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Squamous Cell Carcinoma Antigen 1 (SCCA1) promotes mammary tumorigenesis by inducing the Unfolded Protein Response (UPR) and IL-6 signaling

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Abstract of the Dissertation

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SCCA1 is a member of the serine/cysteine protease inhibitor (serpin) family whose intracellular targets include lysosomal cathepsins L, S and K. Its expression correlates with resistance to cell death resulting from lysosomal membrane permeabilization and cathepsin release in response to radiation and chemotherapy. Elevated SCCA1 expression has been reported in a wide array of cancers including squamous cell carcinomas of the lung, head and neck, skin, cervix, as well as hepatocellular carcinoma. Our laboratory recently found that increased expression of SCCA1 also correlates with high-grade, poorly differentiated breast carcinoma and poor prognosis. However, despite SCCA1's clear relevance in human cancer, its molecular and biological function remain largely unclear. In my dissertation research, I find that ectopic expression of SCCA1 in the non-tumorigenic mammary epithelial cell line MCF10A leads to epithelial-mesenchymal transition (EMT), a key molecular program that is co-opted by invasive and metastatic cancer cells. Correlatively, SCCA1 leads to oncogenic transformation in cultured cells as well in tumor xenografts. Silencing of endogenous SCCA1 in multiple breast cancer cell lines led to attenuation of cell growth. Mechanistically, SCCA1 interferes with cellular protein homeostasis resulting in a persistent yet sub-lethal level of endoplasmic reticulum stress leading to the activation of the unfolded protein response (UPR). This chronic stress results in NF-κB activation and induces the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) that drives EMT and oncogenic transformation. In the MMTV-Neu mouse mammary tumor model, expression of SCCA1 leads to an invasive phenotype with an exaggerated stress response and immune cell infiltration. These findings uncover a previously unknown role for SCCA1 in promoting tumorigenesis by inducing UPR, NF-κB activation, and pro-tumorigenic cytokine production.

Dedication Page

I dedicate this work to my dear aunts, Mrs. Jaya Arnoldussen and Mrs. Prema Surendar who have faced their encounter with breast cancer positively, demonstrating that perseverance is the key to winning this war.

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List of Abbreviations

AR	Amphiregulin
AT	Anti-trypsin
ATF	Activating transcription factor
BiP	Binding protein
BMK	Baby mouse kidney
BSA	Bovine serum albumin
BTC	Betacellulin
CHOP	CCAAT-enhancer binding protein
Cldn	Claudin
CSC	Cancer stem cells
CTC	Circulating tumor cells
CXCL	(C-X-C) motif ligand 1
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal Transition
EPR	Epiregulin
ER	Endoplasmic Reticulum
ER	Estrogen receptor
ERAD	Endoplasmic reticulum associated degradation
ERK	Extracellular signal-regulated kinases
GRP	Glucose regulated protein
HB-EGF	Heparin binding epidermal growth factor
HGF	Hepatocyte growth factor
HIF	Hypoxia inducing factor
HSP	Heat shock protein
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis

IRE	Inositol requiring enzyme
IκB	Inhibitor of kappa B
JNK	c-Jun N-terminal kinases
LMP	Lysosomal membrane permeabilization
MAPK	Mitogen activated protein kinase
MMTV	Mouse mammary tumor virus
MOMP	Mitochondrial outer membrane permeabilization
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf	Nuclear factor erythroid2-related factor 2
Ocln	Occludin
PARP	Poly ADP ribose polymerase
PDI	Protein disulphide isomerase
PERK	PKR-like ER kinase
PR	Progesterone receptor
ROS	Reactive oxygen species
RSL	Reactive site loop
SCCA	Squamous Cell Carcinoma Antigen
STAT	Signal transducer and activator of transcription
TERS	Transmissible endoplasmic reticulum stress
TGF	Transforming growth factor
TIC	Tumor initiating cell
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
UPR	Unfolded Protein Response
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
XBP1	X-box binding protein 1
Zeb	Zinc-finger E-box binding homeobox

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Publications

1. **Sheshadri N***, Catanzaro JM* (equal contribution), Bott A, Sun Y, Ullman E, Wu H, Chen EI, Pan J, Crawford HC, Zhang J, Zong WX. Squamous cell carcinoma antigen 1/SerpinB3 promotes epithelial-mesenchymal transition and oncogenic transformation by activating unfolded protein response and IL-6 signaling. (In revision for Cancer Research)

