neoplastic tissue and contribute to disease progression (Hanahan and Coussens 2012). Interestingly, the tumor microenvironment directly contributes to some of the other hallmarks of cancer. Inflammation can supply both angiogenic and growth factors, which in turn limit cell death and promote proliferation respectively, as well as modify the extracellular matrix to promote invasion (Hanahan and Weinberg 2011; Hanahan and Coussens 2012). Taken together, it has become increasingly clear that the development and progression of cancer is a well-orchestrated process between both the incipient neoplastic cells and the surrounding stromal tissue.

(C) Inflammation contributes to cancer development and progression

The stromal compartment of the tumor microenvironment is made up of three distinct components: endothelial cells, infiltrating immune cells, and cancer-associated fibroblasts (CAFs). Nearly every leukocyte type has been shown to be present in the population of infiltrating immune cells, with ratios varying between different cancer subsets. Macrophages though, are almost universally the predominant immune infiltrate. Referred to as tumor-associated macrophages (TAMs), these cells are recruited to sites of tumor growth by various cytokines and chemokines. While TAMs have the ability to become anti-tumor, they have also been shown to contribute to numerous hallmarks. One of the earliest reports demonstrated TAM supplied MMP-9 to be crucial to the development of HPV16 oncogene-driven squamous cell carcinoma of the epidermis. Loss of TAM supplied MMP-9 resulted in decreased kerotinocyte hyper-proliferation, as well as fewer incidences of invasive tumors (Coussens et al. 2000). This effect was most likely due to the release of mitogenic factors by TAM supplied MMP-9, that aided in tumor cell growth.

More recently, immune infiltrate was shown to be essential in promoting the progression of early pancreatic intraepithelial neoplasias (PanINs) to pancreatic ductal adenocarcinoma (PDAC). Adult mice expressing the oncogenic K-Ras^{G12D} allele in the acinar cell compartment of the pancreas were susceptible to PanIN and PDAC development only when they experienced inflammation associated with pancreatitis. It was demonstrated that inflammation abrograted the tumor-suppressive senescent barrier observed in low grade PanINs. Indeed, senescent PanINs were observed in patients with chronic pancreatitis who were treated with anti-inflammatory

drugs (Guerra et al. 2011). While which immune cell type was directly contributing to senescence bypass and how they triggered this response is currently unknown, the results definitively implicate inflammation directly in tumor development and progression.

Undoubtedly, the most well-appreciated stromal mediator of cancer cell growth are endothelial cells. The process of generating new vasculature, known as angiogensis, has been known to be a limiting factor in the progression of hyperplasia to neoplasia for over 20 years (Folkman et al. 1989). Endothelial cells of the stroma have been most closely associated with the production of new blood vessels. In fact, the FDA approved drug Avastin is a monoclonal antibody that targets vascular endothelial growth factor A (VEGF-A) to inhibit angiogenesis. As a mitogen, VEGF stimulates the proliferation of endothelial cells and in turn promotes neovascularization. Highlighting the importance of endothelial cells and angiogenesis, Avastin has been approved for the treatment of colon, lung, renal, and brain tumors. More recently, endothelial cells and the tumor vasculature have been shown to promote a immuno-suppressive environment (Fisher et al. 2006). Simply, intra-tumor vessels do not exhibit characteristics of a traditional chronic inflammatory site and thus are difficult for anti-tumor T-cells to extravasate. These findings further complicate the burgeoning field of immune stimulating therapeutics.

The last stromal sub-population, that like immune cells can be further subdivided, are cancer-associated fibroblasts. Once normal and healthy fibroblasts, CAFs, as a result of being exposed to malignant cells, are converted to tumor-promoting fibroblasts. In comparison to normal fibroblasts, CAFs exhibit enhanced proliferation, collagen production, and secretion of both growth factors and extracellular matrix modulators (Madar et al. 2013). Similar to both

endothelial and immune cells, CAFs have been shown to contribute to numerous hallmarks. Through the secretion of TGF-β, CAFs can promote epithelial to mesenchymal transition (EMT), as well inflammation through the secretion of cytokines and chemokines (Hanahan and Coussens 2012). Perhaps the most conclusive evidence of fibroblasts promoting tumor growth demonstrated that xenograft tumors grew at an increased rate when co-injected with CAFs. Through the secretion of SDF-1, CAFs supported tumor growth through both the recruitment of endothelial cells to induce angiogenesis, as well directly stimulating tumor cell proliferation through the direct binding of SDF-1 (Orimo et al. 2005).

While the contribution of stromal cells to the development and progression of cancer has been well-studied, why and how these cells are recruited to sites of tumor growth is less understood. There is limited data suggesting that in fact the tumor cells themselves secrete proinflammatory cytokines and chemokines to recruit endothelial cells, immune cells, and fibroblasts to aid in their growth. If this is true, understanding how malignant cells activate these pro-inflammatory transcriptional programs may offer new therapeutic opportunities.

(D) SCCA identification and function

Squamous cell carcinoma antigen 1 and 2 are members of the serpin family of endogenous serine/cysteine protease inhibitors. SCCA was first isolated and identified in the 1970's from the serum of a patient with metastatic squamous cell carcinoma of the cervix (Kato and Torigoe 1977). Originally identified as a single band through SDS gel electrophoresis, SCCA was thought to be a single protein. Further studies using isoelectric focusing found two distinct proteins, one that migrated at a neutral pH (SCCA1) and another that migrated at a more acidic pH (SCCA2) (Kato et al. 1984). During these initial studies it was found that SCCA1 was expressed primarily in non-malignant epithelial cells of the cervix, while SCCA2 was up-regulated in patients with squamous cell carcinoma (Kato and Torigoe 1977). It is now known that SCCA1 and SCCA2 are co-expressed in normal tissues that include the squamous epithelium of the tongue, tonsil, esophagus, cervix, and vagina as well as areas of the thymus and skin and both have been found to up-regulated in numerous cancers (Cataltepe et al. 2000).

Thought to be the result of a gene duplication event, SCCA1 and SCCA2 are tandemly arrayed on chromosome 18q21.3 and are 98% homologous at the nucleotide level. Importantly, this homology extends into their promoter regions (Hamada et al. 2001), as SCCA1 and SCCA2 have nearly identical promoter activities in luciferase promoter assays (Suminami et al. 2005). As protease inhibitors, both SCCA1 and SCCA2 function in an identical manner. The reactive site loop (RSL) domain of the protein acts as a mimic of their target protease substrates. Through a "bait and trap" mechanism, the protease recognizes the RSL region, binds SCCA and cleaves SCCA. Since SCCA is cleaved by its target protease, it is a one-time, one-use inhibitor and is

thus termed a “suicide-substrate” inhibitor. Prior to cleavage, SCCA undergoes a conformational change that locks the protease in place. Following cleavage, a covalent serpin-enzyme complex is formed through an ester bond (Schick et al. 1998). The fate of the cleaved serpin-enzyme complex is currently unknown.

While SCCA1 and SCCA2 are highly homologous at the amino acid level, differences within their RSL regions dictate different substrate specificities. Despite being part of the serine protease inhibitor family, the most well-characterized substrates of SCCA1 are the lysosomal cysteine proteases cathepsins S, L, and K (Schick et al. 1998). SCCA2 remains true to the serpin family and inhibits chymotrypsin-like serine proteinases cathepsin G and human mast cell chymase (Schick et al. 1997). As members of the clade B serpins, SCCA1 and SCCA2 do not contain N-terminal signal peptides that target them for secretion and thus are thought to remain intracellular.

Importantly, SCCA1 and SCCA2 do not have true homologs within the mouse serpin family. SCCA1/2 and mouse serpins b3a-b3d evolved after humans and rodents diverged and because of this, the function of one human serpin may be done by mutliple mouse serpins (Silverman et al. 2010). This problem will be discussed later on when we attempted to find the mouse serpin that behaves similar to SCCA.

(E) SCCA's involvement in cancer

SCCA's involvement in human cancer has been known since it was first identified in the serum of a patient with squamous cell carcinoma of the cervix. Since its identification, SCCA has now been found to be elevated in cancers of the lung, head and neck, and liver (Cataltepe et al. 2000; Vassilakopoulos et al. 2001; Guido et al. 2008). In fact, SCCA has even been developed as diagnostic marker and predictor of various clinical outcomes. Despite the predicted and reported intracellular localization of SCCA, it is often quantified in the serum of patients when used as a diagnostic marker. The presence of SCCA in the serum is thought to occur through the passive release from dying cancer cells, and not through an active secretory process. In cervical cancer, elevated levels of serum SCCA correlated directly with disease stage. While only ~14% of patients with cervical intraepithelial neoplasia (CIN) exhibited elevated serum SCCA levels, ~96% of patients with stage III/IV displayed elevated SCCA levels. Furthermore, serum SCCA levels were predictive of disease recurrence with elevated levels returning 4.3 months prior to clinically diagnosed recurrence (Brioschi et al. 1991).

In addition to being able to relate to clinical stage, SCCA serum levels have been shown to be predictive of reponse to radiotherapy. Patients with cervical cancer had their serum SCCA levels measured before and after receiving a round of radiation, and response to therapy was evaluated. Patients who retained higher levels of serum SCCA were more likely to have residual tumors upon cervival biopsy than patients where serum SCCA levels subsided following one round of radiation (Ngan et al. 1990). Furthermore, serial monitoring of serum SCCA levels was

a valuable predictor of clinical response to chemotherapy in patients with cervical cancer (Scambia et al. 1991).

While the predictive value of SCCA levels in various cancers has been well-reported, a direct role for SCCA within cancer development and progression has not been well-studied. Not suprisingly, given SCCA's predictive value in therapeutic response, many studies have implicated SCCA in only one of the hallmarks of cancer, resisting cell death. The over-expression of SCCA1 was first reported to inhibit both TNFα and activated natural killer (NK) cell induced cell death (Suminami et al. 2000). Depletion of SCCA1 also sensitized squamous cell carcinoma cells to etoposide, and it was reasoned that SCCA1 inhibited proteases upstream of caspase 3 cleavage. More recently, SCCA2 has also been shown to inhibit both TNFα and NK cell mediated cell death, and like SCCA1, the protease inhibitory activity was implicated in this ability (de Koning et al. 2011).

In searching to understand SCCA's role in cancer, we uncovered a novel proinflammatory function for SCCA downstream of the Ras oncogene, as well as its involvement in breast, colorectal, and pancreatic cancers. Our study of SCCA in human breast cancer will first be discussed. This will be followed by work exploring SCCA's oncogenic regulation and its implications in colorectal and pancreatic cancer.

Chapter 2: Identification of SCCA up-regulation in high-grade, advanced stage human breast cancer

(A) Validation of SCCA antibodies

To begin our study of SCCA, we tested three commercially available antibodies that have been previously described for immunoblotting and immunohistochemistry (IHC) analysis (Cataltepe et al. 2000). According to the manufacturer's instruction, one antibody recognizes both SCCA1 and SCCA2 (Santa Cruz Biotechnology, Inc. Clone FL- 390), one specifically recognizes SCCA1 (Santa Cruz, Clone 8H11), and another specifically recognizes SCCA2 (Santa Cruz, Clone 10C12). We characterized these three antibodies using 293T cells transfected with Flag-SCCA1 or Flag-SCCA2 expression constructs. While Clone FL-390 recognized both SCCA1 and SCCA2, and Clone 10C12 specifically recognized SCCA2 as described by the manufacturer, Clone 8H11 failed to recognize SCCA1 and instead recognized SCCA2 (Fig. 1A). The specificity of the antibodies was further examined by immunocytochemistry using paraffin-embedded 293T cells expressing Flag-SCCA1 or Flag-SCCA2. Similar to the immunoblotting analysis, Clone FL-390 recognized both SCCA1 and SCCA2, while Clone 10C12 recognized only SCCA2 (Fig. 1B). The 8H11 antibody, which was described to specifically recognize SCCA1 (Santa Cruz Biotechnology Product Information) (Cataltepe et al. 2000), failed to do so in our hands. These results indicated that Clone FL-390 is a reliable and more efficient antibody for recognizing both SCCA1 and SCCA2. Indeed, when FL-390 was tested on paraffin-embedded normal human tissues, it revealed SCCA expression in the ciliated pseudostratified columnar epithelial of the bronchus, in suprabasal and basal epidermal keratinocytes, and in the

suprabasal keratinocytes of the stratified squamous epithelial of the anal mucosa (Fig. 1C). This is consistent with reports in literature describing SCCA expression patterns (Cataltepe et al. 2000). Therefore, although efforts have been reported to individually detect SCCA1 and SCCA2 (Cataltepe et al. 2000; Nustad et al. 2004), we chose to use Clone FL-390 for the subsequent immunoblotting and IHC assays, because 1) Clone FL-390 has better efficiency for both immunoblotting and IHC analysis; and 2) based on current clinical studies, an assay recognizing both SCCA1 and SCCA2 is recommended for optimal clinical sensitivity.

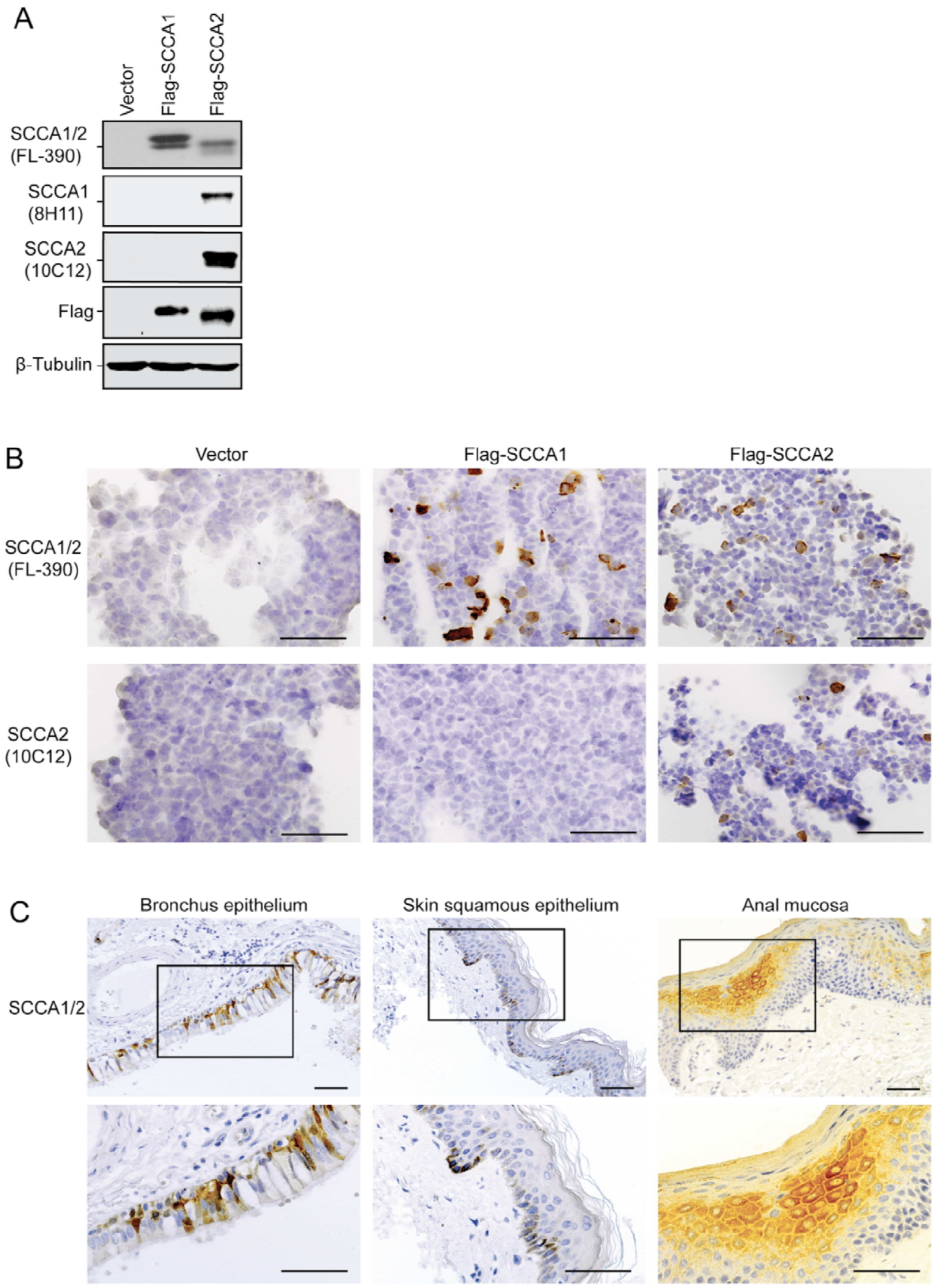

A
Vector
Flag-SCCA1
Flag-SCCA2
SCCA1/2 (FL-390)
SCCA1 (8H11)
SCCA2 (10C12)
Flag
β-Tubulin
B
Vector
Flag-SCCA1
Flag-SCCA2
SCCA1/2 (FL-390)
SCCA2 (10C12)
C
Bronchus epithelium
Skin squamous epithelium
Anal mucosa
SCCA1/2

Figure 1. Validation of SCCA antibodies. 293T cells were transfected with either empty-vector, Flag-SCCA1, or Flag-SCCA2 plasmids. **(A)** Cells were subjected to immunoblot analysis using three SCCA antibodies: FL-390 for SCCA1/2, 8H11 for SCCA1, and 10C12 for SCCA2, as well as Flag antibody and β-tubulin antibody. **(B)** Cells were fixed and embedded in paraffin. IHC was performed with FL-390 and 10C12 antibodies. **(C)** IHC was performed on normal human tissue using the antibody Clone FL-390. Scale bars = 50 μm.

(B) *In vitro* screen of cancer cell lines for SCCA expression

There have been numerous studies reporting SCCA's involvement in cancers of epithelial origin (cervix, head and neck, lung). We therefore examined if SCCA was involved in other epithelial cancers. In searching for evidence that SCCA may be associated with other epithelial cancers, we first compared SCCA expression levels among a number of tumor cell lines including a non-neoplastic breast epithelial cell line MCF10A, breast cancer lines (T47D, MCF7, MDA-MB-468, SK-BR-3, Hs578T, and MDA-MB-231), pancreatic cancer lines (CFPAC-1, MIA PaCa-2, PANC-1), osteosarcoma lines (U-2 OS and SAOS-2), and ovarian cancer lines (OVCAR-4 and OVCAR-5). SCCA was detected at various levels in 5 out of 6 of the breast cancer cell lines (Fig. 2), indicating that SCCA expression is elevated in certain types of breast cancers. It remains to be determined why the bands on the immunoblots appeared to migrate differently in these breast cancer cell lines (Fig. 2). Possible explanations include the different isoforms of SCCA or the proteolytic cleavage of SCCA. Interestingly, the 5 positive cell lines (T47D, MCF7, MDA- MB-468, SK-BR-3, and MDA-MB-231) were derived from metastatic invasive ductal carcinomas (Cailleau et al. 1978; Keydar et al. 1979), whereas the Hs578T cell line was derived from a patient with primary carcinosarcoma (Hackett et al. 1977). Taken together, these results indicate that expression of SCCA is elevated in certain breast cancers and may correlate with invasive ductal carcinoma.

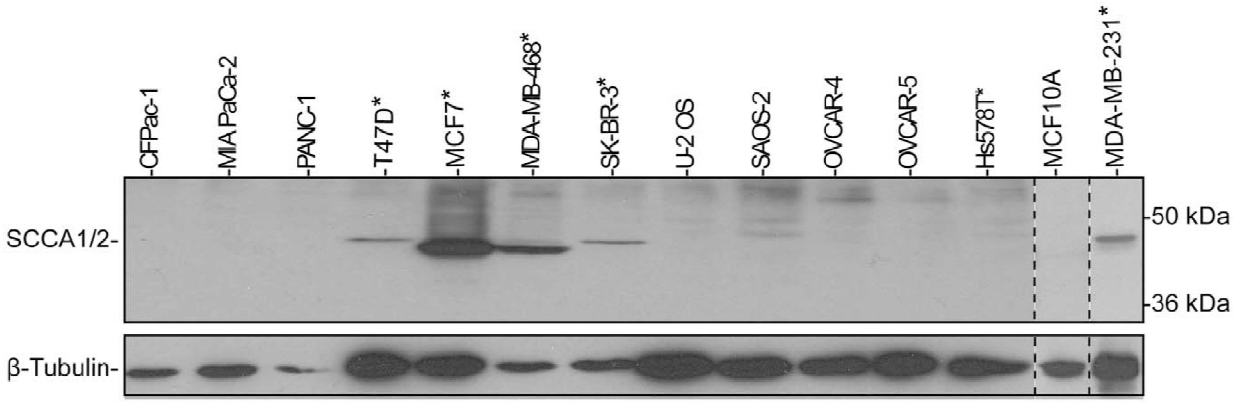


Figure 2. Elevated SCCA expression is found in human breast cancer cell lines. Whole cell lysates from a panel of human cancer cells were probed for SCCA expression by western blot. Five out of the six breast cancer cell lines (denoted with an asterisk) were positive for SCCA expression.