2. Catanzaro JM*****, **Sheshadri N*** (equal contribution), Pan J, Sun Y, Shi C, Li J, Powers S, Crawford HC, Zong WX. Oncogenic Ras induces inflammatory cytokine production by up-regulating squamous cell carcinoma antigens, SerpinB3/B4. Nature Communications 5, Article 3729 (2014)

3. Catanzaro JM, Guerriero JL, Liu J, Ullman E, **Sheshadri N**, Chen JJ, Zong WX. Elevated Expression of Squamous Cell Carcinoma Antigen (SCCA) Is Associated with Human Breast Carcinoma. PLoS One 6:e19096 (2011)

I. Introduction

One of the most fundamental features that distinguish cancer cells from normal cells is their ability to sustain chronic proliferative activity. Normal cells have a tightly controlled mechanism for production of growth promoting factors that regulate entry and progression through the cell cycle, thus achieving homeostasis of cell number and architecture. Cancer cells on the other hand, by deregulating these signals achieve a state of self-sufficiency. Since the identification of the first oncogenes and tumor suppressor genes, the field of molecular oncology has expanded rapidly and has greatly enhanced our understanding of the evolving characteristics of tumor cells.

Several cell autonomous properties that provide pro-tumorigenic capabilities have been identified, namely, the production of autocrine growth factor ligands, genomic amplification of growth factor receptors, activating mutations that promote constitutive signaling or disruption of negative feedback mechanisms that attenuate proliferative signaling. The growing tumor additionally extends angiogenic cues that allow for development of the blood vessel network to ensure constant nutrient input. The access to the vasculature provides a route for disseminating cancer cells from the site of the primary tumor, thus gaining entry into the bloodstream. It is popularly theorized that carcinoma cells gain metastatic potential by turning on the epithelial-mesenchymal transition (EMT) program which allows cells to escape the barrier of the basement membrane and intravasate. Circulating tumor cells then lodge in a suitable secondary site where they may remain dormant for extended periods of time. Upon receiving the right environmental cues these cells may colonize the secondary organ to develop metastatic foci.

One of the major complications associated with treating cancer is the recurrence of metastatic disease which is responsible for 90% of cancer-related deaths. Metastatic tumors are frequently non-responsive to previous treatment modalities, making them especially life threatening. Research over the last decade has been focused on gaining a better understanding of mechanisms of invasion and metastasis to help in development of effective therapeutics.

The efforts to dissect out the essential players contributing to tumor progression has revealed that several non-cell autonomous factors play significant roles. It is now believed that heterogeneous population of tumor cells interact closely with stromal cells to establish a distinct tumor-promoting microenvironment. Tumor cells are known to secrete factors that recruit immune cells to the primary site. Historically, immune cells were thought to eradicate tumor cells but an increasing body of knowledge provides evidence for their ability to enhance tumor

progression. Conversely, cancer cells have evolved mechanisms to evade destruction by immune cells and evolved mechanisms to program immune cells to become tumor promoting.

The following study is a molecular characterization of the tumor promoting function of Squamous Cell Carcinoma Antigen 1 (SCCA1) in mammary tumorigenesis. While SCCA1 has been used as a diagnostic marker for advanced squamous carcinomas, its biological functions are largely unknown. Upon a recent finding in our laboratory that SCCA expression correlates with high grade breast carcinoma and is associated with poor prognosis, I started to evaluate its role in tumor progression using an immortalized but non transformed human mammary epithelial cell system. This study provides a new perspective on the tumor-promoting role of SCCA1 via inflammatory cytokine production, besides its previously known role in the inhibition of cell death. It implicates SCCA in the regulation of protein homeostasis and demonstrates that its upregulation is critical for the function of oncogenes such as Ras. It provides molecular rationale to explain the sensitivity of SCCA expressing cells to treatment with proteotoxic drugs, thus offering a viable therapeutic option for treating tumors with elevated SCCA.

1. Squamous Cell Carcinoma Antigen (SCCA)

SCCA was identified as a tumor specific antigen (TA-4) associated with squamous cell carcinoma of the uterine cervix (1). Its expression was found to be linked to advanced metastatic disease and its levels increased with grade and stage (2,3). Over the years it has been used as a biomarker for the diagnosis as well as prognosis of the disease (4). After its initial identification, TA-4 was found to be a mixture of two isoforms with almost identical molecular weight (390 amino acids, 45 kDa) which were then separated by iso-electric focusing into a neutral form SCCA1 (pI 6.4) and an acidic form SCCA2 (pI 5.9) (5). Genomic analysis identified that these paralogous genes were encoded from independent loci which arose by tandem gene duplication (6-8). It was found that SCCA1 and SCCA2 are 98% and 92% identical at the nucleotide and amino acid levels respectively.

Detailed histological analysis revealed that SCCA 1 and 2 are normally co-expressed in squamous epithelial cells of tongue, esophagus, tonsils, epidermal hair follicles, lung and uterus, while becoming highly up regulated in squamous carcinomas of these organs (9-11). Recent evidence also suggests that SCCA expression is not limited to cancers of squamous origin but also extends to adenocarcinoma of the lung, breast and hepatocellular carcinoma (12-15). Despite such a broad expression profile, its physiological function remains elusive.

A. Classification within the serpin superfamily

The serpin family comprises of a large number of structurally similar but functionally distinct set of proteins that are evolutionally conserved from viruses, plants to higher metazoans. They have a distinct structural entity that utilizes a covalent interaction to bind its cognate protease and functions as a suicide-substrate inhibitor (16). They have been identified to exist in clustered loci spread across the genome indicating that they have expanded and diversified to inhibit a variety of targets. While some have acquired the ability to inhibit cysteine proteases (cross-class serpins) others have completely lost inhibitory functions (non-inhibitory serpins). In humans, 37 serpins have been described so far of which 26 show inhibitory function (17). Functionally, serpins have been demonstrated to participate in several important physiological processes such as development, clotting and fibrinolytic cascades, inflammation and complement

activation, microbial infection, angiogenesis, extracellular matrix (ECM) remodeling and apoptosis inhibition. The function of several other serpins remain elusive and are yet to be validated.

In higher animals serpins are classified into nine clades (A-I) of which A and B are the largest with 13 members each. While Clade A consists of extracellular serpins that share similarity to α1-anti-trypsin (α1-AT), Clade B serpins are mostly intracellular resembling ovalbumin (ov-serpins) (18).

Cloning and sequence homology revealed that SCCA was a member of a large family of serine protease inhibitors also known as serpins (6). SCCA1 was then designated SerpinB3 and SCCA2 as SerpinB4. SCCA1 and SCCA2 belong to clade B serpins, characterized shorter N- and C-terminal extensions than the proto-typical α1-AT and the lack of a cleavable secretory signal peptide. They are located within the serpin cluster located at the genomic locus of 18q21 (19,20).

B. Structure and mechanism of action

Serpins are characterized by 3 β sheets, at least 7 alpha helices and a distinct reactive site loop (RSL). The RSL is a stretch of about 17 amino acids located at the C-terminus of the protein, suited to trap target proteases by functioning as a pseudo-substrate. The bait peptide bond P1-P1' which mimics the substrate is attacked by the serine protease to form an acyl-enzyme intermediate which is a covalent bond. The RSL inserts into the body of the protein and results in an irreversible intramolecular conformation change which distorts the proteinase and renders it functionally inactive and at the same time itself losing further inhibitory activity. This mechanism of action is therefore termed as single use or suicide-substrate inhibitor (16).

Although SCCA1 and 2 are very homologous, they exhibit different inhibitory profiles. A comparison of the sequence of the RSL indicates 6 dissimilar residues out of the 13 amino acids (21). This accounts for the altered substrate preferences; while SCCA1 inhibits cysteine proteases such as lysosomal Cathepsin L, S and K (22,23) , SCCA2 inhibits serine proteases namely Cathepsin G, mast cell chymase and granzyme M (24). SCCA1 is a unique member of the serpin family whose targets include papain-like cysteine proteinases as opposed to serine proteases for which it is also referred to as a cross-class inhibitor. While both serine and cysteine

protease targets of other cross class inhibitors have been demonstrated, a serine protease target for SCCA1 is yet to be discovered before it is termed a dual inhibitor (23). SCCA1 has a distinct inhibitory mechanism in which it does not form a covalent complex with its target (25).

C. Modulation of cell death

Proteolysis is a key feature of events leading to cell death. A variety of external as well as cell-intrinsic stimuli can lead to the activation of intracellular proteinases to eventually cause cell death. Thus, effective control of proteolysis regulates the balance between life and death of cells. In the context of tumors, apoptosis plays a significant role in the clearance of cells harboring potentially harmful genetic mutations. Failure to clear these cells can result in the survival of cells which have aberrant activation of oncogenes or loss of tumor suppressor genes which can lead to the development of lesions. Cancer cells have evolved mechanisms to evade apoptosis to allow the survival and proliferation of such damaged cells (26). Primary tumors may also facilitate tumorigenicity by activating another cell death pathway, necrosis, which is known to release bio-active molecules including cytokines that recruits tumor promoting immune cells.