(C) Assessment of SCCA expression in human breast cancer

We next examined the expression of SCCA in human breast cancer samples. A breast carcinoma progression tissue microarray (TMA) was obtained from the Cooperative Human Tissue Network (CHTN) at the University of Virginia. This TMA contained 7 cases of non-neoplastic breast epithelium from healthy subjects, 7 cases of non-neoplastic breast epithelium from subjects with breast cancer, and 42 cases ranging from low-grade ductal carcinoma in situ (DCIS) to metastatic breast carcinoma. Immunohistochemical staining using Clone FL-390 was semi-quantitatively scored on a tiered scale (0-3) as a percentage of positive tumor cells (Fig. 3). The expression levels were recorded as percent of tumor cells with SCCA expression 0 (no positive staining), 1 (positive staining in less than 10% of cells), 2 (positive staining in 10-50% of cells), and 3 (positive staining in over 50% cells). Tumor tissues with a score higher than 1 were counted positive for SCCA expression. Elevated expression of SCCA was detected in 4 high-grade and one low-grade tumor, and was not present in normal breast tissue (Fig. 4). These results further support the notion that SCCA expression is elevated in certain breast cancers.

To further investigate the involvement of SCCA in human breast cancer, we obtained two large-scale TMA case sets from the NCI Cancer Diagnosis Program (CDP). One is the 2nd generation breast cancer progression TMA, and the other is the CDP 2008 breast cancer prognostic TMA that contains three non-metastatic TNM stages I-III as defined by AJCC Manual for Staging of Cancer. SCCA expression was once again detected through IHC using Clone FL-390. In the NCI CDP progression TMA, IHC analysis revealed that all of the normal breast tissue specimens were negative for SCCA expression. When compared with the grading

information provided for the 291 cases, SCCA was found in only Grade II (n=5) and Grade III (n=8) specimens (p=0.016) (Table 1). These results indicate that elevated SCCA expression is associated with breast carcinomas but not normal breast epithelium, and this expression correlates with the grade of the invasive cancer.

In the prognostic TMA case sets, SCCA expression was also detected primarily in Grade II and Grade III tissue specimens, only 1 Grade I sample screened positive ($p<0.0001$) (Table 2). Strikingly, 68.5% of the positive specimens corresponded with Grade III breast carcinoma even though Grade III specimens were under-represented in these TMA case sets (only 28.9% of the total breast carcinoma tissue samples). Cumulatively, among the CDP progression (Table 1) and the prognostic (Table 2) TMAs, all 124 non-neoplastic specimens were found to be SCCA negative, whereas 1 of the 330 (0.3%) Grade I cases, 16 of the 638 (2.5%) Grade II cases and 37 of the 392 (9.4%) Grade III cases were SCCA-positive ($p<0.0001$) (Table 3). Importantly, SCCA positivity also correlated with stage of the disease, as 8.6% of Stage III tissue samples tested positive for SCCA expression, whereas only 2.4% and 3.1% of Stage I and II samples, respectively, showed SCCA expression ($p = 0.0005$) (Table 4). In addition, our study encompassed 1,138 breast cancer samples with ductal/lobular classification. While 40 of the 1,029 ductal carcinoma specimens were SCCA-positive, only 1 SCCA-positive case was found in the 109 lobular carcinoma specimens.

Moreover, while no statistical difference was detected for the mean age of diagnosis ($p = 0.38$), the mean size of tumors was 2.41 cm for SCCA-negative versus 3.58 cm for SCCA-positive tumors ($p<0.0001$). The progression TMA also came with information for the

expression of estrogen receptor (ER) and progesterone receptor (PR), but not Her2/neu status. Out of the 13 SCCA positive breast carcinoma specimens, 9 cases (69%) were classified as double negative (DN) for the expression of both ER and PR, whereas only 24% of the SCCA-negative tumor specimens were DN ($p=0.0009$). This is consistent with the notion that tumors negative for both hormone receptors are more likely to be Grade III and to have a larger mean tumor size. Taken together, the IHC results obtained from both the progression and prognostic TMAs indicate that SCCA expression correlates with high-grade and advanced stage breast carcinomas.

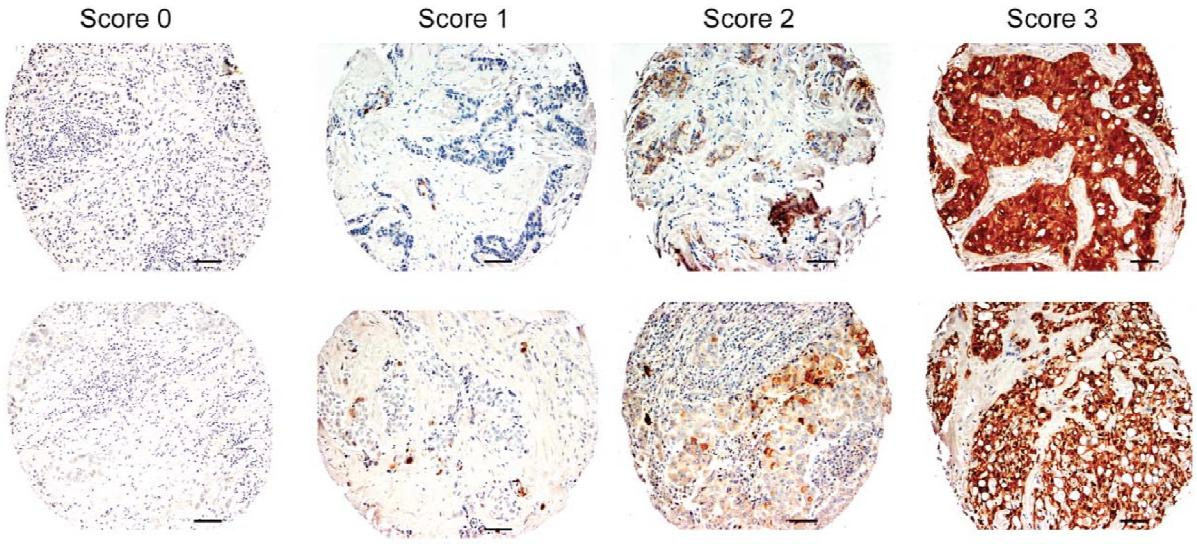


Figure 3. Immunohistochemistry Scoring System. IHC staining was performed on the NCI CDP breast cancer progression and the prognostic TMAs using antibody FL-390. SCCA staining was scored on a tiered-scale (0–3). A representative panel is shown. Scale bars = 50 μm.

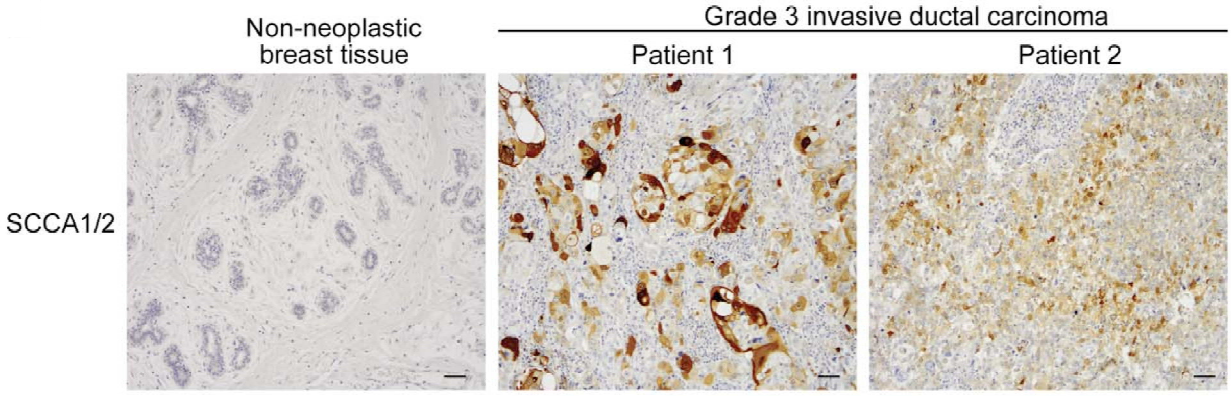


Figure 4. Identification of SCCA in high-grade breast cancers. IHC analysis was performed on an array of breast carcinomas and normal breast tissue obtained from CHTN, using antibody FL-390. Representative images of normal breast tissue and sections with Grade III invasive ductal carcinoma are shown.

	Case Set 3	Case Set 5	Case Set 7	**Total**
Normal tissue(SCCA Positive)	23(0)	23(0)	23(0)	**69(0)**
Grade 1(SCCA Positive)	18(0)	9(0)	9(0)	**36(0)**
Grade 2(SCCA Positive)	34(2)	34(2)	36(1)	**104(5)**
Grade 3(SCCA Positive)	21(2)	33(3)	28(3)	**82(8)**

$p<0.05$

Table 1. SCCA expression correlates to high-grade breast cancer in the CDP progression TMA. The three CDP progression TMA case sets (designated by CDP) were probed for SCCA expression and scored. The grading information was provided by CDP in 291 accountable samples.

	Case Sets 9-13 (Stage I)	Case Sets 14-17 (Stage II)	Case Sets 18-19 (Stage III)	**Total**
Normal tissue(SCCA Positive)	25(0)	20(0)	10(0)	**55(0)**
Grade 1(SCCA Positive)	197(0)	73(0)	24(1)	**294(1)**
Grade 2(SCCA Positive)	268(4)	175(1)	91(6)	**534(11)**
Grade 3(SCCA Positive)	108(10)	143(11)	59(8)	**310(29)**

$p<0.0001$

Table 2. SCCA expression correlates to high-grade breast carcinoma in the CDP prognostic TMA. The Stage I (Case Sets 9–13), Stage II (Case Sets 14–17), and Stage III (Case Sets 18–19) prognostic TMAs were obtained from CDP, containing 598, 411, and 184 tissue specimens, respectively. IHC was performed against SCCA. The tissue was scored and the SCCA-positive cases are shown in parentheses against the number of cases in each grade. Note that all of the normal tissue were SCCA-negative, whereas 1 out of 294 Grade I, 11 out of the 534 Grade II, and 29 out of 310 Grade III cases were SCCA-positive.

	# SCCA Positive/Total Breast Carcinoma Specimen
Normal/Non-Neoplastic	0/124
Grade I	1/330(0.30%)
Grade II	16/638 (2.51%)
Grade III	37/392 (9.44%)

$p<0.0001$

Table 3. SCCA expression correlates to high-grade breast carcinomas. The cumulative results of SCCA positivity against the breast carcinoma grading from the CDP progression and prognostic TMAs are shown. SCCA is negative in the 124 normal or non-neoplastic cases of breast tissue. SCCA is positive in 1 of the 330 (0.3%) Grade I cases, 16 of the 638 (2.5%) Grade II cases, and in 37 of the 392 (9.4%) Grade III cases.

	# SCCA Positive/Total Breast Carcinoma Specimen
Normal/Non-Neoplastic	0/55
Stage I	14/573 (2.44%)
Stage II	12/391 (3.07%)
Stage III	15/174 (8.62%)

p=0.0002

Table 4. SCCA expression correlates to advanced stage breast carcinomas. The Stage I, Stage II, and Stage III prognostic TMAs were obtained from CDP. IHC against SCCA was performed. The tissue was scored and the SCCA-positive cases are shown in parentheses against the number of cases for each stage. Note that all of the normal tissue were SCCA-negative, whereas 14 out of 573 (2.4%) Stage I, 12 out of the 391 (3.1%) Stage II, and 15 out of 174 (8.6%) Stage III cases were SCCA-positive.

(D) Correlation between SCCA expression and overall survival (OS) and recurrence-free survival (RFS)

Following the proposal by Hudis et al., survival analyses were carried out to compare both overall survival (OS) and recurrence-free survival (RFS) using the information included with the prognostic TMAs. SCCA positivity correlated with decreased OS and RFS (Fig. 5A,B) (OS: hazard ratio (HR), 2.75; 95% CI, 1.62–4.68; Log-rank p = 0.0002) (RFS: HR, 4.64; 95% CI, 2.26–9.55; Log-rank p<0.0001). Furthermore, comparing only Grade II and III breast cancers, patients with SCCA expression had a decreased OS (HR, 2.08; 95% CI, 1.26–3.44; Log-rank = 0.004). The median OS was 155.0 months for SCCA-negative patients, with a 5-year survival rate of 79.1%, whereas the median OS was 88.0 months, with a 5-year survival rate of 54.2% for patients positive for SCCA (Fig. 5C). SCCA expression also correlated with a worse RFS (HR, 3.08; 95% CI, 1.59–5.98; Log-rank p = 0.0009). Grade II and III SCCA-negative patients had a 5-year RFS of 74.4%, while SCCA-positive patients had a 5-year RFS of 42.2% and a median time to recurrence of 52 months (Fig. 5D). These results further support the notion that SCCA expression correlates with high-grade breast cancers with a worse overall outcome.

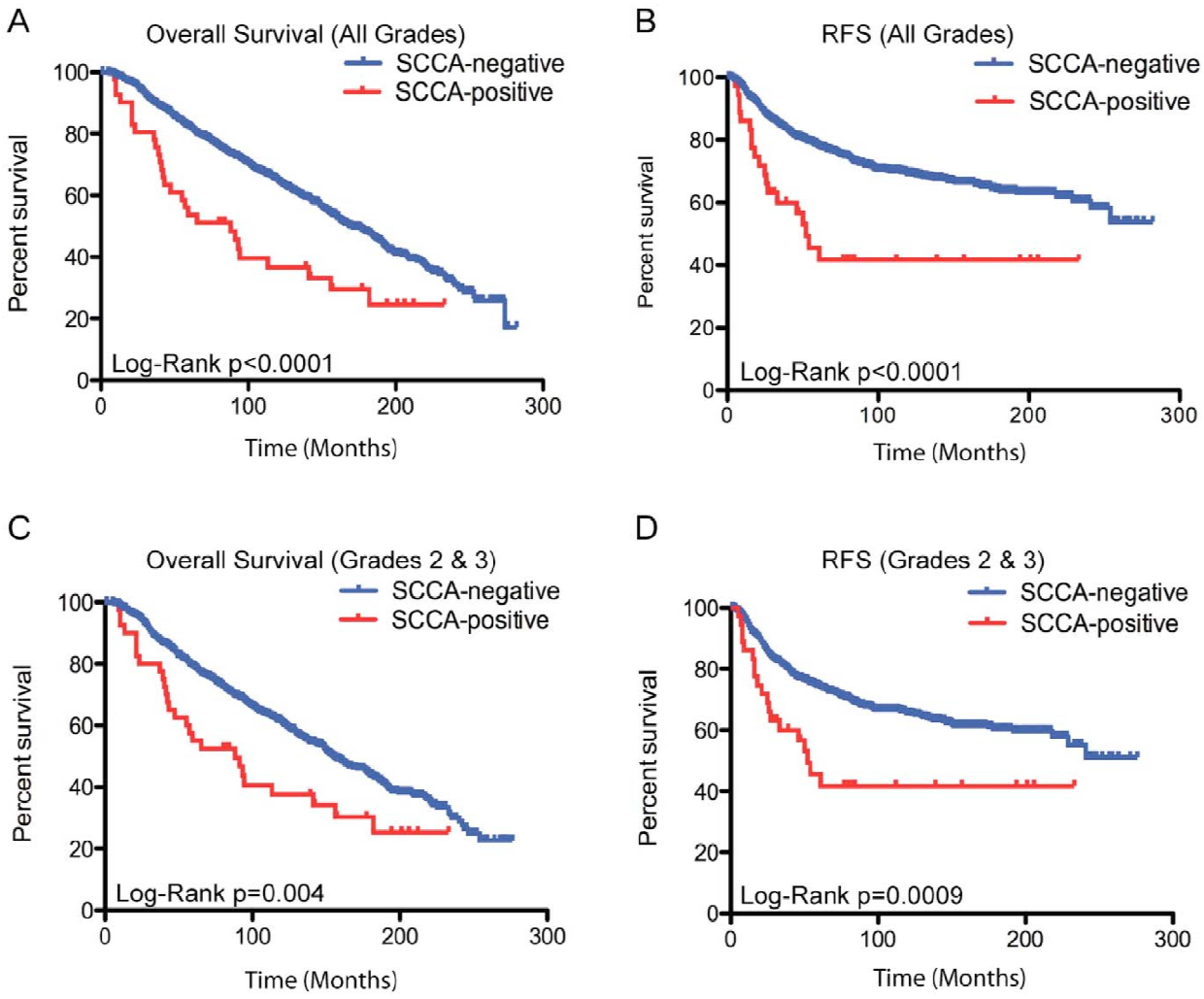


Figure 5. SCCA expression correlates to a decreased overall survival and recurrence-free survival. IHC was performed using the SCCA antibody FL-390 on the CDP prognostic TMAs. Kaplan-Meier survival curves for all patients **(A,B)** and only Grade II and Grade III patients **(C,D)** with SCCA-positive and negative tumors were compared for overall survival **(A,C)** and recurrence-free survival **(B,D)**.

Chapter 3: Introduction II: Oncogenic Ras

(A) The Ras family of GTPases

The Ras family of small GTPases were originally discovered as viral oncogenes of the Harvey and Kirsten rat sarcoma viruses, v-H-Ras and v-K-Ras respectively, that had the ability to transform cells *in vitro* (Ellis et al. 1981). Shortly after the discovery of the viral oncogenes, their cellular homologs were identified in human cancers (Der et al. 1982; Parada et al. 1982; Shimizu et al. 1983). The H-Ras gene was identified in a bladder cancer cell line, while K-Ras was discovered in a lung cancer cell line. The third member of the Ras gene family, N-Ras, was identified in a neuroblastoma cell line, and like H-Ras and K-Ras, was capable of transforming NIH 3T3 cells (Taparowsky et al. 1983). H-Ras, K-Ras, and N-Ras are highly conserved and 85% homologous at the amino acid level, with most of the differences occurring in the C-terminal CAAX domain of the protein. At the endoplasmic reticulum (ER), the CAAX domain is the target of post-translational hydrophobic modifications that directs the Ras proteins to cellular membranes. Not surprisingly, the Ras isoforms are differentially modified and presumably confers each Ras protein with distinct cellular membrane compartments. For all isoforms though, membrane localization is essential for Ras activation.

Despite being localized to different membrane compartments, all three proteins function in an identical manner. As GTPases, the Ras proteins cycle between the inactive GDP-bound and active GTP-bound state that acts as an on/off switch for Ras activity. In wild-type cells, Ras cycles between GDP and GTP from signaling cues upstream. In a normal cell, when a growth

factor binds its cognate receptor (i.e. EGF binds EGFR), the receptor becomes active and phosphorylated. These phosphorylation sites provide a docking site for several adaptor proteins, including SOS and Grb2, that facilitate Ras activation. SOS is a guanine exchange factor (GEF) that enables inactive GDP-bound Ras to replace GDP for GTP and thus become active. Once GTP-bound, Ras undergoes a conformational change and now has the ability to interact with downstream effector proteins that promote cell growth, differentiation, and cytoskeletal rearrangement. Since Ras' intrinsic GTPase ability is rather inefficient, it requires the assistance of GTPase activating proteins (GAPs) to hydrolyze GTP to GDP and thus become inactive. The upstream signal that directs GAPs to inactivate Ras is currently unknown. Due to this on/off signaling, Ras acts as a molecular switch to activate several signaling pathways.

Oncogenic Ras is a constitutively active version of its normal wild-type counterpart. Mutations often in either codons 12, 13, or 61 render Ras unable to hydrolyze GTP and thus remain constantly active and signaling to downstream effector pathways. Mutations in codon 12 and 13 have been shown to disrupt the interaction between Ras and GAP proteins that decrease the rate of GTP hydrolysis, while mutations at codon 61 directly impair Ras' ability to hydrolyze GTP (Pylayeva-Gupta et al. 2011). The capability of oncogenic Ras to promote cellular transformation is due in large part to its ability to constitutively signal to downstream pathways that drive proliferation and survival. Owing to this function, mutant Ras is thought to be present in 30% of all human cancers (Bos 1989). Though the incidence of mutation between the three members of the Ras family varies between cancer types, they have been found to associate with certain cancers. For instance, mutant K-Ras is found in 95% of all pancreatic cancers, H-Ras in 10% of bladder cancers, and N-Ras in 15% of melanomas (Downward 2003). While some

studies have shown that one Ras mutant can substitute for another suggesting overlapping pathological functions (To et al. 2008), the reason for these varying distributions, and why mutant K-Ras is found in nearly all pancreatic cancers while mutant H-Ras is absent is currently unknown.

Shortly after their identification, Ras' ability to promote cellular proliferation by stimulating entry in the S-phase of the cell cycle in the absence of growth stimuli was soon appreciated (Feramisco et al. 1984). It does this by directly up-regulating transcription factors required for cell cycle progression and indirectly by up-regulation of growth factors such as EGF (Pylayeva-Gupta et al. 2011). While the Ras protein's most well-recognized contributions to the hallmarks of cancer may be sustaining growth signaling and evading growth suppressors, they have been shown to participate in many of the other hallmarks. Through both the down-regulation of pro-apoptotic proteins (Ahmed et al. 2008) and up-regulation of anti-apoptotic proteins (Wu et al. 2010), Ras has been shown to modulate cell death pathways. Moreover, mutant Ras, primarily through HIF1α, has been shown to promote glycolysis to deregulate cellular energetics (Chen et al. 2001). The ability of Ras to engage multiple hallmarks of cancer make it a remarkably powerful oncogene. Its ability to modulate the tumor microenvironment will be discussed shortly.