Serpins control the activity of cytotoxic proteinases such as caspases, cathepsins and granzymes are therefore critical modulators of cell death pathways of apoptosis and necrosis. Several serpins have been implicated in protecting cells against apoptosis. CrmA, a viral serpin has been reported to inhibit Fas induced cell death by cytotoxic T lymphocytes and protect the virally infected host cells from immune surveillance (27). Similarly, SerpinB9a is granzyme B inhibitor which protects antigen presenting cells from cytotoxic effects of misdirected granzyme B (28). In fact the nematode intracellular serpin, SRP-6 has been shown to inhibit necrosis resulting from massive lysosomal permeabilization in response to hypoxia, oxidative stress and heat shock (29).

In line with this idea, the first biological role reported for SCCA was to effectively neutralize cathepsins upon lysosomal membrane damage by the treatment of squamous cell carcinoma cells with anti-cancer agents (30). While it conceivable that SCCA expression in normal cells safeguards it from the cytotoxic effects of lysosomal membrane permeability, its elevated levels in cancer cells might offer an unwarranted survival advantage to cancer cells exposed to chemotherapeutic agents.

a. Lysosomal Cell death

Lysosomes are organelles equipped with hydrolytic enzymes that serve to degrade macromolecules within the cell and recycle the constituents for biosynthetic purposes. The autophagosome sequesters cytoplasmic contents within a double membrane structure which fuses with the lysosome to mediate a critical role in eliminating toxic protein aggregates and recycling of amino acids to maintain protein homeostasis (31). The lysosomes protects its limiting membrane from hydrolytic damage by a lining of heavily glycosylated transmembrane proteins called lysosomal-associated membrane protein1 and 2 (LAMP-1 and -2). Several stress agents are capable of inducing lysosomal membrane permeabilization (LMP) results in a leakage of lysosomal proteases, namely cathepsins, into the cytosol. Cathepsins are sub-optimally active at the neutral pH of the cytosol and begin to activate proteolytic cascades involved in cell-death. Based on degree of permeabilization as well as the cellular context, lysosomal cell death may have apoptotic or necrotic features associated with it (32,33). A large panel of agents have been named to trigger LMP, ROS being one of the most studied mechanism in addition to caspases, p53, sphingosine, photodamage, radiation and chemotherapeutic drugs including DNA damaging agents and microtubule poisons (31).

The specific role of SCCA in apoptosis resistance was confirmed by the observation that the genetic ablation of SCCA1 in cervical cancer cells rendered them sensitive to TNF-α as well as the DNA damaging agent, etoposide induced apoptosis (30). Following this study, both SCCA1 and SCCA2 was also shown to inhibit apoptosis induced by ultraviolet and γ-radiation by decreasing the activity of initiator as well as effector caspases -3 and -9 (34). While the precise mechanism for such inhibition was not clear, suppression of phosphorylation and activation of the p38 MAPK pathway in response to UV and radiation stress was implicated. More recently, SCCA2 was reported to protect tumor cells from natural killer cells and cytotoxic T lymphocytes by inhibiting Granzyme M (24). Although the precise mechanism for SCCA's inhibition of cell death is yet to be established, it has been reported to interfere with caspase activation as well as mitochondrial cytochrome c release (35).

While the above mentioned reports attribute this function of SCCA to its protease inhibitory effects, there exists a parallel study suggesting it might have an additional scaffolding function. SCCA1 expression level is highly elevated upon sun exposure and UV irradiation,

during which c-Jun NH_2 terminal kinase (JNK) pathway activation is thought to mediate cell death. This stress response pathway was found to be attenuated in keratinocytes expressing SCCA thereby protecting these cells from UV-induced apoptosis (36). The observed anti-apoptotic activity was independent of protease inhibition activity involving either direct or indirect interaction of SCCA1 with c-Jun NH_2 terminal kinase 1 (JNK1) and translocation to the nucleus mediated suppression of apoptosis mediated via c-Jun.

b. Cell death by proteotoxic stress

Various lines of evidence suggest that SCCA is pro-survival molecule which functions to protect cells from death induced by agents that damage the integrity of lysosomes. These findings were further accentuated in studies from our own group which suggested that SCCA1 protects cells from lysosomal membrane permeabilization induced cell death by DNA alkylating agents and hypotonic shock (37). Surprisingly, SCCA1 expression sensitized cells to death by proteotoxic stress inducing agents such as the proteasomal inhibitor MG132 and ER stress inducers, Tunicamycin and Thapsigargin. This study also revealed that SCCA1 impaired global protein turnover by attenuating lysosomal and proteasomal protein degradation pathway. Aggregation induced activation of caspase-8 was described to be the underlying mechanism for cell death due to proteotoxicity.

These data prompted us to explore the function of SCCA in regulation of protein turnover in greater depth. This mechanism offers a promising avenue to understand mechanisms by which cell death can be induced in cells which are otherwise resilient to multiple stress inducing agents. This vulnerability of cancer cells expressing SCCA can be exploited as a therapeutic option to rationalize a treatment regimen of proteotoxic drugs. In fact, the use of proteasome inhibitors is currently approved for the treatment of multiple myeloma which demonstrates pronounced protein turnover (38,39).

The above referenced literature supports the role of SCCA as an anti-apoptotic molecule. However, a previous study from our group reported the clinical association of SCCA specifically with poorly differentiated advanced stages of breast carcinoma indicating a possible role in tumor progression (13). Poorly differentiated tumors frequently manifest aggressive phenotypes with increased invasiveness and resistance to apoptosis by conventional chemotherapy

presenting poor prognosis. A parallel clinical report described that SCCA was a marker of incomplete pathological response to anthracycline based chemotherapy in breast cancer patients (14). We therefore decided to evaluate the role of SCCA in breast cancer progression.

2. Breast Cancer

American Cancer Society reports for 2014 indicate that breast cancer is the second most commonly diagnosed form of cancer in women after skin cancer. Breast cancer ranks the second leading cause of cancer related mortality in women after lung cancer. Death rates for breast cancer in women have steadily decreased since 1990, with larger decreases in younger women from 2006-2010 (3% in women younger than 50 and 1.8% in women older than 50) owing to the early detection and development of more effective therapeutics. The 5 year survival rate for all breast cancers during the period of study from 2004-2010 was 89.2%. Overall, 61% of breast cancers are diagnosed at a relatively early stage with no spread to lymph nodes or other locations outside the breast (localized stage), for which the five year survival rate is 99%. If the cancer has spread to the tissues or lymph nodes under the arm (regional stage), the survival rate is 84%. If the spread included lymph nodes around the collar bone and distant node or organs (distant stage), the survival rate drops to 24%. These data clearly suggests the need for additional research for the better understanding of the mechanism involved invasion and metastasis of breast cancer and development of therapeutic strategies to prevent and treat these advanced stages of the disease.

Breast cancers are broadly classified as carcinomas or sarcomas based on the epithelial or stromal origin of the cancer. Carcinomas of the breast arise from lobular units or terminal ducts of the mammary gland. Sarcomas are rare cancers that arise from myofibroblasts and supporting cells and account for only about 1% of all primary breast cancers. The carcinomas are further classified as “in situ” or invasive based on the aggressiveness of the cancer. Carcinoma in situ refers to a pre-invasive stage wherein cancer cells start to proliferate within existing lobules and ducts without breaching the boundary of the basement membrane. Invasive cancers have cells infiltrating the connective tissues with the possibility of regional or distant metastatic spread. Approximately 80% of all breast carcinomas are invasive ductal carcinoma and 15% comprise

on invasive lobular carcinoma and they have distinct pathologies, prognoses and treatment options.

A. Pathological staging, grading and biomarker testing

Abnormal masses in the breast are usually identified during mammography or radiological imaging, after which a tissue sample is obtained by either fine needle aspiration, core needle biopsy or excisional biopsy. The specimen is then evaluated for signs of benign tumors, fibro adenomas or malignant cancer based on standardized staging and grading protocols. The American Joint Committee published a revised protocol of TNM for the staging of specimens from patients with invasive cancer in 2010 (40). The pathological state of the tumor tissue is examined for the characteristics of the tumor such as size (T), the presence of lymph node metastasis (N) or distant metastasis (M).

The grade of breast cancer represents the aggressive potential of the tumor and is broadly classified as low grade and high grade cancer based on the aggressiveness of the cancers. There are different scoring systems available for determining the grade of breast cancer. The most common one is the Nottingham Histological Scoring system which takes into account the following considerations: 1) the percentage of glandular structures (differentiation), 2) nuclear pleomorphism and 3) mitotic activity of the cells (41). Each of these are scored from 1-3. Then each value is added to get a total score ranging from 3-9. Grade 1 tumors have a score of 3-5, Grade 2 with 6-7 and Grade 3 with a score of 8-9.