(B) Oncogenic Ras and its effector pathways

The ability of Ras to promote a variety of phenotypic outcomes is due in part to its ability to signal to multiple downstream effector pathways. The first to be characterized and the most extensively studied is the Raf-MEK-ERK/MAPK signaling pathway. Upon exchange of GDP for GTP, active Ras now has the ability to bind and activate the Raf serine/threonine kinases that activates the MAPK signaling cascade. Once active and bound to the plasma membrane, the Raf kinases signal downstream by phosphorylating and activating the mitogen-activated protein kinases kinases 1 and 2 (MEK1 and MEK2), which in turn phosphorylate and activate the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). The active ERKs translocate to the nucleus and phosphorylate a variety of transcription factors that control cell cycle progression. Owing to the MAPK pathway's importance downstream of mutant Ras, constituitively active mutants of both Raf and MEK have been shown to fully recapitulate mutant Ras in several cell settings. In regards to the data presented here, the activity of the Ets transcription factor PEA3 has been shown to be modulated by MAPK signaling. Activation of MAPK signaling promotes sumoylation of PEA3 to enhance its transcriptional activity (Guo and Sharrocks 2009).

The next most well-characterized signaling pathway downstream of Ras is the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Containing Ras binding domains, the catalytic subunits of PI3K (i.e. p110α) can interact directly with Ras that results in PI3K activation. As lipid kinases, PI3K, now membrane bound, phosphorylates its lipid substrates which act as second messagers. Protein kinases such as AKT and PDK1 contain pleckstrin

homology (PH) domains that bind the phospho-lipids, become membrane bound, activated, and signal to downstream pathways. For instance, upon membrane binding AKT is phosphorylated at threonine 308 (by PDK1) and serine 473 (by mTORC2) and activated. Among the many targets of AKT, the pro-apopotic Bcl-2 family member BAD has been shown to be phosphorylated by AKT which inhibits BADs ability to promote cell death. Similar to the MAPK pathway, the PI3K signaling pathway has been shown to modulate a number of cellular events that include cell survival, cellular metabolism, and transcription. The PI3K arm of Ras signaling also provides a connection between Ras and Rho GTPases (Rho, Rac, and Cdc42). The production of phospho-lipids by PI3K can stimulate Rho activity through the activation of Rho GEFs. It is through Rac signaling that Ras mediates cytoskeletal rearrangement to promote cellular motility. Like MAPK and PI3K, Rac signaling has been shown to be crucial to mutant Ras-mediated transformation.

The third effector pathway to be described downstream of Ras involves the closely related RAL proteins. Through the interaction with RAL guanine nucleotide exchange factors, Ras can indirectly stimulate RAL activity that in turn plays an important role in Ras' transforming capabilities. While the RAL effector arm of Ras signaling is not completely elucidated, it has been shown to be crucial to mutant Ras function (Hamad et al. 2002). The ability of mutant Ras to engage multiple effector pathways enables it to promote various phenotypic outcomes that can contribute to tumor development. Its ability to activate numerous signaling pathways is also partially the reason why targeting mutant Ras therapeutically remains so difficult.

(C) Targeting mutant Ras and its effector pathways

Since the discovery of the Ras family members and the extensive involvement of their mutant counterparts in human cancers, the identification of a small molecule that effectively inhibits Ras activity has remained elusive. Despite a clear understanding of Ras' structure and biochemistry, targeting Ras directly through therapeutics has proven immensely challenging. Studies demonstrating that Ras activity is essential to tumor maintenance supports the notion that targeting mutant Ras remains an ideal therapeutic strategy. Depletion of mutant Ras through either genetic ablation, doxycycline-regulation, or shRNA have been shown to impair tumor growth in multiple tumor models (Chin et al. 1999; Brummelkamp et al. 2002). Initially the logical approach was to screen for small molecules that blocked loading of GTP onto inactive GDP-bound Ras, similar to already successful small molecules that inhibited ATP binding onto protein kinases. Unfortunately this approach was proven unsuccessful because unlike ATP/protein kinase binding, GTP has an extremely high affinity for Ras.

Crucial to Ras function is its ability to localize to different membrane compartments. It was reasoned that by blocking the binding of Ras to the plasma membrane, Ras proteins would be unable to exchange GDP for GTP and thus would be unable to signal to downstream effectors. For membrane localization, the Ras proteins must first be modified through the addition of hydrophobic moeities to the CAAX domain of the protein. This process is broadly referred to as farnesylation, and is accomplished through farnesyltransferases and geranylgeranyltransferases. In support of this therapeutic approach, mutagenesis studies have shown that mutation of the cysteine residue in the CAAX motif keeps Ras cytosolic and unable

to promote transformation (Jackson et al. 1990). Initially small molecules directed against these transferases showed great promise, but like many other early successes, failed in human clinical settings. It was later realized that sensitivity to these inhibitors did not correlate with Ras mutation status and furthermore the most biochemically active inhibitors effectively inhibited mutant H-Ras but not K-Ras or N-Ras (Sepp-Lorenzino et al. 1995; James et al. 1996; Whyte et al. 1997).

The next logical step in developing therapeutics against Ras-driven malignancies was to target its downstream effector pathways. This approach is supported by the fact that both Raf kinases (MAPK) and p110α (PI3K) are frequently mutated in cancer and that both effector pathways have been shown to be crucial to Ras-mediated transformation (Roberts and Der 2007; Wong et al. 2010). The importance of the MAPK pathway in cancers harboring mutant Ras is highlighted by the fact that mutations within MAPK (i.e. Raf^{V600E}) are nearly mutually exclusive with mutations within Ras suggesting nearly identical functions (Dhomen and Marais 2007). While specific and effective inhibitors of the MAPK pathway (i.e. Vemurafenib: B-Raf inhibitor) have been developed and shown to be effective in mutant Raf cancers (Flaherty et al. 2010), they have proven ineffective in treating mutant Ras cancers (Hatzivassiliou et al. 2010). This is due to the fact that mutant Ras has the ability to engage multiple effector pathways and also complicated by negative feedback mechanisms (Pratilas et al. 2009).

Currently, the most extensively studied therapeutic approaches are dual combination therapy that includes either conventional chemotherapy or radiation together with targeted therapy or dual targeted therapy. Combining classical chemotherapeutics with targeted small

molecules has shown great promise. A clinical trial treating patients with advanced hepatocellular carcinoma with both doxorubicin and the Raf inhibitor sorafenib improved both overall survival and progression-free survival (Abou-Alfa et al. 2010). While similar studies have yielded comparable results, they are complicated by possible antagonistic effects one therapy could have on another. For instance, MAPK inhibition through either small molecule inhibtion of MEK or ERK blocked cisplatin-induced apoptosis of HeLa cells (Wang et al. 2000). Furthermore, drug dosage and treatment regimen can determine whether the two treatments can potentiate each others effects.

For these reasons, it is of the utmost importance to identify druggable targets that mediate Ras' oncogenic properties. Recently, the ability of mutant Ras to up-regulate EGFR has been shown to be essential for Ras-driven pancreatic epithelial cell transformation (Ardito et al. 2012; Navas et al. 2012). The authors demonstrate that up-regulation and activation of EGFR is required for robust Ras activity and amplification of MAPK signaling that is required for metaplasia and neoplasia. Given the availability of effective EGFR inhibitors (cetuximab and erlotinib), the authors show that treatment with these EGFR inhibitors efficiently disrupted the onset of PDAC, but had no effect on its progression. These results provide rationale for treating at-risk patients with EGFR inhibitors, but also highlights the importance of identifying mediators of oncognic Ras that can be targeted therapeutically.

(D) Modulation of the tumor microenvironment by mutant Ras

While mutant Ras' cell autonomous effects have been well documented, the ability of mutant Ras to affect the tumor microenvironment has also been reported. The interaction between cancer cells and the normal host stroma (fibroblasts, endothelial, and inflammatory cells) has begun to be appreciated and the idea of targeting the tumor stroma as a therapeutic option is beginning to be explored. For example, while the genetic lesions responsible for the development of pancreatic cancer are well understood, they remain one of the most deadly cancers due in large part to the lack of effective therapeutics. It was reasoned, that chemotherapies are ineffective in treating PDAC because they are unable to access the tumor cells because of the large stromal reaction signature of PDAC. Indeed, it was recently shown that depletion of stromal tissue through inhibition of Hedgehog signaling sensitized KPC mice (K-$Ras^{LSL\text{-}G12D/+}$; $p53^{LSL\text{-}R172H/+}$; Pdx1-cre) to gemcitabine (Olive et al. 2009).

One of the earliest reports to describe the alteration of the tumor microenvironment by mutant Ras demonstrated a Ras-dependent neovascularization through the production and secretion of CXCL8/IL-8 (Sparmann and Bar-Sagi 2004). The authors reported that through its effector pathways, mutant Ras transcriptionally up-regulates CXCL8/IL-8 and in a non-cell autonomous manner recruits both inflammatory and endothelial cells to aid in angiogenesis. The depletion of CXCL8/IL-8 using a neutralizing antibody resulted in decreased tumor growth that was accompanied by both fewer endothelial and inflammatory cell infiltration.

In a similar manner, a separate report implicated IL-6 as a crucial secretory factor in mutant Ras-driven tumorigenesis (Ancrile et al. 2007). The authors demonstrated that depletion of IL-6 impaired the ability of mutant Ras-transformed human kidney cells to form xenograft tumors. Moreover, IL-6-null mice were resistant to DMBA/TPA induced papillomas, a model where mutant Ras occurs at high frequency. The reduction in tumor growth, like IL-8, was attributed to a reduction in angiogenesis.

Most recently, mutant Ras was shown to promote the production and secretion of GM-CSF at the earliest stages of pancreatic neoplasia (Pylayeva-Gupta et al. 2012). Excitingly, the authors demonstrated that through the up-regulation and secretion of GM-CSF, mutant Ras elicits an immunosuppresive response to promote tumor growth. Moreover, depletion of GM-GSF through shRNA triggered the infiltration of anti-tumor $CD8^+$ T cells that resulted in tumor cell death and regression. While all of these studies clearly demonstrate the ability of mutant Ras to promote cytokine/chemokine production, the exact mechanism of how Ras promotes this secretory response remains unknown. Therefore, a better understanding of Ras-induced cytokine production is warranted and may uncover new therapeutic targets.

In the 2nd part of our study on SCCA, we sought to identify the oncogenic regulation of SCCA and uncovered a novel, proinflammatory role for SCCA downstream of mutant Ras.

Chapter 4: Identification of oncogenic Ras-mediated up-regulation of SCCA

(A) Small-scale oncogenic screen of SCCA regulators

Following the finding that SCCA expression is elevated in advanced stage, high-grade human breast cancers, we became interested in how SCCA expression may be regulated. To address this question, we performed a small scale oncogenic screen to identify an oncogene that may mediate up-regulation of SCCA expression. We chose to perform this initial screen in the IMR90 primary human lung fibroblast cell line. The IMR90 cell line is a primary non-immortalized cell line that allows for the study of the immediate effects of oncogene activation in a pure wild-type background.

Using this approach we ectopically expressed the H-Ras^{V12}, myr-AKT, and c-Myc oncogenes using a stable retrovirus transduction. While myr-AKT and c-Myc failed to induce SCCA up-regulation, H-Ras^{V12} expression resulted in a marked increase in SCCA expression levels (Fig. 6 and Fig. 7A). As mentioned previously, the SCCA antibody utilized in our studies was unable to distinquish between SCCA1 and SCCA2 (Cataltepe et al. 2000; Catanzaro et al. 2011). To differentiate between SCCA1 and SCCA2, we performed quantitative RT-PCR (qRT-PCR) and found a drastic increase in SCCA1 and SCCA2 transcript levels in reponse to H-Ras^{V12} (Fig. 7B). This is not suprising as the promoter of SCCA1 and SCCA2 are highly homologous (Hamada et al. 2001; Suminami et al. 2005). To exclude cell line specificity, we ectopically expressed H-Ras^{V12} in the BJ primary foreskin fibroblast cell line and assessed

SCCA expression. Similar to IMR90 cells, the introduction of H-Ras^{V12} resulted in up-regulation of both SCCA1 and SCCA2 at both the protein and transcript level (Fig. 7C,D).

In both IMR90 and BJ cells, the introduction of H-Ras^{V12} elicits the tumor suppressive response known as oncogene-induced senescence (OIS) (Serrano et al. 1997). To exlcude the possibility that senescence was a prerequisite for SCCA expression, we ectopically expressed Ras^{V12} in several cancer cell lines that express wild-type Ras (HT-29 and Caco-2) and HeLa cells. In these cell lines, the introduction of oncogenic Ras does not trigger OIS (Sparmann and Bar-Sagi 2004; Kikuchi et al. 2009). For these set of experiments, we utilized K-Ras^{V12} as it is much more frequently mutated in human cancers (Downward 2003) and also allowed us to exclude the possibility that Ras^{V12} up-regulation of SCCA was not specific to H-Ras^{V12}. In all three cell lines, ectopic expression of K-Ras^{V12} resulted in up-regulation of SCCA at both the protein and transcript level (Fig. 8A,B). These findings indicate that Ras^{V12} up-regulation of SCCA is not due to senesence but due directly to expression of Ras^{V12}. Also, the ability of Ras^{V12} to up-regulate SCCA is conserved between H-Ras and K-Ras and may be a general phenomena of the Ras family.

In support of the idea that SCCA up-regulation by Ras^{V12} in IMR90 and BJ fibroblast is not a consequence of the senescence response, we chose to induce premature senescence independent of Ras^{V12} and assess SCCA expression. To this end, IMR90 cells were treated with the DNA damage inducing topoisomerase inhibitor etoposide (10 μM, 48 hr) and H_2O_2 (10 μM, 1 hr) and assayed 7 days post-treatment. Despite undergoing senescence, as seen through β-gal positivity and p21 up-regulation, premature senescence induced by etoposide or H_2O_2 failed to

up-regulate SCCA expression (Fig. 9A,B). Similarly, IMR90 cells undergoing replicative senescence, triggered by continually passaging the cells, failed to exhibit up-regulation of SCCA (Fig. 9C,D).

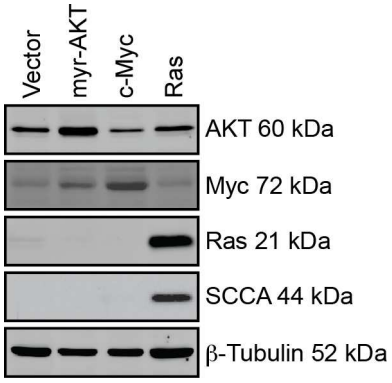


Figure 6. Ras^{V12} but not myr-AKT or c-Myc induces SCCA expression. Indicated oncogenes were stably expressed in IMR90 cells. Whole cell lysates were obtained and analyzed by western blot with indicated antibodies.

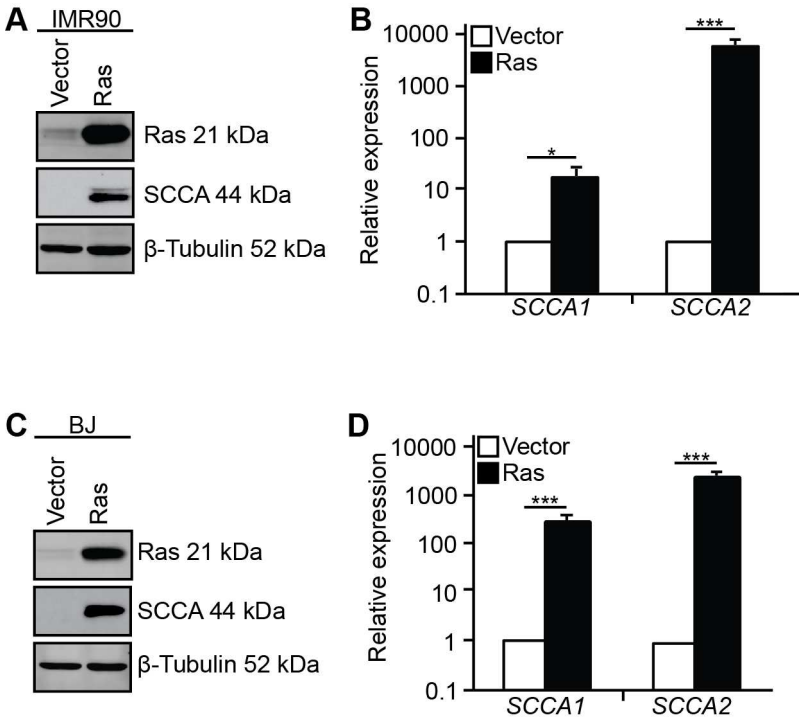


Figure 7. Oncogenic Ras induces SCCA expression in primary human cell lines. IMR90 and BJ primary human fibroblasts were stably transduced with either vector control or H-Ras^{V12} and harvested 7 days post-selection. **(A,C)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B,D)** Total RNA was extracted and 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. *p<0.05; ***p<0.001 by t-test.

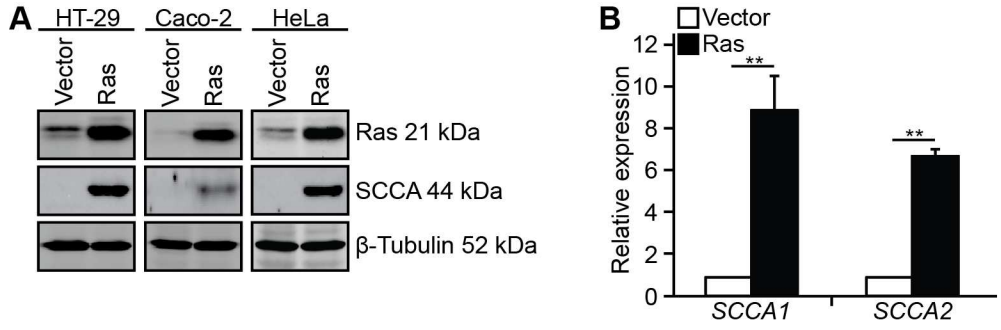


Figure 8. Oncogenic Ras induces SCCA expression in human cancer cell lines with wild-type Ras. Caco-2, HT-29, and HeLa cells were stably transduced with either vector control or K-Ras^{V12} and harvested 7 days post-selection. **(A)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B)** Total RNA from HeLa cells was extracted and 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. **$p<0.01$ by t-test.

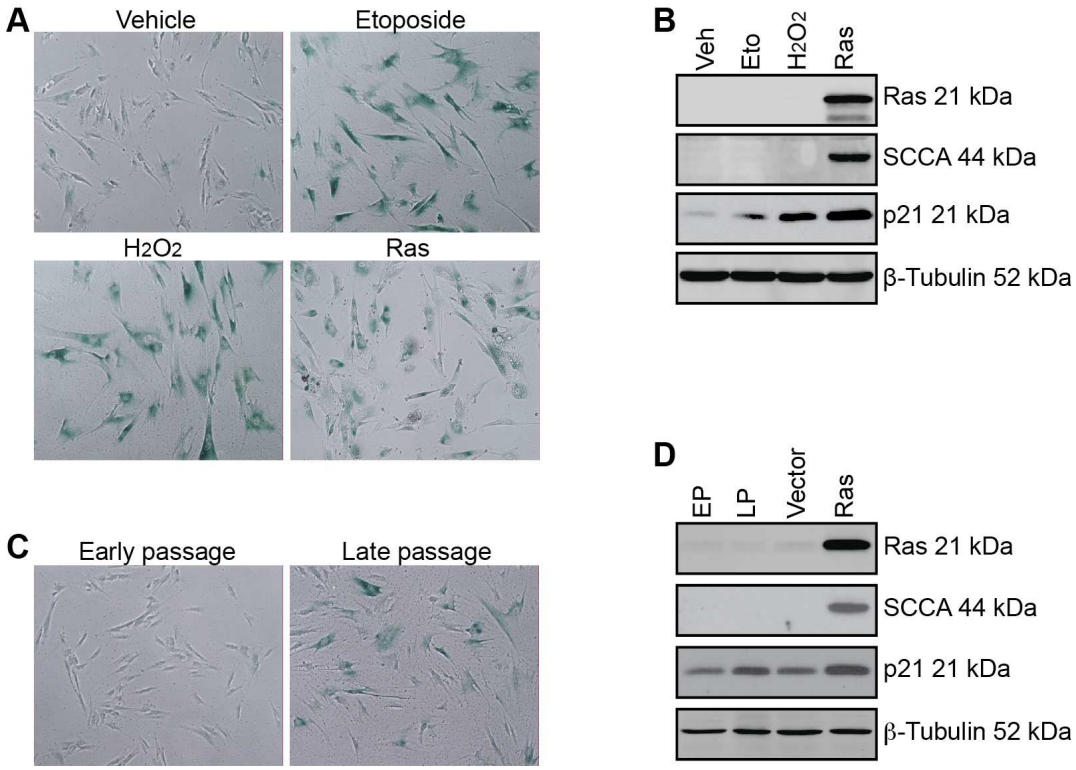


Figure 9. SCCA expression is induced by Ras^{V12}, but not by premature senescence induced by DNA damage or replicative senescence. (A,B) IMR90 cells were treated with vehicle-control, etoposide (10 μM) for 48 h, H_2O_2 (10 μM) for 1 h, or stably transduced with Ras^{V12}, and then analyzed 7 d post-treatment. (**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.

(B) Ras^{V12}-mediated up-regulation of SCCA is MAPK/PEA3-dependent

The above results suggest that active Ras signaling can directly promote SCCA transcription. To ascertain whether Ras^{V12} signaling or a possible Ras^{V12}-mediated epigenetic alteration was regulating SCCA transcription, we utilized the estrogen receptor (ER):H-Ras^{V12} fusion protein. Here, H-Ras^{V12} can easily be turned on/off since it is fused to a modified estrogen receptor that is only stable in the presence of 4-hydroxytamoxifen (4-OHT) (Young et al. 2009). In the absence of 4-OHT, Ras transcript and protein are constitutively transcribed and translated, but the protein is quickly degraded as the ER domain is unstable without 4-OHT bound to it. Upon addition of 4-OHT, the protein is quickly stabilized and H-Ras^{V12} can effectively signal to downstream pathways.

Using this approach, cells stably expressing ER:Ras^{V12} were treated with 4-OHT for 8 days to induce Ras^{V12} protein expression. As expected, addition of 4-OHT turned on Ras^{V12}, followed by the up-regulation of SCCA (Fig. 10A,B). The removal of 4-OHT resulted in a drastic reduction in Ras^{V12} protein levels and diminished downstream signaling indicated by decreased levels of phosho-ERK. This was accompanied by a reduction in SCCA protein and transcript levels (Fig. 10A,B). These results suggest that SCCA up-regulation by Ras^{V12} does not occur through an epigenetic mechanism, but rather sustained Ras^{V12} signaling is required to maintain SCCA expression.

Since the above results indicate that SCCA up-regulation is mediated directly through signaling pathways downstream of Ras^{V12}, we chose to selectively inhibit these pathways to elucidate which may be directly regulating SCCA transcription. The two most well-characterized pathways downstream of Ras^{V12} are the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K), both of which have effective, specific inhibitors. Vector-control or Ras^{V12}-expressing cells were treated 7 days post-selection with either vehicle-control (DMSO), AKTi (10 μM, 24 hr) to inhibit PI3K pathway or U0126 (10 μM, 24 hr) to inhibit MAPK signaling and SCCA levels were assessed. Despite effective inhibition of PI3K signaling as seen through decreased amounts of phospho-AKT, this inhibition had little to no effect on Ras^{V12}-induced SCCA up-regulation (Fig. 11A,B), suggesting that PI3K signaling does not activate SCCA transcription downstream of Ras^{V12}. However, successful inhibition of MAPK signaling, as seen through decreased amounts of phospho-ERK, resulted in a significant reduction in both SCCA1 and SCCA2 transcript levels (Fig. 11C,D). Simlar results were obtained in Ras^{V12}-expressing HeLa cells (Fig. 12). Taken together, these results indicate that activation of SCCA transcription downstream of Ras^{V12} is mediated through MAPK signaling.

We next sought to address what transcription factors may be acting downstream of MAPK signaling to activate SCCA transcription. We chose to focus on the Ets transcription factor family member PEA3, as it has been shown to activate SCCA transcription and be modulated by MAPK signaling (Iwasaki et al. 2004; Guo and Sharrocks 2009). Vector or Ras^{V12}-expressing cells were stably transduced with either a control short-hairpin (shNTC) or shRNA targeting PEA3 (Fig. 13). Silencing of PEA3 resulted in a drastic decrease of both SCCA1 and SCCA2 at protein and transcript levels (Fig. 13A,C). Importantly, PEA3 depletion did not affect

MAPK signaling, as the levels of phospho-ERK were equal in all Ras^{V12} expressing cells (Fig. 13A). Similar results were obtained in Ras^{V12}-expressing HeLa cells (Fig. 13D-F). These results indicate that expression of Ras^{V12} leads to SCCA up-regulation that is dependent on the Ets family transcription factor PEA3.

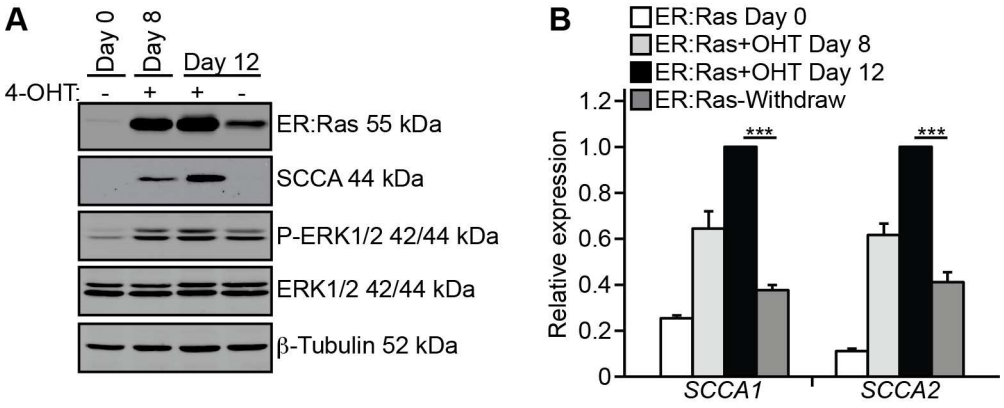


Figure 10. Sustained Ras signaling is required for SCCA up-regulation. (A,B) IMR90 cells expressing the ER:Ras^{V12} fusion protein were treated with 4-OHT for 8 d, split and either cultured in media containing 4-OHT or withdrew 4-OHT for additional 4 d. **(A)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B)** Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to that of Day 12 ER:Ras with 4-OHT cells. Data shown are mean + SEM of three independent experiments performed in triplicate. ***$p<0.001$ by t-test.

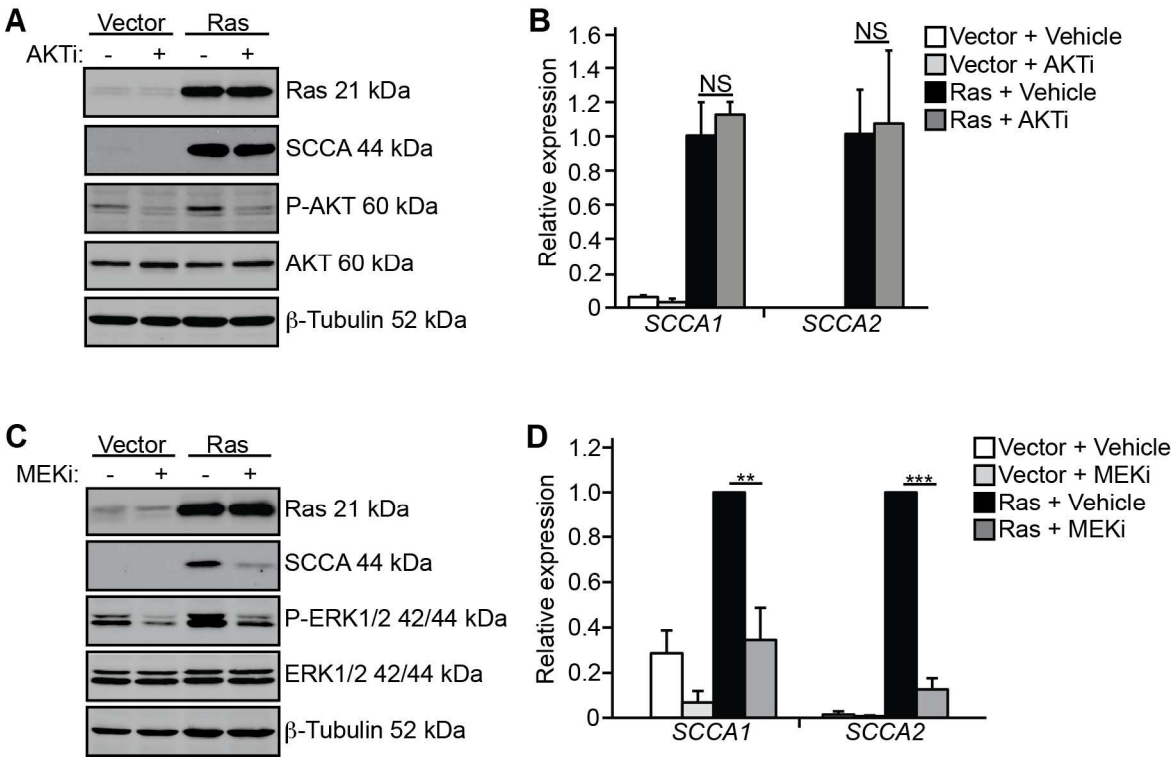


Figure 11. SCCA up-regulation is dependent on active MAPK signaling, but not AKT in IMR90 cells. (A-D) Vector-control or Ras^{V12}-expressing IMR90 cells were treated with either vehicle control (DMSO), AKTi (10 μM), or U0126 (MEKi, 10 μM) for 24 h. **(A,C)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B,D)** Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to that of Ras^{V12}-expressing cells treated with vehicle. Data shown are mean + SEM of two independent experiments for AKTi and three independent experiments for MEKi performed in triplicate. **p<0.01; ***p<0.001: NS, non-significant by t-test.

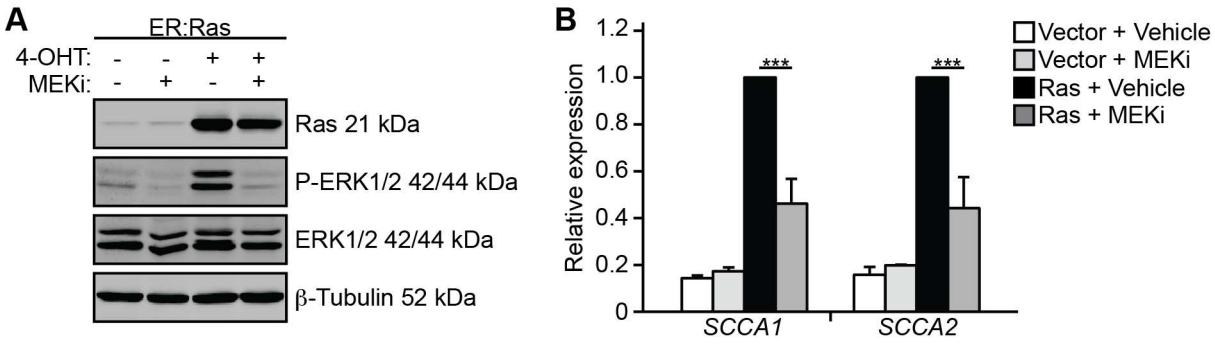


Figure 12. Ras-induced SCCA expression is sensitive to the inhibition of MAPK in HeLa cells. (**A,B**) ER:Ras^{V12}-expressing HeLa cells were treated with either vehicle control or U1026 (MEKi, 10 μM) for 24 h. (**A**) Whole cell lysates were analyzed by western blot with indicated antibodies. (**B**) Total RNA was extracted and SCCA1 and SCCA2 transcript levels were analyzed via qRT-PCR, and normalized to Ras^{V12}-expressing cells treated with vehicle control. Data shown are mean + SEM of two independent experiments performed in triplicate. ***p<0.001 by t-test.

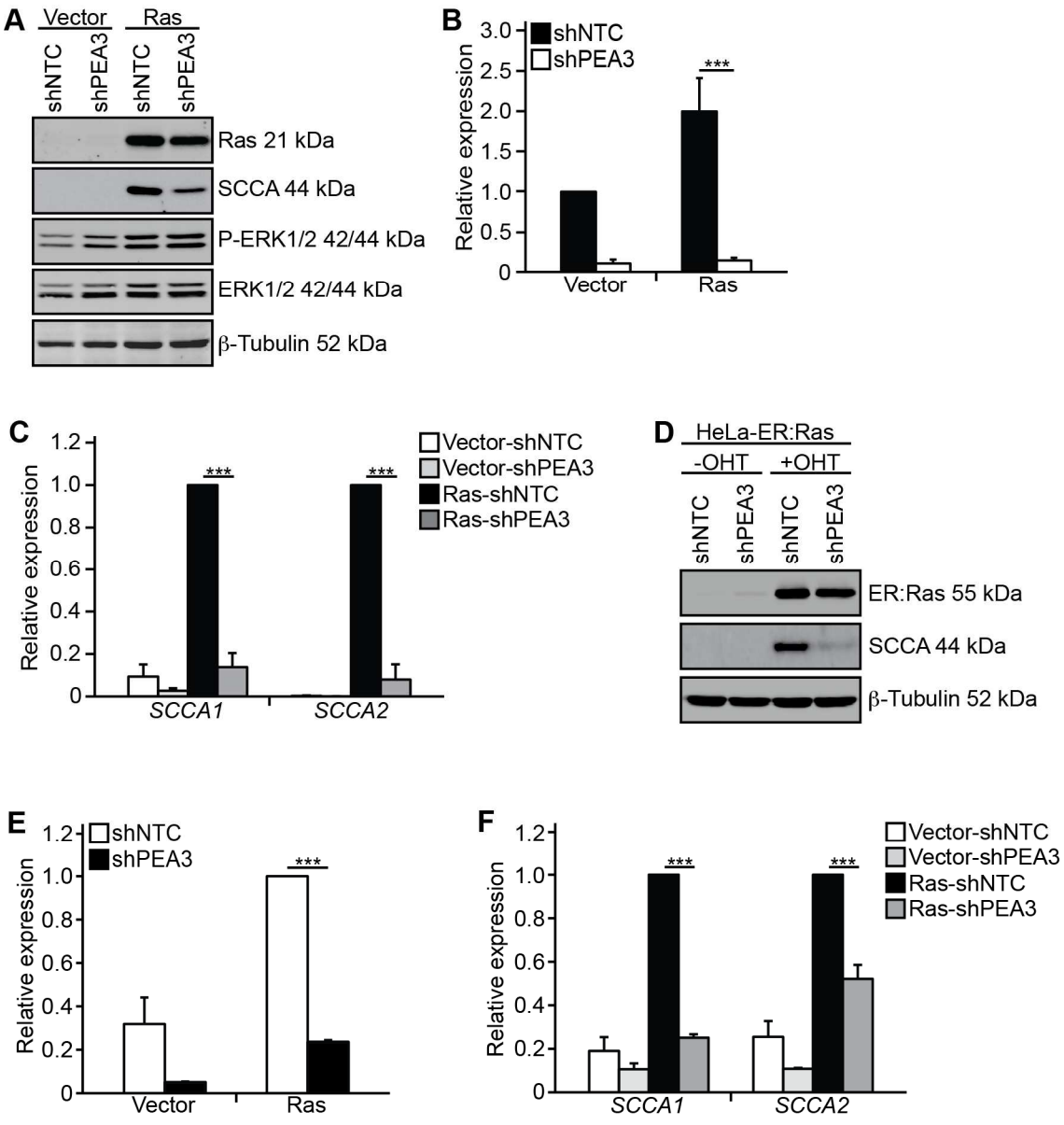


Figure 13. Ras-induced SCCA expression is mediated by the Ets transcription factor PEA3. **(A-C)** Vector-control or Ras^{V12}-expressing IMR90 cells were stably transduced with shNTC (non-target control) or shPEA3. **(A)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B,C)** Total RNA was extracted and the transcript levels of **(B)** PEA3 and **(C)** SCCA1 and SCCA2 were analyzed via qRT-PCR. Transcript levels were normalized to Ras-shNTC cells. Data shown are mean + SEM of three independent experiments performed in

triplicate. **(D-F)** K-Ras^{V12} HeLa cells were stably transduced with either shNTC or shPEA3. (**D**) Whole cell lysates were analyzed by western blot with indicated antibodies. (**E,F**) Total RNA was extracted and the transcript levels of **(E)** PEA3 and (**F**) SCCA1 and SCCA2 were analyzed via qRT-PCR. Transcript levels were normalized to Ras-shNTC cells. Data shown are mean + SEM of two independent experiments performed in triplicate. ***$p<0.001$ by t-test.

(C) Ras^{V12}-induced up-regulation of SCCA mediates cytokine production

We next sought to identify a functional role for SCCA downstream of Ras^{V12}. We chose to again utilize the IMR90 primary fibroblast cell line, as this cell line has a very robust phenotype in response to Ras^{V12}. After an initial proliferative burst, cells expressing Ras^{V12} undergo a stable arrest of the cell cycle referred to as oncogene-induced senescence (OIS) (Serrano et al. 1997). This senescent response is characterized by a DNA damage response (DDR) and a pro-inflammatory secretory profile known as senesence-associated secretory phenotype (SASP) (Di Micco et al. 2006; Coppe et al. 2008; Kuilman et al. 2008). Using this model allows for the study of the immediate effects of Ras^{V12} activation in a wild-type background.

Initially, we examined the senescence response triggered by Ras^{V12} in cells where SCCA had been silenced. It has been well reported that in IMR90 cells Ras^{V12} activates the p53, Rb, and p16 tumor suppressor networks to block cell cycle progression and induce the senescence response. The senescent response is triggered by mutant Ras's oncogenic properties and its ability to promote replication. We reasoned that if SCCA was functioning downstream of Ras^{V12}, then it may alter the ability of Ras to promote proliferation and thus induce senescence. Vector-control or Ras^{V12}-cells expressing either a control short-hairpin (shNTC) or two independent shRNAs targeting SCCA were harvested and analyzed 7 days post-selection. It is important to note that the hairpins targeting SCCA target regions where SCCA1 and SCCA2 are highly homologous and thus efficiently silence SCCA1 and SCCA2 (Fig. 14A). Cells were cultured with BrdU (10 μM, 6 hr) 7 days post-selection and assessed for cell proliferation through a BrdU

incorporation assay. As expected, Ras-shNTC cells incorporated BrdU at a decreased rate than vector-control cells indicating a senescent growth arrest (Fig. 14B). Senescence cells can also be quantified through senescence-associated heterochromatic foci (SAHF), as DAPI staining exhibits a punctate pattern when cells undergo OIS. Indeed, Ras-shNTC cells exhibited a far greater percentage of SAHF-positive cells, again indicating a senescent phenotype (Fig. 14B). Loss of SCCA had a minimal effect on Ras^{V12}-induced senescence, as they incorporated BrdU and showed SAHF-positivity at similar rates of Ras-shNTC cells (Fig. 14B). Furthermore, Ras^{V12}-expressing cells where SCCA was silenced displayed up-regulation of the CDK inhibitors p16 and p21 to similar levels of Ras^{V12}-expressing control cells (Fig. 14C).

Despite the finding that loss of SCCA does not affect the growth arrest phenotype of Ras^{V12}-induced senescence, this allowed us to study other possible functions for SCCA independent of any difference in cell cycle arrest. We chose to next focus our attention on the downstream pathways activated by mutant Ras (AKT, MAPK, NF-κB). Utilizing phosphorylation specific antibodies, we assessed signaling pathways downstream of Ras^{V12} following SCCA silencing. While loss of SCCA had little to no effect on the ability of mutant Ras to activate AKT and MAPK phosphorylation (Fig. 15A), loss of SCCA dramatically diminished Ras^{V12}-induced RelA/p65 phosphorylation that is indicative of NF-κB activation (Fig. 15A). This effect was further confirmed by assessing the relative amounts of nuclear localized RelA/p65 through immunoblot (Fig. 15B) and by utilizing a NF-κB luciferase reporter construct (Fig. 15C).

Activation of NF-κB signaling has been attributed to cytokine production downstream of Ras activation (Chien et al. 2011). Therefore, we examined whether SCCA plays a role in Ras-induced cytokine production. We initially performed an ELISA against IL-6 to quantify the amount of secreted IL-6. As reported, Ras-shNTC cells exhibited robust secretion of IL-6 when compared to vector-control cells (Fig. 15D). In agreement with NF-κB signaling, silencing of SCCA suppressed IL-6 secretion 10-100-fold when compared to Ras-shNTC cells (Fig. 15D). We next performed a quantitative cytokine array analysis using conditioned cell culture media. As reported in literature (Coppe et al. 2008; Kuilman et al. 2008), 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. 16A,B). Consistent with the observed decrease in NF-κB signaling (Fig. 15A-C), this increased cytokine expression was significantly abrogated upon SCCA silencing (Fig. 16A,B).