The percentage area of glandular or tubular differentiation is determined for the first criterion, nuclear morphology is categorized based on variability in size, nuclear shape, vesicular nature and uniformity in chromatin and finally, the mitotic activity is estimated by the number of dividing cells per field.

Another important aspect in the pathological evaluation of breast cancer is the biomarker testing for the accurate assessment of the estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor status of the cancer (42). Biomarkers are useful in determining prognostic features of risk of recurrence and mortality as well as in predicting the response to a given therapy. The presence of steroid hormone receptors ER and PR pose a weak prognostic but strong predictive propensity for a patient's breast cancer. These patients are likely to benefit

from endocrine therapy such as tamoxifen. The over expression of the oncogene HER2 is associated with diminished prognosis with increased risk of recurrence but predicts that the patient is likely to benefit from anthracycline and taxane based chemotherapies and the targeted therapy toward HER2 (Trastuzamab).

B. Molecular subtypes of breast cancer

Advances in molecular techniques, particularly in global gene expression profiling, has greatly refined the molecular classification of breast cancers to assess prognosis and response to therapy. Characterization of cDNA microarray data across surgical specimen from human breast cancer revealed five major subtypes including two hormone receptor positive cancers, luminal A and luminal B groups, two hormone receptor negative sub-groups, HER2 and basal-like as well as the normal breast-like tumors (43). Variations in the transcriptional programs govern the biological diversity of cancer cells, indicating that breast cancer is a complex disease in terms of incidence rates, cell of origin, survival, treatment options and prognosis for each subtype (44-46).

The Luminal A and Luminal B types comprise of 70% of all breast cancer cases and are known to respond well to endocrine therapy like tamoxifen and aromatase inhibitors. They generally present favorable prognosis. The highlight of this study was the identification that ER/PR negative breast cancers include two distinctly different classes of disease, HER2 positive and basal-like breast cancers. The HER2 enriched subgroup frequently includes an amplification in the HER2 gene and responds well to targeted therapy (Trastuzumab) and anthracycline based chemotherapy. However, it is associated with poor prognosis and increased risk of recurrence. The basal-like breast cancer sub-type, on the other hand, accounts for the remaining 15% of all breast cancers characterized by poor differentiation status and prognosis, with the lack of expression of ER, PR and HER2 receptors. While they show some response to broad spectrum chemotherapeutics such as PARP inhibitors and platinum based agents, a targeted approach to treating this type of cancer is currently unavailable due to the lack of a distinct identifying biomarker (47).

Based on new molecular findings the subtyping has continued to evolve over the years. The term “Triple negative breast cancers” (TNBC) is used for a group of breast cancers that closely resemble basal like breast cancers in the fact that they lack ER, PR and HER2. About

20% of basal like breast cancers might express ER and HER2. But genetically these two classes are remarkable distinct (48). TNBC encompasses a Claudin-low subtype which is believed to be enriched in cells that have properties similar to that of cancer stem cells, with features of epithelial-mesenchymal transition (49). Both basal-like and TNBC are more frequently found in young African-American and Hispanic women. Of note is the involvement of breast cancer susceptibility gene BRCA1, with approximately 75% of women carrying a mutation in this gene have a triple-negative, or basal-like phenotype or both (50). Another ER negative subtype with androgen receptor positivity, responsive to bicalutamide, has recently been identified within the TNBC group (51).

C. Clinical association of SCCA with breast cancer

Strikingly, from a detailed analysis of tissue microarray sample sets, we have identified that elevated SCCA expression correlates to advanced human mammary carcinoma (13). While non-neoplastic tissue showed undetectable levels, 9.4% of poorly/undifferentiated Grade III samples were positive for SCCA staining by immunohistochemistry. Additionally, its expression was associated with estrogen and progesterone receptor negative status (ER-/PR-) suggesting poor prognosis. SCCA showed stronger association with advanced stage based on TNM classification of tumors. Moreover, SCCA positivity suggested poor overall survival as well as recurrence free survival indicating its use as a potential prognostic biomarker. A parallel clinical report from another group has suggested that SCCA expression can be used as a predictive biomarker for incomplete pathological response to anthracycline based chemotherapy further confirming its association with cancers with poor clinical outcome (14). Despite such a strong correlation between SCCA and advanced disease, its molecular role in breast cancer progression remains unknown.