We next wondered whether loss of SCCA was inhibiting secretion of these proteins or whether this observation was because of transcriptional differences. To address this question, we harvested total RNA from cells 7 days post-selection and performed qRT-PCR against those cytokines that exhibited a difference in the cytokine array. In direct agreement with the array results, while Ras-shNTC cells exhibited robust cytokine production, SCCA silencing severely abrogated cytokine transcription (Fig. 16C). The introduction of oncogenic Ras in IMR90 elicits a DNA damage response that has been reported to mediate cytokine production (Coppe et al. 2008; Rodier et al. 2009). However, silencing of SCCA had virtually no effect on Ras-induced DNA damage indicated by the phosphorylation of H2A.X (Fig. 17A,B). Taken together, these

results indicate that SCCA plays an essential role in Ras-mediated NF-κB activation and inflammatory cytokine production that is independent of the DNA damage response.

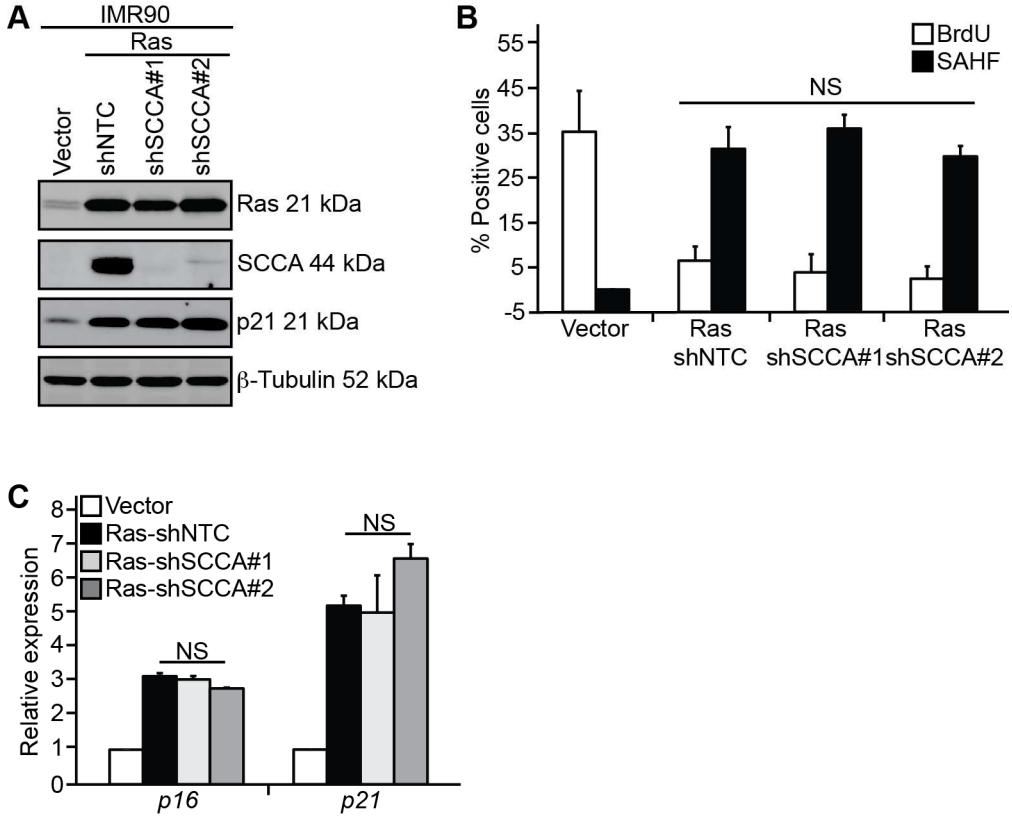


Figure 14. SCCA silencing does not effect Ras-induced senescence. (A-C) Vector-control or Ras^{V12}–expressing IMR90 cells were stably transduced with lentiviral shRNA control (shNTC) or two independent hairpins targeting SCCA. **(A)** Whole cell lysates were analyzed by western blot with indicated antibodies. **(B)** Cells were cultured with BrdU (10 μM) for 6 h and immunofluorescence against BrdU was performed. Quantification of BrdU-positive and senescence associated heterochromatic foci (SAHF)-positive cells are shown. Data shown are mean + SEM of three independent experiments. **(C)** Total RNA was extracted and the expression level of p16 and p21 was analyzed via qRT-PCR, and normalized to vector-control cells. Data shown are mean + SEM of three independent experiments performed in triplicate. NS, non-significant by t-test.

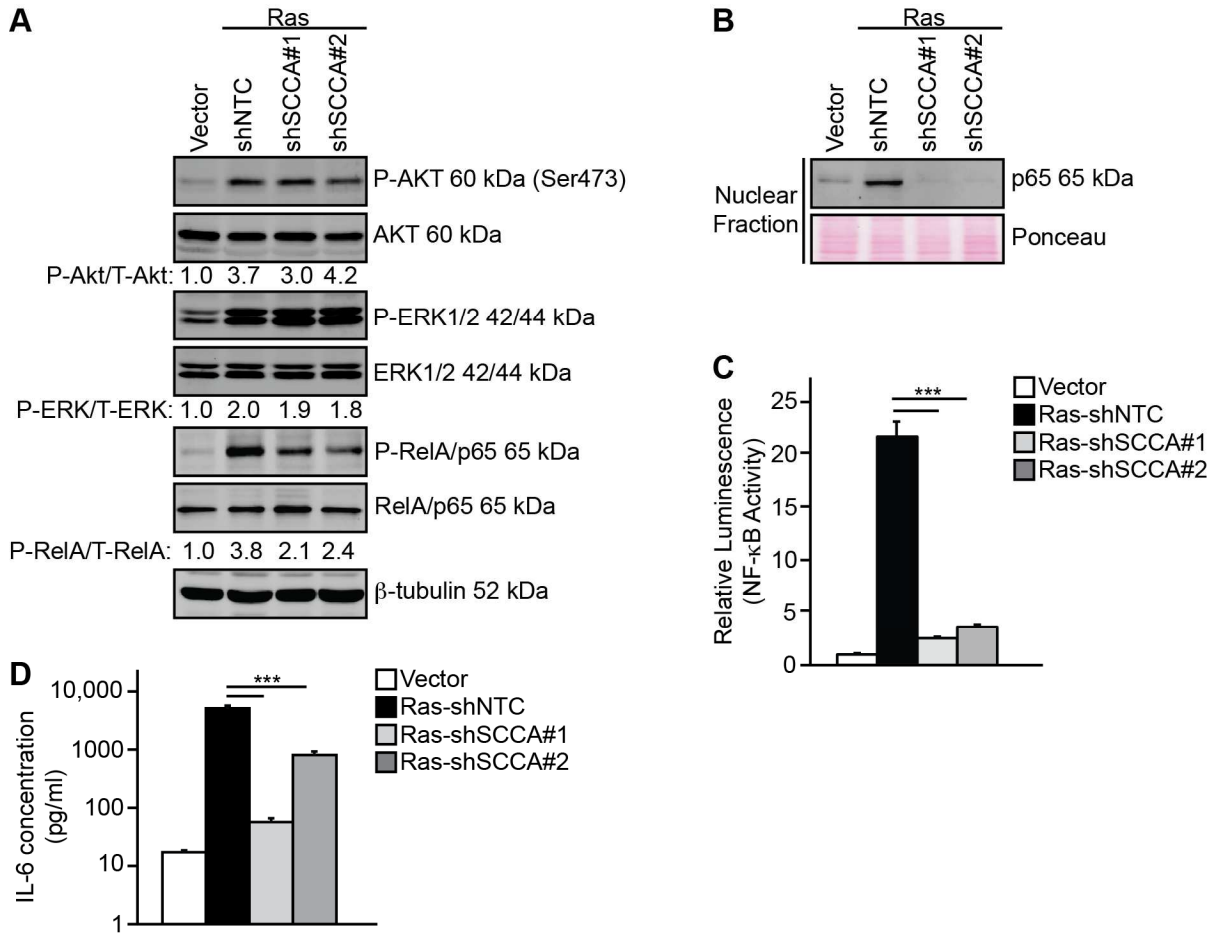


Figure 15. SCCA silencing abrogates NF-κB activation and IL-6 production. (A-D) Vector-control or Ras^{V12}–expressing IMR90 cells were stably transduced with shRNA control (shNTC) or two independent hairpins targeting SCCA and analyzed 7 days post-selection. Whole cell lysates **(A)** and nuclear-localized proteins **(B)** 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 an ELISA against IL-6. Data shown are mean + SEM of three independent experiments performed in triplicate. ***p<0.001.

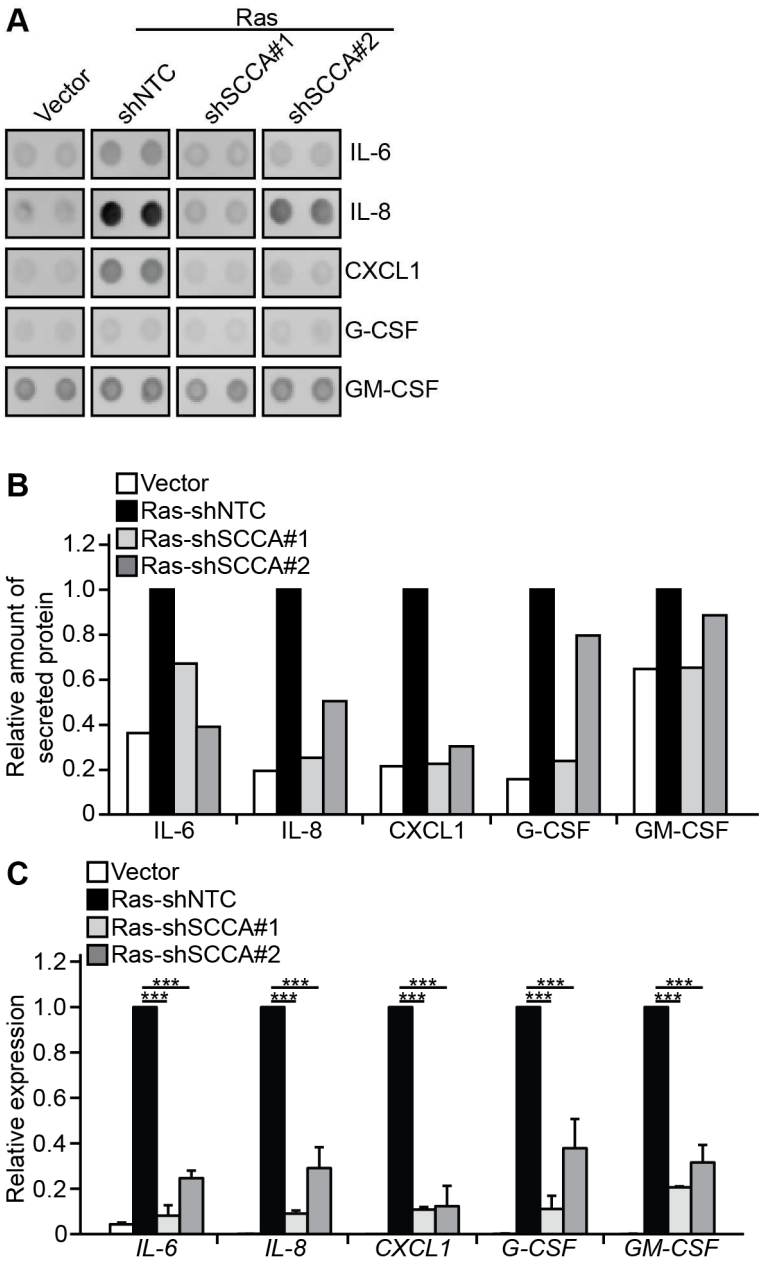


Figure 16. SCCA modulates Ras-induced cytokine production. (A-C) Vector-control or Ras^{V12}–expressing IMR90 cells were stably transduced with shRNA control (shNTC) or two independent hairpins targeting SCCA and analyzed 7 days post-selection. **(A)** Culture media were collected and subjected to a cytokine antibody array. The blots of indicated cytokines are shown and are representative of two independent experiments. **(B)** The relative amount of cytokines was quantified and normalized to that of Ras^{V12}-shNTC cells. **(C)** 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. ***$p<0.001$ by t-test.

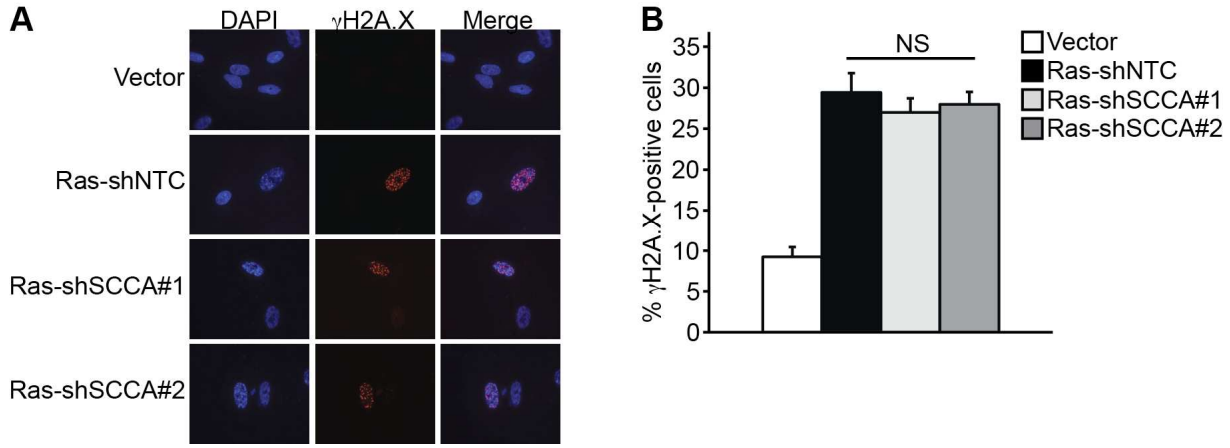


Figure 17. Silencing of SCCA does not interfere with the DNA damage response. (A,B) Vector-control or Ras^{V12}-expressing IMR90 cells were stably transduced with shRNA control (shNTC) or two independent hairpins targeting SCCA and analyzed 7 days post-selection. **(A)** Immunofluorescence against γH2A.X was performed. Representative images are shown. **(B)** Quantification of percent γH2A.X-positive cells is shown. Note that silencing of SCCA does not compromise Ras^{V12}-induced DNA damage. Data shown are mean + SEM of two independent experiments. NS, non-significant by t-test.

(D) SCCA promotes cytokine production by inducing the unfolded protein response (UPR)

Our focus then turned to how SCCA may be activating NF-κB signaling downstream of Ras^{V12} to promote cytokine production. Previous work in our lab has shown that ectopic expression of SCCA increases basal level ER-stress and sensitizes cells to proteotoxic therapeutics (Ullman et al. 2011). Though limited, there are reports suggesting that ER-stress can promote NF-κB signaling (Garg et al. 2012). Interestingly, the ability of oncogenic Ras to elicit an ER-stress response has been reported, although the exact mechanism by which Ras does this is currently unknown (Denoyelle et al. 2006). We therefore suspected that by up-regulating SCCA, Ras^{V12} was triggering a sub-lethal ER-stress response to activate NF-κB signaling. The ER-stress response caused by oncogenic Ras has been associated with the vacuolization of the cell (Denoyelle et al. 2006), and we noticed that Ras-shNTC cells exhibited a dramatic vacuolization that was completely lost in cells where SCCA silenced (Fig. 18A). Interestingly, the Ras^{V12}-expressing cells that stained most robustly for SCCA through immunofluorescence were those cells that were highly vacuolized (Fig. 18B). Our previous work has shown that SCCA inhibits degradation through the proteasome, although not through inhibition of proteolytic activity. Indeed, Ras-shNTC cells appeared to have impaired proteasome function as seen through immunoblot analysis against total ubiquitin. This inhibition was partially relieved upon SCCA knockdown (Fig. 18C). To further characterize the ER-stress response in Ras^{V12} cells, we probed for several effectors of the ER-stress response through immunoblot analysis. The appearance of cleaved ATF6, as well as the spliced active form of XBP1 in Ras-shNTC cells indicated an active UPR (Fig. 18D). The UPR as evidenced through the appearance of ER-stress effectors was severely reduced in SCCA silenced cells (Fig. 18D).

We next wondered whether the ER-stress response was mediating activation of NF-κB signaling to promote cytokine production. To this end, we silenced ATF6 and XBP1 using shRNA in Ras^{V12}-expressing cells and examined cytokine production. Indeed, the silencing of both ATF6 or XBP1 (Fig. 19A) resulted in decreased levels of NF-κB signaling as seen through phospho-p65 (Fig. 19C) and drastic reductions of IL-6, IL-8, CXCL1, G-CSF, GM-CSF transcript levels (Fig. 19B). In order to see if SCCA was activating NF-κB signaling via the ER-stress response, we ectopically expressed the transcriptionally active spliced form of XBP1 (XBP1s) in cells where SCCA was silenced (Fig. 20A). As previously noted, SCCA knockdown in Ras^{V12} expressing cells resulted in diminished cytokine production that was accompanied by reduced amounts of spliced XBP1 (Fig. 20A,B). The restoration of XBP1s levels nearly completely rescued SCCA silencing and restored cytokine levels to that of Ras-shNTC (Fig. 20B). Taken together, these results suggest that through up-regulation of SCCA, Ras^{V12} elicits an ER-stress response that activates NF-κB signaling to promote cytokine production.

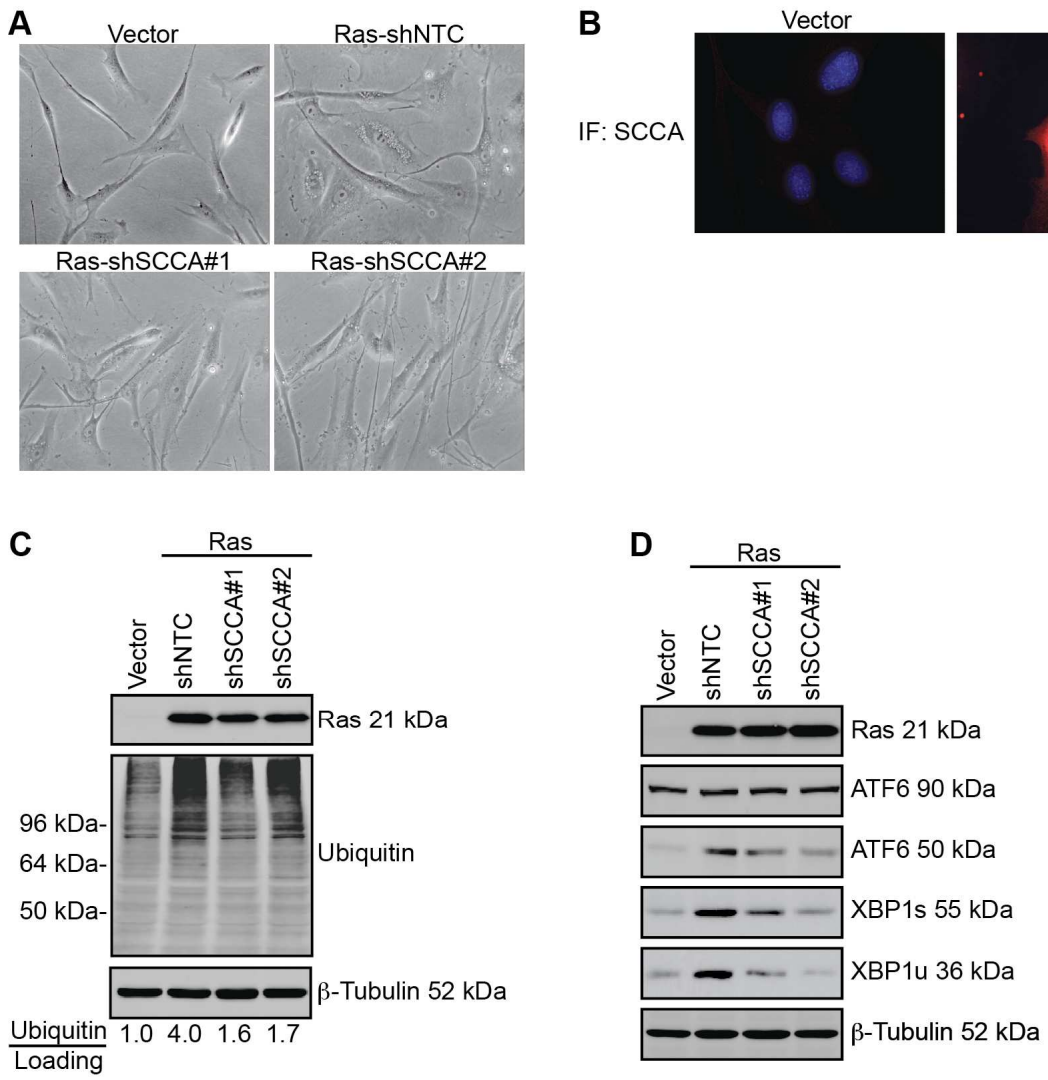


Figure 18. SCCA silencing relieves Ras-induced ER-stress. (A-D) Vector-control or Ras^{V12}-expressing IMR90 cells were stably transduced with shRNA control (shNTC) or two independent hairpins targeting SCCA and analyzed 7 days post-selection. **(A)** Representative phase-contrast images of indicated cell lines are shown. Note loss of vacuoles in SCCA knockdown cells. **(B)** Immunofluorescence against SCCA was performed; cells were counterstained with DAPI. Note highly vacuolized, SCCA-positive Ras-expressing cell. **(C,D)** Whole cell lysates were obtained and analyzed by western blot with indicated antibodies.

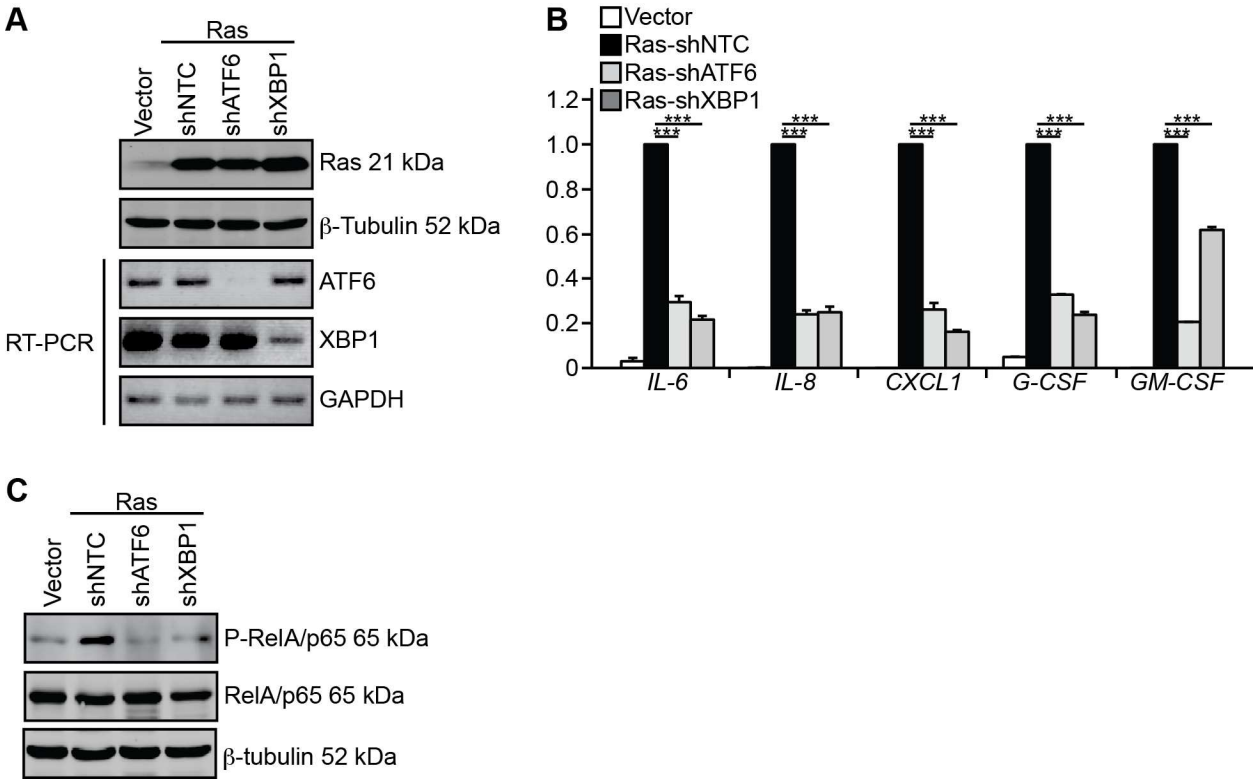


Figure 19. Silencing of ER-stress effectors ATF6 or XBP1 abrogates Ras-induced cytokine production. (A,B) Vector control or Ras^{V12}-expressing IMR90 cells were stably transduced with shRNA control (shNTC) or shRNA hairpins targeting ATF6 or XBP1. **(A,C)** Whole cell lysates were obtained and analyzed by western blot with indicated antibodies. **(B)** 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. ***$p<0.001$ by t-test.

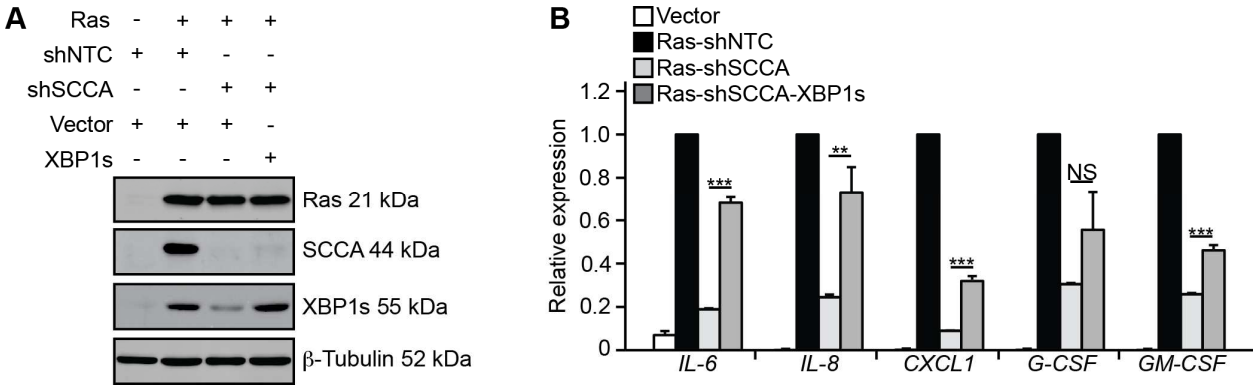


Figure 20. Ectopic expression of XBP1s rescues cytokine production in SCCA silenced cells. **(A,B)** Vector-control or Ras^{V12}-expressing IMR90 cells were stably transduced with shRNA control (shNTC) or shSCCA, together with vector control or XBP1s-expressing construct. **(A)** Whole cell lysates were obtained and analyzed by western blot with indicated antibodies. **(B)** 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.

(E) Up-regulation of SCCA in human colorectal cancer

Thus far, all of our data has been from human cell lines. We attempted to identify the mouse serpins that responded in a similar manner as SCCA1 and SCCA2 did to Ras^{V12}, in hopes of utilizing the well-established LSL-K-Ras^{G12D} mouse model. While SCCA1 or SCCA2 do not have true mouse homologs, there are orthologs within the mouse serpin family (Askew et al. 2004). Our efforts to identify the mouse serpin that behaved similar to SCCA downstream of Ras^{V12} were unsuccessful, as mutant Ras failed to up-regulate Serpinb3a or Serpinb3b in NIH 3T3 fibroblasts (Fig. 21A) and primary mouse embryonic fibroblasts (Fig. 21B). We therefore sought to find an *in vivo* connection between mutant Ras and SCCA using available patient data in The Cancer Genome Atlas (TCGA). The TCGA database offers an easily accessible bank of tumor data including mutational status, gene expression, and copy number variation divided into cancer types. Of the available datasets, colorectal cancer offered the most ideal cancer type, as mutant K-Ras has been reported to be present in 45% of cancer samples (Downward 2003). Using this approach, we found a statistically significant correlation between the presence of mutant Ras and up-regulation of SCCA (Fig. 22). This finding supports our *in vitro* finding that suggests mutant Ras can directly up-regulate SCCA gene expression.

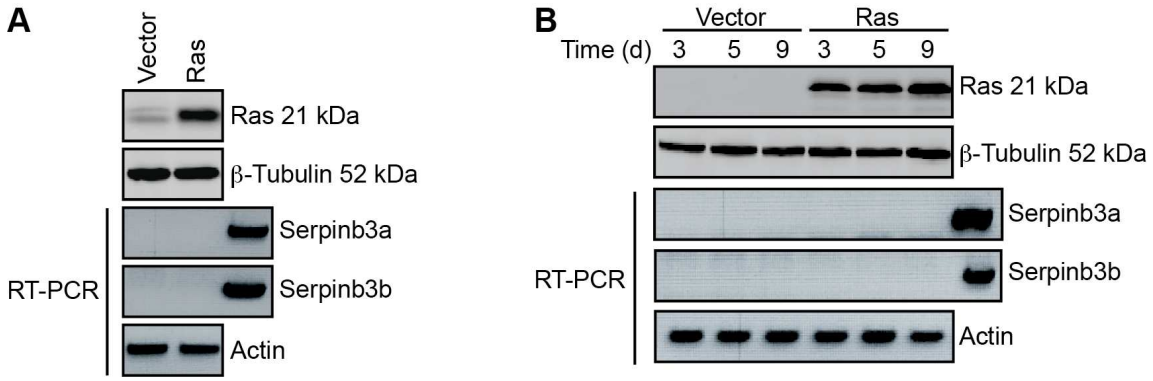


Figure 21. Oncogenic Ras fails to induce Serpinb3a and Serpinb3b in murine cells. (A) NIH 3T3 cells stably expressing vector-control or Ras^{V12} were harvested 7 d post-selection. Whole cell lysates and total RNA were obtained and analyzed by western blot with indicated antibodies or semi-quantitative RT-PCR for Serpin expression. Note that since antibodies against murine Serpinb3a and Serpinb3b are not available, semi-quantitative RT-PCR was performed. **(B)** Primary MEFs were stably transduced with vector-control or Ras^{V12}, and harvested 3, 5, and 9 d post-selection. Whole cell lysates and total RNA were obtained and analyzed by western blot with indicated antibodies or semi-quantitative RT-PCR for serpin expression. Serpinb3a and Serpinb3b expression constructs were used as positive controls for RT-PCR.

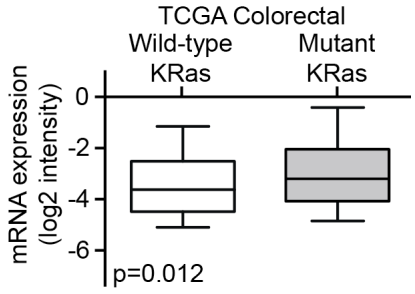


Figure 22. SCCA expression levels correlate with the presence of mutant K-Ras in human colorectal cancer. TCGA human colorectal cancer data of somatic mutation and RNA expression from Broad Institute's Genome Data Analysis Center were analyzed. There were 207 human colorectal tumors that have both somatic mutation and mRNA expression data available. K-Ras was mutated in 87 out of the 207 samples. By comparing SCCA mRNA expression level of the groups with wild-type and mutated K-Ras, SCCA expression was found to be significantly higher in the group with K-Ras 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) K-Ras samples are shown. p = 0.012 by Wilcoxon Rank Sum test.

(F) Involvement of SCCA in pancreatic cancer progression

We chose to extend our *in vivo* approach by examining SCCA expression in human pancreatic ductal adenocarcinoma (PDAC) samples. Pancreatic cancer offers a unique tumor model because the incidence of mutant K-ras has been reported as high as 95% (Downward 2003). The pancreas also offers the opportunity to study the early neoplastic lesions that precede the development of PDAC. Pancreatic intraepithelial neoplasias (PanINs) are the earliest precursor lesions to PDAC and have a defined, well-characterized progression that can be pathologically graded (I-III). It is well-accepted that mutant K-Ras is often the initiating hit in PDAC and drives the formation of the earliest PanIN lesions (Maitra and Hruban 2008).

We began this approach by utilizing the Oncomine database that contains numerous pancreatic cancer gene expression datasets. In doing so, we identified SCCA gene expression to be up-regulated in five independent datasets when comparing normal pancreatic tissue to pancreatic cancer (Fig. 23A,B). Across the five datasets, SCCA1 showed an average 3.25-fold up-regulation, while SCCA2 showed a 2.33-fold up-regulation. Furthermore, in a separate dataset both SCCA1 and SCCA2 were both significantly up-regulated in pancreatic cancer when compared against chronic pancreatitis samples (Fig. 23C).

To confirm the results obtained through Oncomine, we obtained a human pancreatic tissue microarray that contained normal pancreatic tissue, PanINI-III lesions, and PDAC samples. IHC against SCCA was performed and samples were scored for SCCA positivity. While all non-neoplastic/normal pancreatic samples were negative for SCCA expression, SCCA

positivity was seen throughout pancreatic cancer progression. The incidence of SCCA positivity progressively increased as pancreatic cancer progressed: in 2 out of 17 (11.8%) PanIN I lesions, 5 out of 19 (26.3%) PanIN II lesions, 8 out of 15 (53.3%) PanIN III lesions, and 20 out of 30 (66.7%) PDAC samples (Fig. 24A,B).

Our *in vitro* data indicated that Ras^{V12}-induced up-regulation of SCCA promotes cytokine production. We continued this approach by utilizing that same panel of pancreatic tissue that had been screened for SCCA positivity and performed IHC against IL-6 to assess to correlation between SCCA positivity and IL-6 positivity. Consistent with our *in vitro* results, IHC against IL-6 showed a positive correlation between IL-6 staining intensity and SCCA-positivity in both PDAC (Fig. 25A,B) and PanIN samples (Fig. 25C,D).

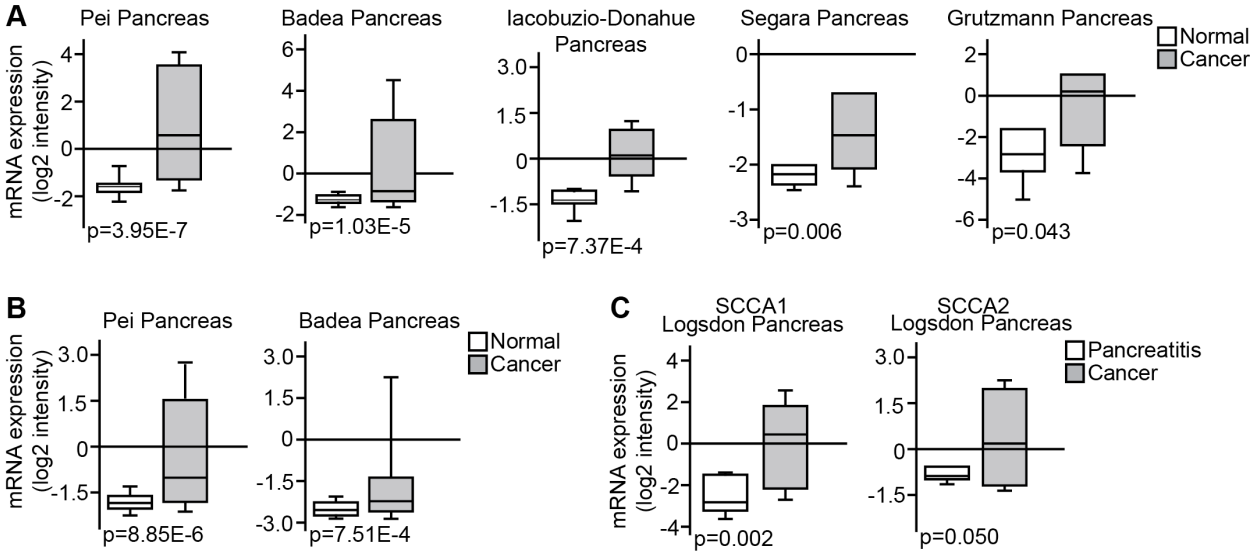
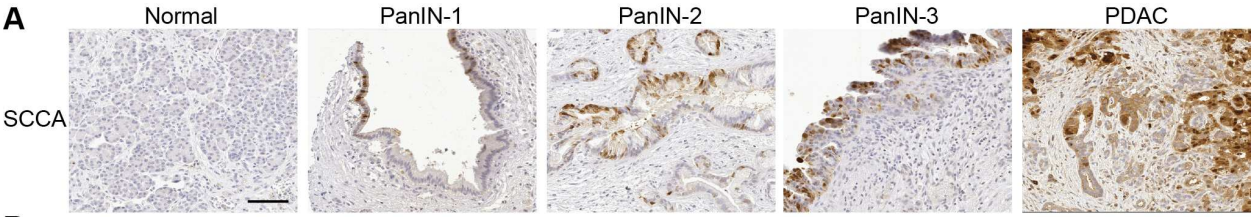


Figure 23. SCCA expression is up-regulated in human pancreatic cancer. (A-C) Oncomine (www.oncomine.org) datasets were analyzed for SCCA1 **(A)** or SCCA2 **(B)** mRNA expression levels in normal pancreatic tissue and pancreatic cancer, or for SCCA1 and SCCA2 mRNA expression levels in chronic pancreatitis and pancreatic cancer **(C)**. 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.



B

	SCCA-positive/Total specimen
Normal	0/48 (0%)
PanIN1	2/17 (11.8%)
PanIN2	5/19 (26.3%)
PanIN3	8/15 (53.3%)
PDAC	20/30 (66.7%)

p<0.0001

Figure 24. Incidence of SCCA expression increases along pancreatic cancer progression. **(A,B)** IHC against SCCA was performed on pancreatic tissue microarrays. **(A)** Representative images of normal pancreatic tissue and SCCA-positive PanIN1, PanIN2, PanIN3, and PDAC samples are shown. **(B)** SCCA-positive samples in different disease stages was quantified. Chi-squared test for trend was used to determine significance, $p < 0.0001$.

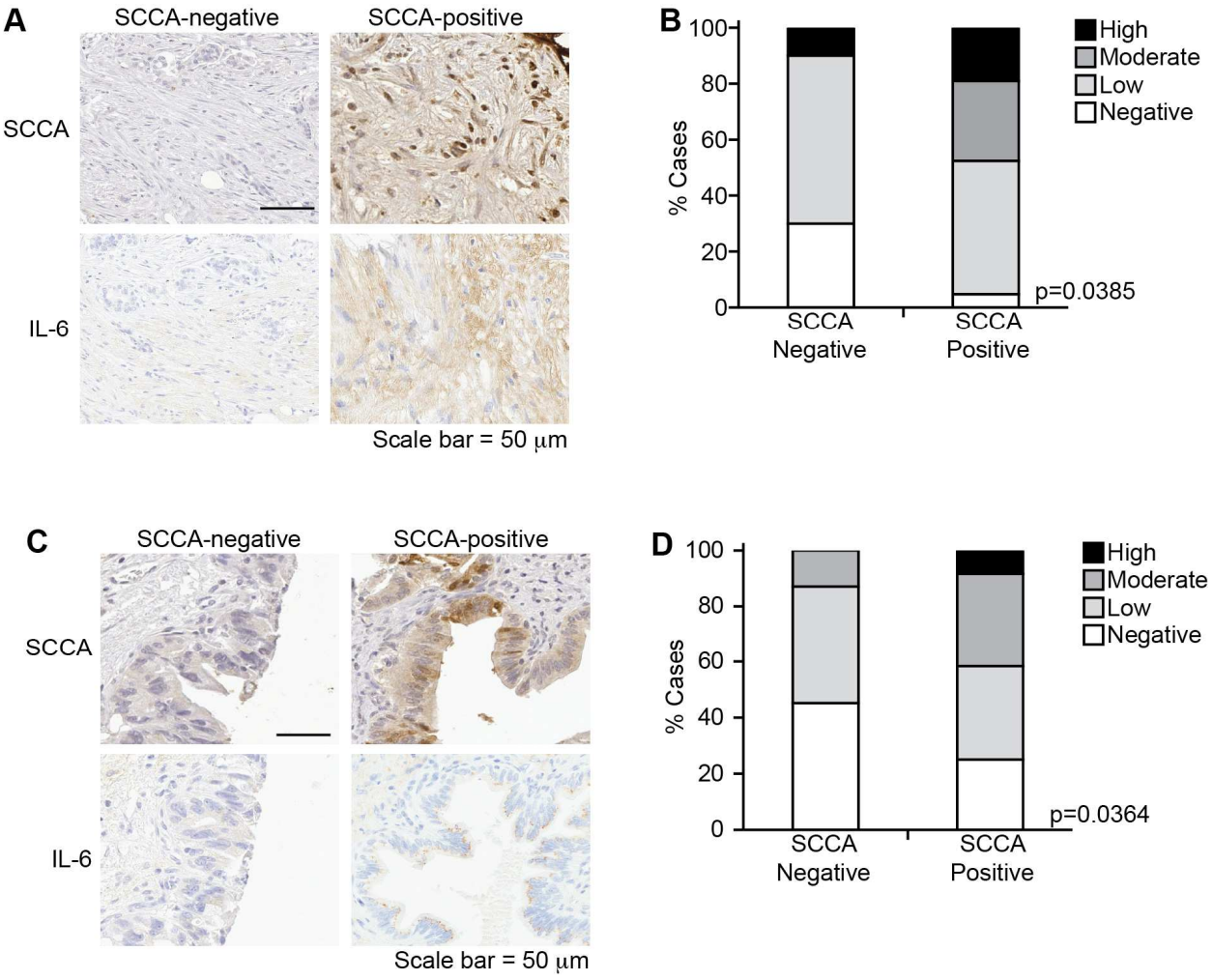


Figure 25. SCCA expression correlates with IL-6 expression in human pancreatic cancer. **(A-D)** IHC against IL-6 was performed on corresponding serial pancreatic tissue microarrays. **(A)** Representative images of SCCA/IL-6-negative and SCCA/IL-6-positive grade III PDAC samples. **(B)** Quantification of IL-6 staining in SCCA-negative and SCCA-positive PDAC samples. Chi-squared test for trend was used to determine significance, p=0.0385. **(C)** Representative images of serial sections of SCCA/IL-6-negative and SCCA/IL-6-positive PanIN samples are shown. **(D)** Quantification of IL-6 staining in SCCA-negative and SCCA-positive PanIN samples. Chi-squared test for trend was used to determine significance, p=0.0346.