D. Progression of breast cancer

Tumor metastasis is a multi-step phenomenon involving dissemination of cells from the primary site, migration to a secondary site via the blood-stream where the tumor re-establishes itself, best summarized by the following sequence of events: local invasion, intravasation,

transport, extravasation and colonization. Tumor cells are known to employ an evolutionarily conserved developmental program termed epithelial-mesenchymal transition (EMT) to facilitate epithelial-derived carcinoma metastasis. The loss of epithelial cell differentiation has long been considered a marker for invasive phenotype of breast cancer. In terms of functionality, cancer cells adopt the EMT program to allow stationary epithelial cells to migrate and invade the surrounding tissue to promote metastatic dissemination (52).

a. EMT and de-differentiation

Epithelial and mesenchymal cells have distinct cell morphology, functional properties and organization within tissues. While epithelial cells display apical-basal polarized cells arranged on a basement membrane which are held together as sheets by cell-cell adhesion molecules, tight junctions and desmosomes, mesenchymal cells are found dispersed in the stromal compartments with a front/back polarity designed for motility.

EMT was first described by the pioneering work of Elizabeth Hay while studying chicken embryogenesis (53). It was later observed epithelial cells in culture lose the expression of adhesion proteins and gain cytoskeletal protein vimentin upon exposure to TGF-β (54). While EMT is a well-accepted concept in embryogenesis, its role in carcinoma metastasis was a topic of intense debate for the lack of clinically relevant evidence (55). While EMT was clearly documented in breast cancer cell lines and mouse models of cancer (56,57), its occurrence in human patient samples was not easily detectable. Unsupervised hierarchical clustering revealed that the appearance of EMT markers was preferentially associated with basal like breast cancers (58), thereby solidifying the link between poorly differentiated cancers and an EMT signature. In the recent years, circulating breast cancer cells with simultaneous expression of epithelial and mesenchymal features have been identified indicating the dynamic interchange between the two states, with the appearance of multiple intermediate stages (59).

b. Molecular mediators of EMT

The EMT trans-differentiation program is a complex event mediated by the coordinated action of multiple signaling pathways and molecular regulators. Based on the functionality of

these molecules during EMT they are classified as effectors, core regulators and inducers (60). The EMT effector molecules are mostly defined by structural molecules which maintain the epithelial or mesenchymal identity of cells. This molecules are important in execution of the EMT program. Epithelial cell adhesion molecules and tight junction proteins are down regulated during the process of EMT. The family of claudins and occludins make up the majority of the tight junction proteins while desosomal structures link the cell-adhesion molecules to the intracellular cytokeratins (61). E-cadherin is believed to be the gate-keeper of the epithelial state. It is reported to undergo transcriptional down regulation, epigenetic silencing and/or proteolytic degradation. Cells in turn upregulate a mesenchymal cadherin N-cadherin in a phenomenon termed cadherin switching (62). Cadherin switching is believed to drive the migration of cells away from the site of the primary tumor. Cells undergo a massive cytoskeletal re-modelling to facilitate motility, for instance intermediate filaments change from cytokeratin expression to vimentin expression.

EMT involves the transcriptional re-programming of cells executed by the core regulators that directly bind to the promoters of genes regulating cell-adhesion and mesenchymal differentiation to promote migratory and invasive behavior of cancer cells. These transcription factors are classified into three families; the Snail zinc finger family which includes SNAI-1 and SNAI-2 (63), the zinc finger E-box-binding homeobox family proteins Zeb1 and Zeb2, and the basic helix loop helix (bHLH) family of transcription factors comprising of Twist1, Twist2 and E12/E47. All of these transcription factors can repress E-cadherin gene expression either alone or co-operatively, in addition to up regulating mesenchymal specific genes.

Several extracellular factors in the tumor micro-environment are thought to regulate EMT and these are referred to as EMT inducers. These signals require the integrated activity of multiple pathways. Cytokines, growth factors and hypoxia have been identified as effective EMT inducers. One of the best characterized inducers of EMT is the TGF-β family of cytokines (64), known to play a significant role in various physiological contexts such as embryogenesis, wound healing, fibrosis and cancer metastasis. It can act directly via SMAD pathway as well as engaging other developmental pathways which are known to participate in EMT such as Wnt, Hedgehog, and Notch pathways (65).