(G) Loss of SCCA inhibits xenograft tumor growth of pancreatic cancer cells

To this point, all of our *in vitro* data suggesting that Ras^{V12} up-regulates SCCA expression levels has been *in vitro* expressing mutant Ras at supra-physiological levels. Our finding that SCCA-positivity increases through PDAC development suggests that SCCA plays a functional role in the progression of PanIN lesions to PDAC. To address this question, we screened a panel of pancreatic cancer cell lines harboring K-Ras mutation (AsPC-1, Capan-1, Capan-2, CFPAC-1, L3.6, HPAF-II, Panc-1, PL-45, S2-013) for SCCA expression. Of the 9 cell lines screened, 5 displayed detectable levels of SCCA expression (Fig. 26A). Interestingly, the incidence of SCCA-positivity *in vitro* (5 out of 9, 56%) is similar to what was observed *in vivo* (20 out of 30, 66.7%).

We next sought to uncover whether the SCCA expression observed in the pancreatic cancer cell lines was dependent on mutant K-Ras signaling. To this end, using a shRNA approach we silenced K-Ras expression and assessed SCCA expression levels. Not surprisingly, several cell lines, including the SCCA-positive HPAF-II cell line, did not tolerate K-Ras depletion and appeared to undergo cell death. This is not not surprising, as multiple cell lines have been shown to be dependent on mutant K-Ras for survival. Of the SCCA-positive cell lines that tolerated K-Ras knockdown, Capan-1, CFPAC-1, and L3.6 all exhibited reduced levels of SCCA expression upon K-Ras depletion (Fig. 26B).

We next sought to determine whether the SCCA expression observed in the pancreatic cancer cell lines was mediating IL-6 production. To address this, we silenced SCCA expression

using two independent hairpins targeting SCCA and assessed IL-6 production via qRT-PCR. In agreement with both our earlier *in vitro* studies and our finding that SCCA-positivity and IL-6-positivity correlated in human PDAC, loss of SCCA diminished IL-6 production in CFPAC-1, L3.6, and HPAF-II cells (Fig. 28A). To rule out the posibility of shRNA off-target effects, we utilized the same shRNAs targeting SCCA in PANC-1 cells which have undetectable levels of SCCA. Depletion of SCCA in PANC-1 cells had little to no effect on IL-6 production, suggesting the reduction in IL-6 observed in CFPAC-1, L3.6, and HPAF-II cells was caused specifically by SCCA silencing.

Lastly, we wondered whether SCCA depletion affected the ability of pancreatic cancer cells to form *in vivo* xenograft tumors. To this end, we injected CFPAC-1 cells, which had the highest level of SCCA expression, harboring control shRNA (shNTC) or shSCCA into the flanks of athymic nude mice and monitored tumor growth. It is important to note that SCCA silencing did not affect cell growth *in vitro*. While control cells formed robust tumors, silencing of SCCA nearly abolished the tumor forming capacity of CFPAC-1 cells (Fig. 27B).

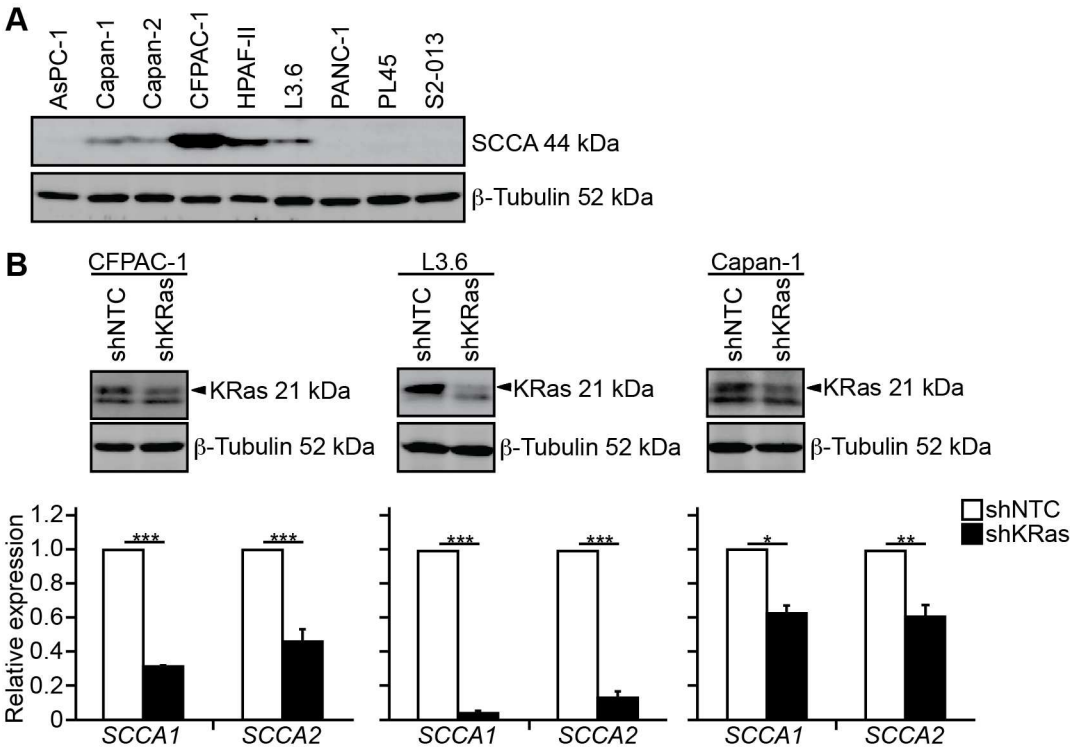


Figure 26. SCCA expression is up-regulated in a subset of pancreatic cancer cell lines and is K-Ras dependent. (A) Whole cell lysates from a panel of pancreatic cancer cells were obtained and analyzed through western blot with indicated antibodies. **(B)** Indicated cell lines were stably transduced with 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 K-Ras 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. *$p<0.05$; **$p<0.01$; ***$p<0.001$ by t-test.

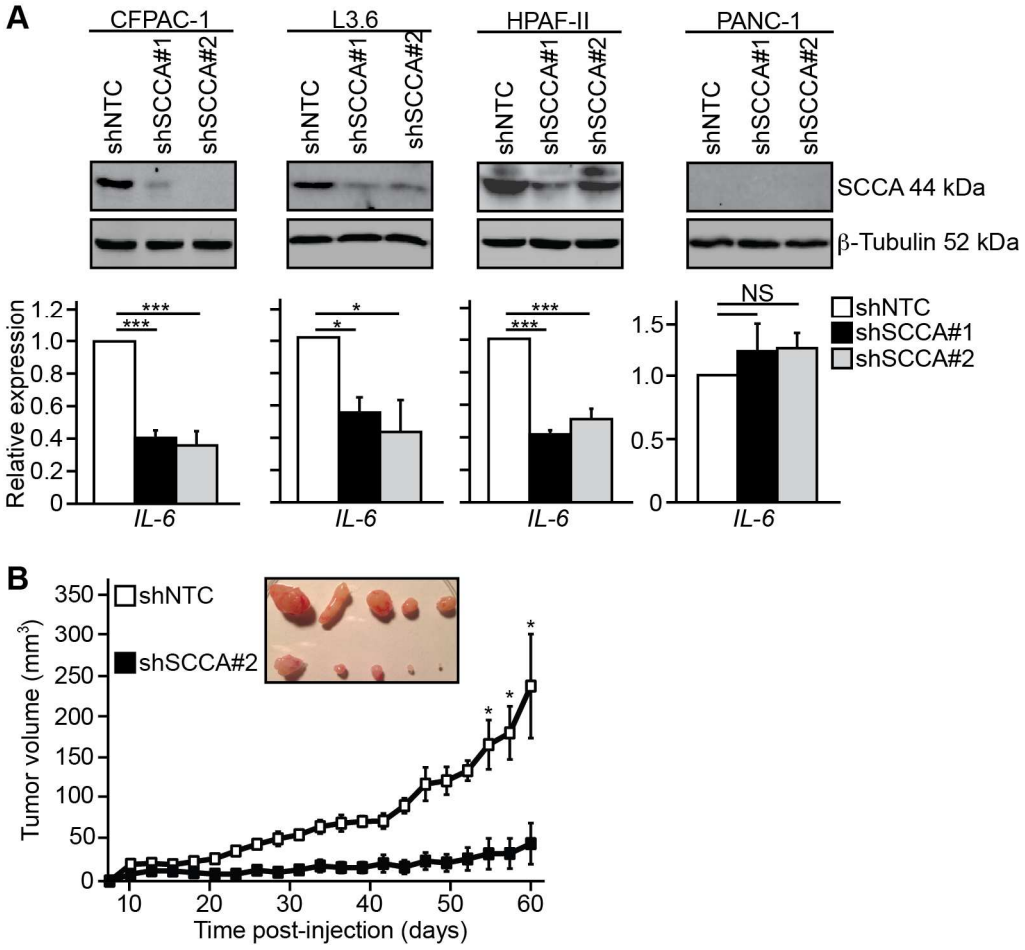


Figure 27. SCCA silencing abrogates IL-6 production and impairs xenograft tumor growth in pancreatic cancer cells. (A) Indicated cell lines were stably transduced with shNTC or shSCCA. Whole cells lysates were analyzed through western blot with indicated antibodies. Total RNA was extracted and IL-6 transcript 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. **(B)** CFPAC-1 cells were injected into the flanks of athymic nude mice and monitored for tumor

growth. n = 5. Representative images of tumors and the tumor growth curve ±SEM are shown. *p < 0.05; ***p<0.001; NS, non-significant by t-test.

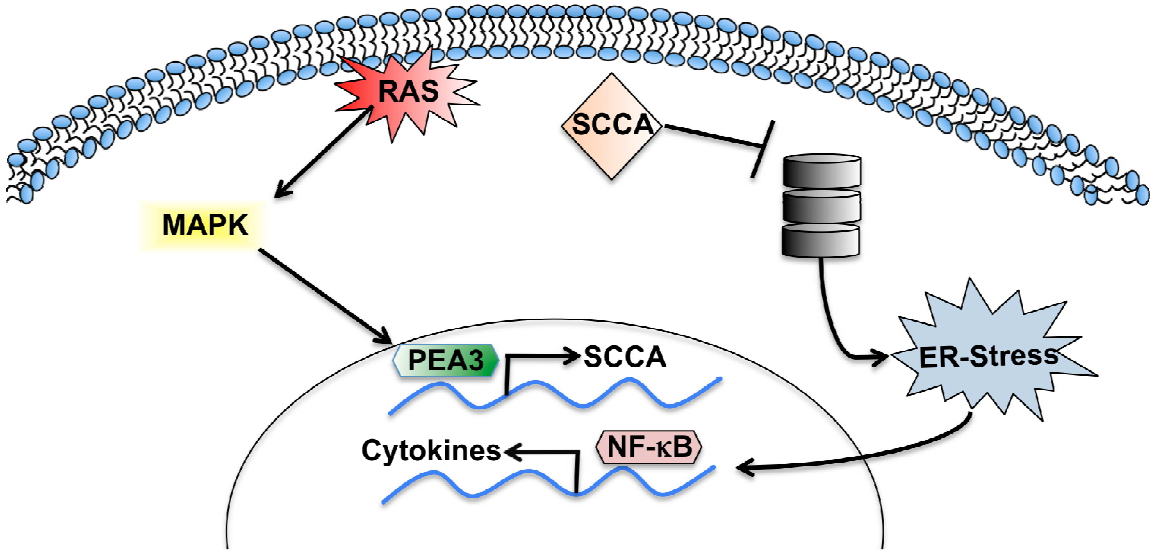


Figure 28. Model for the role of SCCA downstream of oncogenic Ras. Upon activation of mutant Ras, SCCA is up-regulated through a MAPK/PEA3-dependent mechanism. Up-regulation of SCCA promotes a proteotoxic environment that appears to be caused by an inhibition of degradation through the proteasome. This proteotoxictiy elicits an unfolded protein response that in turn activates NF-κB signaling and cytokine production. In addition, I have identified SCCA to be involved in human breast, colorectal, and pancreatic cancer. Importantly, up-regulation of SCCA appears to play a role in the development and progression of pancreatic cancer.

Chapter 5: Conclusions and Perspectives

(A) Implications of SCCA's involvment in human breast cancer

The finding that SCCA expression is up-regulated in high-grade, advanced stage human breast cancers should be explored further. First, our finding that those with SCCA-positive tumors have a worse clinical outcome, including decreased OS (Fig. 5A,C) and RFS (Fig. 5B,D) are consistent with other studies that have shown high levels of SCCA correlate to disease recurrence (Brioschi et al. 1991). Additionally, SCCA serum levels have been regarded as a valuable marker of patient response to both radiation and chemotherapy (Ngan et al. 1990; Yazigi et al. 1991). While our study links SCCA tissue expression levels with disease state, overall survival and recurrence, it hints at the possibility of utilizing SCCA serum levels in breast cancer patients to determine clinical outcome and monitor response to therapy.

Expression of SCCA in human breast carcinoma may also represent a valuable biomarker for small subsets of breast cancers including triple-negative breast cancers. Lacking expression of the estrogren receptor (ER), progesterone receptor, and Her2, triple-negative breast cancers are resistant to commonly utilized therapeutics used to treat many breast cancers (i.e. tamoxifen and herceptin). Interestingly, a small fraction of the samples included in our TMA analysis contained data for the expression of the estrogen receptor (ER) and progesterone receptor (PR), but not Her2/neu status. Out of the 13 SCCA positive breast carcinoma specimens, 9 cases (69%) were classified as double-negative for the expression of both ER and PR, whereas only 24% of the SCCA-negative tumor specimens were double-negative ($p=0.0009$). The idea that SCCA

may represent a biomarker for triple negative breast cancers is also consistent with the finding that *in vitro*, ectopic expression of SCCA can lead to down-regulation of Her2 expression (data not shown).

A small fraction of human breast cancers are squamous cell carcinomas. A number of reports have shown that squamous cell carcinoma of the breast is an aggressive disease behaving like poorly differentiated breast adenocarcinoma (Cornog et al. 1971; Dejager et al. 1995). Our finding that SCCA positivity is associated with high-grade breast carcinoma (Table 3) is consistent with this notion. In addition, the features of the SCCA-positive cases we identified in our study are very similar to those obtained from a study carried out at M.D. Anderson Cancer Center, where 33 breast SCC patients identified from 1985–2001 were analyzed and shown to correlate with worse OS and RFS (Hennessy et al. 2005). While further characterization of SCCA's involvement in both triple negative and squamous cell carcinoma of the breast is warranted, the idea of exploiting SCCA expression in the treatment of human breast cancer should also be explored. Previous work in the lab has shown that expression of SCCA sensitizes cells to proteotoxic therapies, such as tunicamycin and bortezomib (Ullman et al. 2011). This finding, together with the finding that SCCA is up-regulated in high-grade, advanced stage breast cancers suggest that treating SCCA-positive breast cancers with proteotoxic therapies may be a valuable option.

(B) Advantages and limitations of tissue microarrays

Tissue microarray technology remains an easy, attractive approach to quickly identify potential diagnostic markers across a large panel of patient samples. The ability to spot as many as 1,000 tissue samples on a single slide allows for high-throughput identification of both diagnostic and prognostic biomarkers. Using TMA technology, we were able to probe for SCCA expression in ~1,300 human breast cancer samples. This approach allowed us to identify SCCA in high-grade, advanced stage human breast cancer. Furthermore, often times tissue microarrays are available with clinical patient data that includes surivival data, tumor size, lymph node involvement, and therapy received. With this data available, we were able to easily correlate SCCA-positivity with both a worse overall survival (OS) (Fig. 5A,C) and recurrence-free survival (RFS) (Fig. 5B,D). The large sample size also improves the quality and reliability of the data. This is especially true for biomarkers that represent only a small fraction of the patient population (i.e. SCCA).

The presence of many tissue samples on a single slide allows for uniform identical experimental conditions across ~1,000 patient samples. This removes the staining variability that occurs when perfoming immunohistochemistry on different samples at different times. The ability to analyze an entire patient cohort at a single time removes the staining variability that may occur due to antigen retreival conditions (temperature and time), reagent concentrations, washing time and other various procedures that can vary from experiment to experiment. This ensures that all patient samples can be analyzed using identical staining procedures. This is more

fully appreciated when you consider the amount of variability that would occur if ~1,300 samples were stained individually.

While tissue microarray technology allows for experimental uniformity and minimizes assay time and cost, there are numerous limitations and criticisms against the use of tissue microarrays. One common criticism is that due to the small size of the patient sample, the tissue spotted onto the array may not be a true representation of the entire patient sample. Standard array tissue core sizes of 0.6-mm may not accurately depict the heterogeneity of many cancer types, this is especially true for human breast cancers. Along these lines, in our study the percentage of SCCA-positive patients may be under-represented due to the nature of tissue microarray construction. Many feel that the results obtained through tissue microarray must be validated through whole-section staining. Despite this, some independent studies have shown that a single core sample can accurately respresent whole-section staining (Zhang et al. 2003). This may be due to the fact that pathologists select well-defined regions of the whole tissue to be included in the microarray. At the moment the consensus seems to be the inclusion of two samples per patient per slide to overcome the small size of the patient samples (Camp et al. 2000). While this solution does not substitute for whole section staining, to some degree it allows for the capture of the heterogeneity of cancer.

(C) Significance of SCCA as a target of oncogenic Ras

The ability to successfully target mutant Ras through small molecule inhibition has proven more difficult than initially expected. While pharmacological inhibitors targeting immediate downstream effectors of oncogenic Ras (MAPK and PI3K) have proven effective, resistance is easily acquired. For this reason, continued identification of Ras effectors remains paramount to the development of therapeutics that can successfully treat Ras-driven tumors. As an example, by analyzing a subset of K-Ras-dependent mutant K-Ras colon cancer cell lines, Singh et al. identified a K-Ras-dependent enhancement of Wnt signaling that is dependent on the TAK1 kinase (Singh et al. 2012). Small molecule inhibition of TAK1 in K-Ras-dependent cell lines resulted in apoptotic cell death, and ultimately tumor regression. While this study underscores the importance of cell-type and context dependency, it highlights the importance of identifying mutant Ras effectors that can be targeted therapeutically.

Our finding that SCCA is transcriptionally up-regulated by oncogenic Ras (Fig. 6-8) and that loss of SCCA can inhibit tumor growth (Fig. 27B), suggests a possible therapeautic value in targeting SCCA. Studies in our lab have shown that SCCA's protease inhibitory activity is crucial to its ability to increase basal levels of ER-stress and promote transformation (Ullman et al. 2011; Sheshadri et al. 2014). While SCCA's target proteases have not been clearly identified *in vivo*, biochemical studies have shown that its ability to inhibit protease function is dependent on the flexibility of its RSL domain (Schick et al. 1998). Therefore, we hypothesize that small molecules that restrict the flexibility of the RSL domain can effectively inhibit SCCA's protease inhibitory function. Using structural analysis, we have identified 50 lead compounds in NCI's

drug compound library with high affinity and high specificity for SCCA. Using a fluorogenic assay, we are currently identifying compounds that can effectively inhibit SCCA's protease inhibitory activity.

Previous work in our lab has demonstrated that cells with high levels of SCCA expression are more susceptible to proteoxic stress (Ullman et al. 2011). This is due in part to SCCA's protease inhibitory activity and its ability to increase basal levels of ER-stress. Interestingly, tumors driven by mutant Ras have been shown to be susceptible to proteotoxic therapies, such as the proteasome inhibitor Bortezomib (De Raedt et al. 2011). Whether oncogenic Ras-induced SCCA expression causes this increased susceptibility remains to be seen and warrants further exploration. Along these lines, SCCA expression levels may offer a novel prognostic marker to identify mutant Ras-driven tumors that may be sensitive to proteotoxic stress. This may be especially useful in colorectal cancers, as only wild-type K-Ras colon cancers respond to cetuximab (EGFR inhibitor), a conventional front-line therapeutic (Van Cutsem et al. 2009). Furthermore, the ability to use already FDA approved therapeutics (i.e. Bortezomib) represents an attractive approach.

Lastly, our data supports the notion that mutant Ras can directly promote SCCA up-regulation. However, of the 9 pancreatic cancer cell lines, all of which harbor mutant K-Ras, 4 of the cell lines had no detectable levels of SCCA suggesting a negative regulatory mechanism. One possible mechanism is genetic deletion. SMAD4 is commonly lost through homozygous deletion during pancreatic cancer progression and interestingly is located on chromosome 18q21.1 while SCCA1 and SCCA2 are located on 18q21.3. Whether there is homozygous

deletion of SCCA1 and SCCA2 in those 4 cell lines remains to be determined. One other possible negative regulatory mechanism is through alterations in signaling pathways. Of note, silencing of p53 abrogated mutant Ras-induced SCCA up-regulation in IMR90 and p16 silencing enhanced mutant Ras-induced SCCA expression (data not shown), both of which are commonly altered in pancreatic cancer. These results suggest multiple layers of regulation mediating SCCA expression. The finding that suggests that p53 may mediate Ras-induced SCCA up-regulation warrants further study and also raises the question of how the various p53 mutants may modulate SCCA gene expression.

(D) Over-expression of oncogenic Ras vs. endogenous/physiological levels of oncogenic Ras

One major criticism of mutant Ras studies has been the use of ectopic, over-expression systems. Critics argue that over-expression models generate levels of mutant Ras that are supra-physiological. These criticisms are supported by several studies that demonstrated that cells or tissues expressing endogenous levels of mutant Ras present differently than those expressing supra-physiological levels. While over-expression of Ras^{V12} was shown to induce a senescent growth arrest in both primary human and rodent fibroblasts (Serrano et al. 1997), mutant Ras expressed at endogenous levels failed to give the same phenotype (Tuveson et al. 2004). In fact, expression of endogenous K-ras^{G12D} not only failed to elicit senescence, but instead promoted a transformed phenotype, demonstrating loss of contact inhibition and focus formation.

The idea that endogenous and over-expression models could generate completely opposing phenotypes was further supported *in vivo* using a tet-regulated H-Ras^{V12} expression system in the mouse mammary gland (Sarkisian et al. 2007). Using this approach, the authors administered mice varying concentrations of doxycycline which resulted in varying expression levels of the mutant Ras protein. Similar to what had been previously reported, high levels of mutant Ras expression resulted in the growth arrest of mammary epithelial cells, while mammary epithelial cells expressing mutant Ras at endogenous levels become hyper-proliferative and eventually formed tumors.

While the two aforementioned studies clearly demonstrated the differences between endogenous and supra-physiological levels of mutant Ras, they do not mean that all studies

which use mutant Ras over-expression systems produce artificial phenotypes. For instance, mutant Ras-induced senescence was first reported using an over-expression system (Serrano et al. 1997), and while the two previously mentioned studies argue against the idea that endogenous levels of mutant Ras can elicit senescence *in vivo*, numerous reports have clearly demonstrated that endogenous levels of mutant Ras can trigger senescence *in vivo* (Collado et al. 2005; Young et al. 2009; Guerra et al. 2011).

Given the reported discrepancy in some cell and tissue systems between mutant Ras under the control of its endogenous promoter and an over-expression approach, results obtained through over-expression of mutant Ras should be viewed with caution. Initial experiments utilizing mutant Ras over-expression should be corroborated in systems where mutant Ras is endogenously expressed. As our initial experiments uncovering SCCA as a target of mutant Ras utilized over-expression, we wanted to demonstrate that this finding had some physiological relevance. To this end, we examined both human tissue samples where Ras mutation occurs at a high frequency (Fig. 22-24) and human cancer cell lines that harbor mutant Ras (Fig. 26,27) to confirm our initial finding.

(E) Mechanism of SCCA-induced ER-stress

The ability of SCCA to increase basal level ER-stress has been clearly demonstrated and shown to be dependent on SCCA's protease inhibitory function (Ullman et al. 2011; Sheshadri et al. 2014). The exact mechanism though by which SCCA can induce ER-stress remains unknown. We have previously shown that ectopic expression of SCCA can block protein degradation through the proteasome. While an overall increase in ubiquitinated proteins and an obvious failure to degrade a ubiquitin-tagged luciferase supports the idea that SCCA can impair proteasome degradation, the chymotrypsin-like and trypsin-like activities of the proteasome were not affected by SCCA expression (Ullman et al. 2011). These results suggest that SCCA functions upstream of actual degradation by the proteasome. These results also raise the question of where SCCA is functioning within the cell. Given SCCA's function and reported targets, we would hypothesize that SCCA localizes to the lysosome. There are also reports of SCCA localizing to the nucleus following UV-irradiation (Katagiri et al. 2006). Interestingly, our IHC results against SCCA in human PanIN and PDAC samples suggest that SCCA can indeed localize to the nucleus (Fig. 22). Cellular fractionation was performed in mutant Ras-expressing cells and SCCA was found to be almost exclusively cytosolic (data not shown). The discrepancy between our IHC results and the *in vitro* fractionation remains unknown. This may be due to multiple factors, differing expression levels of SCCA, endogenous levels of mutant Ras vs. over-expression, differing genetic backgrounds, and differing interacting proteins. Given all this, we reason that cytosolic SCCA may have a different functional role than nuclear localized SCCA, especially in the sense of disrupting protein degradation pathways. We are currently performing

immunoprecipatation followed by mass spectrometry to identify SCCA interacting proteins in effort to help understand how exactly SCCA is inducing ER-stress.

Our finding that both SCCA1 and SCCA2 are both transcriptionally up-regulated by oncogenic Ras (Fig. 6-8) raises the question of whether SCCA1 and SCCA2 have overlapping functional roles. While we attempted to silence both SCCA1 and SCCA2 independently, this was difficult due to their high sequence homology. In fact, in our attempt to knockdown SCCA1 we observed a compensatory increase in SCCA2 levels that resulted in greater levels of cytokine production (data not shown). These results suggest that SCCA1 and SCCA2 may have some overlapping function. While SCCA1 and SCCA2 have no reported targets in common to date, the possibility remains that they do share common targets as all studies thus far have been *in vitro* assays using recombinant SCCA and hand-selected proteases.

(F) Distinguishing SASP and oncogene-induced cytokine production

Since the identification of the senescence-associated secretory phenotype it has always been thought of as a consequence of cellular growth arrest, and not the preceding insult that triggers senescence. This idea is supported by several studies that demonstrate that drug-induced senescence, replicative senescence, reactivation of tumor suppressor-induced senescence, and oncogene-induced senescence all are capable of activating this secretory profile (Shelton et al. 1999; Xue et al. 2007; Coppe et al. 2008; Rodier et al. 2009). The possibility remains however, that the initial insult can directly activate cytokine production independent of the senescent response. This may be especially true for the mutant Ras, as it has been shown to modulate various cytokine transcript levels in models of transformation (Sparmann and Bar-Sagi 2004; Ancrile et al. 2007; Leslie et al. 2010; Pylayeva-Gupta et al. 2012).

While the ability to separate the growth arrest phenotype from SASP using a senescence model is difficult, our data suggests that mutant Ras directly mediates this secretory profile. The up-regulation of SCCA by oncogenic Ras was clearly shown to be independent of senescence (Fig. 6-9), yet genetic knockdown of SCCA completely abrogated the SASP response (Fig. 16). These findings suggests that mutant Ras is directly modulating cytokine production independent of the senescent response. This idea is further supported by ongoing work in the lab that demonstrates HeLa cells expressing mutant Ras, which do not undergo senescence, exhibit a robust increase in IL-6, IL-8, CXCL1, GM-CSF, and G-CSF transcript levels.

Since SASP has been demonstrated in both oncogene and drug-induced senescence, but SCCA up-regulation is only observed in response to mutant Ras, our findings suggest that the secretory profiles induced by the various senescence triggers (i.e. oncogene vs. drug) are distinct. That is, the signaling upstream of cytokine production or the secretory profiles differ. This idea is supported by a recent finding that the secretory profiles between Ras-expressing cells and H_2O_2-treated cells are different (Suzuki et al. 2013).

(G) Utilizing TCGA and ONCOMINE databases

The advent of microarray gene expression technology has resulted in large amounts of data being obtained very quickly. Unfortunately, most of this data is analyzed by the group which obtains it for something they are particularly interested, the dataset is published online and rarely examined and utilized by others. Online databases such as The Cancer Genome Atlas (TCGA) and ONCOMINE allow for a user-friendly platform that can be easily accessed and analyzed by anyone. This is important because the handful of laboratories generating vast microarray datasets do not have the capabilities to validate all the potential biomarkers that arise in their studies. For this reason, it is important to make these datasets readily available and easy to understand so that cell biologists and clinicians can work to validate highly significant hits.

While in our study the use of both TCGA and ONCOMINE datasets was used to verify *in vitro* results, they can be used in an unbiased approach to identify potential biomarkers that can aid in the prognosis and diagnosis of various cancers. For instance, the ONCOMINE database can be used to readily identify secreted proteins that are highly over-expressed in lung cancers when compared to normal lung tissue. Within seconds, an easy to understand list of secreted proteins is generated and ordered by level of significance. The protein(s) of interest must then be validated using a cohort of clinical samples. Along these lines, we identified SCCA to be up-regulated in numerous pancreatic cancer datasets (Fig. 23). These results were then validated by obtaining a tissue microarray and performing IHC against SCCA (Fig. 24).

(H) Future Directions

The work described in this dissertation has uncovered several new questions that deserve further exploration. While the ability of ER stress to mediate an inflammatory response has been demonstrated by us and others, exactly how the ER stress effectors talk to NF-κB to promote cytokine production remains unclear. The IRE1α and PERK arms of UPR are thought to promote degradation or inhibit translation of IκB, respectively, but how ATF6 promotes NF-κB activation is unknown (Garg et al. 2012). Currently, ongoing work in the lab is exploring how ATF6 can promote cytokine production in the context of oncogenic Ras.

Our lab has previously demonstrated that high levels of SCCA sensitize cells to proteotoxic therapies (Ullman et al. 2011). As mentioned previously, the idea of targeting pancreatic and colon cancer with high levels of SCCA expression with the proteasome inhibitor bortezomib presents an attractive therapeutic option. It remains to be seen however whether SCCA expression in pancreatic cancer cells confers sensitivity to proteotoxic therapies. The establishment of SCCA silenced pancreatic cancer cell lines that show no obvious growth defects *in vitro* presents a feasible model system to test this idea. Furthermore, we have now established pancreatic cell lines that have no detectable levels of endogenous SCCA expression that ectopically express SCCA. These two model systems can now be utilized to examine whether SCCA expression levels correlate with susceptibility to proteotoxic drugs.

Lastly, pancreatic cancer remains a devastating disease due in large part to the lack of early detection methods. Our results demonstrating that SCCA expression correlates with

pancreatic cancer progression (Fig. 24) and that SCCA expression is up-regulated in PDAC when compared to chronic pancreatitis (Fig. 23C) suggests that SCCA levels may be a valuable biomarker.

Chapter 6: Materials and Methods

(A) Cell lines and culture

293T, T47D, MCF7, MDA-MB-468, SK-BR-3, MDA-MB-231, MCF10A, CFPAC-1, MIA PaCa-2, SAOS-2, U-2 OS, PANC-1, IMR90, BJ, HeLa, HT-29, Caco-2, L3.6, HPAF-II, AsPC-1, Capan-1, Capan-2, PL-45, and S2-013 cells were cultured according to ATCC recommendations. 293T, MCF7, Panc-1, and HeLa cells were cultured in DMEM supplemented with 10% FBS (HyClone). IMR90 and BJ cells were cultured in MEM supplemented with 10% FBS. HT-29 cells were cultured in McCoy's 5a supplemented with 10% FBS. Caco-2 cells were cultured in MEM 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 g/ml streptomycin (Invitrogen).

(B) Plasmids

Retroviral expression vectors for WZL-hygro, WZL-H-Ras^{V12}, pBABE-puro, pBABE-K-Ras^{V12}, and pLNCX-ER:HRas^{V12} have been described previously (Serrano et al. 1997; Guerriero et al. 2008). Human SCCA1 was cloned by RT-PCR from total RNA of MDA-MB-468 cells. Human SCCA2 was cloned by RT-PCR from total RNA of MCF 10A cells. Primers used for cloning of both SCCA1 and SCCA2: Forward primer, contains BamHI restriction site and Flag tag: 5'-CGGGATCCATGGACTACAAGGACGACGATGACAAGACCATGAATTCACTCAG TGAAGCC-3'. Reverse primer: contains XhoI restriction site: 5'-CCCTCGAGCATCTACGGG GATGAGAATCTGCCA-3'. RT-PCR products were ligated into the pCR2.1-TOPO vector (Invitrogen) and verified through sequencing (Stony Brook University DNA Sequencing Facility). Sequences were verified against reported sequences at NCBI GenBank. Both SCCA1

and SCCA2 were then subcloned into the pLPC retroviral expression vector. Human sXBP1 was cloned by RT-PCR from total RNA from IMR90 cells. Primers used were as follows, forward with HindIII restriction and Flag tag: 5'-AAGCTTATGGATTACAAGGATGACGATGACAA GTGGTGGTGGCAGCCGCGCCGAACCC-3' and reverse primer with HindIII restriction site: 5'-AAGCTTTTAGACACTAATCAGCTGGGGAAAG-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: shGFP: 5'-TACAACAGCCACAACGTCTAT-3'; shScramble: 5'-CAACAAGATGAAGAGCACCAA-3'; shSCCA#1: 5'-GCACAACAGATTAAGAAGGTT-3'; shSCCA#2: 5'-CCGCTGTAGTAGGGA TTCGGAT-3'; shPEA3: 5'-GCTCCGATACTATTATGAGAA-3'; shATF6: 5'-GCAGCAACC AATTATCAGTTT-3'; shXBP1: 5'-GCCTGTCTGTACTTCCATTCAA-3'; shKRas: 5'-CAGT TGAGACCTTCTAATTGG-3'.

(C) 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 hours after initial transfection, viral supernatant was harvested, filtered, supplemented with polybrene (10 μg/ml) and used to infect target cells. 48 hrs after last infection, cells were selected with appropriate antibiotics. IMR90 cells were selected for 2 days with 100 μg/ml of hygromycin, 2 μg/ml of puromycin or 1.75 mg/ml of G418. HeLa cells were selected with, 0.5 μg/ml of puromycin for 2 days. CFPac cells were selected with 0.5 μg/ml of puromycin for 2 days.

(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). Nuclear and chromatin isolation were performed as previously described (Mendez and Stillman 2000). Protein expression was examined by western blotting using antibodies against SCCA1/2 (FL-390; Santa Cruz), Flag (M2, Sigma), Ras (Clone Ras10; Millipore), p21 (C-19; Santa Cruz), ERK1/2 (4695; Cell Signaling), P-ERK1/2 (4370; Cell Signaling), AKT (9272; Cell Signaling), P-AKT (4058; Cell Signaling), p53 (FL-393; Santa Cruz), p65 (F-6; Santa Cruz), ATF6 (F-7; Santa Cruz), Xbp1 (M-186; Santa Cruz), Ubiquitin (P4D1; Covance), and β-tubulin (Sigma). All primary antibodies were incubated overnight at 4°C. Horseradish peroxidase or Alexafluor-conjugated goat anti-rabbit (Rockland) or goat anti-mouse (Rockland) antibodies were used as secondary antibodies. Western blots were developed using an ECL detection kit (Thermo Scientific) or an Odyssey Imager (LI-COR).

(E) Senescence assays

Senescence was induced by oncogenic Ras, etoposide (100 μM, 48 hrs), H_2O_2 (100 uM, 1 hr), or long term passaging (replicative senescence). All cells were analyzed 7 days 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 $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 40 mM NaPi, pH 6.0, 1 μg/ml X-Gal) overnight at 37°C. For BrdU staining, cells were cultured with BrdU (10 μM) for 6 hr, fixed with acid ethanol (90% ethanol, 5% acetic acid, 5% H_2O) 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 hr 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 hr at room temperature, washed, stained with DAPI and mounted.

(F) ELISA

Cells were plated 6 days post-antibiotic selection and allowed to recover overnight. Cells were washed once and incubated for 8 hr in serum free medium. Supernatant was collected, cleared by centrifugation, and used immediately. The amount of supernatant used was normalized to cell number and used with the human IL-6 quantikine ELISA kit (R&D Systems).

(G) Cytokine array

Cells were plated 6 days post-antibiotic selection and allowed to recover overnight. Cells were washed once and incubated for 8 hr in serum free medium. 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 and arrays were imaged on an Odyssey Imager.

(H) 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 g of 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: GAPDH: 5'-AAGGTCGGAGTCAACGGATTTG-3' and 5'-CCATGGGTGGAATCATATTGGAA-3'; SCCA1: 5'-AGCCGCGGTCTCGTGC-3' and 5'-GGCAGCTGCAGCTTCTG-3'; SCCA2: 5'-AGCCACGGTCTCTCAG-3' and 5-GCAGCTGCAGCTTCCA-3'; Serpinb3a: 5'-CATTTGTTT GCTGAAGCCACTAC-3' and 5'-CATGTTCGAAATCCAGTGATTCC-3'; Serpinb3b: 5'-ATT CGTTTTCATGCAGCTGATGT-3' and 5'-GAAAGCTGAAGTTAAATTTGTTCG-3'; PEA3: 5'-GGACTTCGCCTACGACTCAG-3' and 5'-CGCAGAGGTTTCTCATAGCC-3'; IL-6: 5'-TCCACAAGCGCCTTCGGTCCA-3' and 5'-AGGGCTGAGATGCCGTCGAGGA-3'; IL-8: 5'-AAGGAAAACTGGGTGCAGAG-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'; p16: 5'-GAAGGTCCCTCAGACATCCCC-3' and 5'-CCCTGTAGGACCTTCGGTGAC-3'; p21: 5'-TGTCCGTCAGAACCCATGC-3' and 5'-AAAGTCGAAGTTCCATCGCTC-3'.

(I) Luciferase assay

NF-κB activity was determined by using a NF-κB 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 hr prior to transfection at $5x10^4$ cells/well of 24-well plate. 250 ng NF-κB-Luciferase vector and 100 ng pCMV-RL were used for transfection. 24 hr post-transfection, cells were washed with PBS and lysed in 100 ul 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.

(J) 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. K-Ras was mutated in 87 out of the 207 samples. SCCA mRNA expression level was compared between the groups with wild-type and mutant K-Ras.

(K) Tissue microarrays

The tissue microarrays (TMAs) used to examine SCCA's involvement in breast cancer were obtained from both the Cooperative Human Tissue Network (CHTN) at the University of Virginia and the National Cancer Institute (NCI) Cancer Diagnosis Program (CDP). The TMA obtained from the CHTN contained 7 cases of non-neoplastic breast tissue from healthy subjects, 7 cases of non-neoplastic breast tissue from subjects with breast cancer, and 42 cases ranging

from low grade DCIS to metastatic breast carcinoma. The progression TMA obtained from the CDP (Sets 3, 5, and 7) were designed by NCI statisticians to provide high statistical power and are suitable for use in the investigation of differences in the prevalence of potential markers in invasive breast cancer. Each TMA consists of 288 cores (0.6 mm) taken from paraffin-embedded specimens that represent a total of 252 breast cancer and normal breast tissue specimens plus 36 controls. The prognostic TMA obtained from the CDP (Stage I, Sets 9–13; Stage II, Sets 14–17; Stage III, Sets 18–19) are designed to examine potential prognostic markers in non- metastatic breast cancer. Each TMA consists of between 100– 150 cores, including 100–120 breast cancer and normal breast specimens plus 5–20 control cores. Pancreatic 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.

(L) Immunohistochemistry

Paraffin-embedded TMA arrays were deparaffinized and rehydrated with graded ethanol. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Antigen retrieval was accomplished using 10 mM citrate buffer (pH 6.0). The sections were blocked with 5% goat serum for one hour at room temperature. SCCA primary antibody (FL-390, 1:200; 10C12, 1:50) was diluted in the blocking solution. Slides were incubated with the primary antibody overnight at 4uC. Slides were then washed and incubated with the appropriate biotinylated secondary antibody (1:1,000) for one hour at room temperature. Following a wash series, tissue was incubated with avidin/biotinylated HRP (ABC Elite kit from Vector Labs) according to manufacturer's instructions. Slides were submerged in diaminobenzidine (DAB)/ H2O2 substrate

solution until the desired staining intensity was obtained and slides were counterstained with hematoxylin. Slides were observed and photographs taken using an Olympus BX41 microscope. For pancreatic TMA IHC was performed on a Ventana XT (Tucson, AZ, USA) autostainer, according to manufacturer's directions.

(M) TMA analysis

Damaged core spots and those that were entirely adipose tissue were eliminated from scoring. The sections were scored indepen- dently by two evaluators blinded to the clinical status of the patients. The results were classified as percent of tumor cells with SCCA expression: 0, no expression; 1, <10%; 2, 10–50%; 3, >50%. Clinical data was then used to correlate SCCA expression with various clinicopathological variables.

(N) Xenograft tumor experiments

Male 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 institutional animal care and use committee. Tumors were established by resuspending 1×10^6 tumor cells in 100 µl 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.

(O) Statistical analysis

Two sample t-tests were used to compare continuous clinical features, such as age and size of invasive tumor between SCCA- negative and positive samples; Chi-squared tests and Fischer's exact tests, when applicable, were used to assess statistical significance of various categorical clinical features between SCCA-negative and SCCA-positive samples. Kaplan-Meier curves for overall survival (OS) and recurrence-free survival (RFS) were constructed for SCCA-negative and positive patients and compared using the Log-rank (Mantel-Cox) test. Hazard ratios and their 95% confidence intervals were derived, together with median survival times and 5-year survival rates. Statistical analyses were performed with GraphPad Prism (Graphpad Software Inc). Two-sided P values <0.05 were considered statistically significant.

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