Several members of receptor tyrosine kinases of EGF, Met, FGF, IGF and PDGF play critical roles in EMT occurring during development as well as cancer progression (66). Hypoxia

is the best known physiological context for EMT induction via multiple mechanisms including upregulation of hypoxia inducible factor 1α (HIF1α), scatter factor or hepatocyte growth factor (HGF), Notch and NF-κB pathways as well as epigenetic mechanisms (67). More recently, microRNAs have been recognized as important regulators of EMT core regulators, with the miR200 and miR205 family members targeting Zeb1 and Zeb2 to control E-cadherin and vimentin gene expression (68).

c. Inflammation as a driver of EMT, transformation and cancer stem cell phenotype

Both clinically and epidemiologically chronic inflammation has been linked to cancer but the precise mechanisms integrating these two phenomena are currently being uncovered (69,70). In the context of breast cancer, inflammatory cytokines more specifically, IL-6, has been associated with estrogen receptor negative status and poorly differentiated cancers (71). In fact, IL-6 is a potent inducer of EMT in human breast cancer cell lines capable of triggering malignant features (72,73). Cell autonomous production of IL-6 in turn activates the NF-κB and STAT3 signaling pathways which sustain positive feedback loops to achieve chronic inflammation which aids tumor progression (74,75).

Research in the EMT field came to the forefront of cancer research in 2008 when two independent groups identified that EMT could generate cells with the cell surface marker profile associated with cancer stems cells (CSC) or tumor initiating cells (TIC) (76,77). Cancer stem cells are defined as cells capable of self-renewal as well as differentiation into multiple cell types forming the bulk of the tumor, characterized by the ability to initiate tumors in serial transplantation assays (78). They have a cell surface marker profile including $CD44^{hi}$ $CD24^{lo}$ in humans. These experimental results were supported by the clinical observations that disseminated breast cancer cells which have undergone EMT, were enriched in $CD44^{hi}$ $CD24^{lo}$ cell populations (79), thus solidifying the link between EMT and the cancer stem cell phenotype.

Diverse extracellular cues including the paracrine signaling inflammatory cytokines regulate epithelial cell differentiation status and induce EMT in the context of development as well as cancer. Such re-programmed cells produce autocrine signaling factors which promote tumorigenicity and metastasis. Recently, autocrine signals have been indicated to contribute to

maintenance of the mesenchymal state as well as the stem-cell like states in mammary epithelial cells (65). Autocrine IL-6 loop has been reported to be essential in the development and maintenance of the mammary cancer stem cell population and mediate resistance to chemotherapy (80-82).

3. Proteotoxic stress and cancer

We have recently reported that ectopic expression of SCCA1 leads to the inhibition of both proteasomal and lysosomal protein degradation (37), suggesting that elevated SCCA1 expression may lead to a disruption in the normal protein homeostasis manifested by an increase in the unfolded protein response (UPR). Since chronic UPR and inflammation contribute to the pathology a variety of diseases such as diabetes, pulmonary fibrosis, colitis (83), we hypothesized that SCCA via inducing chronic misfolded protein stress, might promote inflammation and tumorigenicity. Emerging evidence confirms that UPR effectors are activated in carcinomas of the breast, colon and liver and play essential roles in tumor cell survival (84).

Protein quality control is a coordinated effort mediated by protein chaperones and targeted proteolysis (85). Molecular chaperones are involved in folding the newly synthesized peptides into their cognate conformation for optimal functionality. Protein turnover rates are guided by the intrinsic half-life of the protein as well the regulated activity of the lysosome and the ubiquitin-proteasome system (UPS). Impairment of protein degradation pathways can lead to the dysregulation of cellular protein homeostasis which leads to the accumulation of misfolded or unfolded proteins as well as protein aggregates which can become potentially toxic to cells. Eukaryotic cells have evolved to compartmentalize these functions to specific organelles.

The endoplasmic reticulum (ER) is the second largest organelle after the nucleus and it consists of a network of membranes and flattened sacs extending from the nuclear membrane branching out into the cytosol. The ER membrane constitutes half of the total membrane mass of animal cells. The ER is the primary organelle for protein folding, assembly and quality control of newly synthesized proteins. In addition, it is a store house of free calcium, co-ordinates transport of proteins into the various subcellular destinations, and is site for sterol and lipid biosynthesis (86,87).

The protein folding function in the ER is accomplished by a variety of chaperone proteins such as calnexin and calreticulin apart from member for the heat shock protein family (HSPs) GRP78/BiP and GRP94. Co and post-translational modifications including disulphide bond formation and glycosylation modifications facilitate proper folding and assembly of oligomeric proteins. Despite such an elaborate system, protein misfolding does occur and these proteins are recognized and degraded by a mechanism termed Endoplasmic Reticulum Associated Degradation (ERAD). Misfolded proteins are retro-translocated to the cytosol where they are ubiquitinated and degraded. Sometimes, improperly folded proteins tend to form aggregates that are cleared by an autophagy dependent mechanism (88).

Overall, the functions of the ER are critical for cell survival and thus the cell has evolved a highly sophisticated system to maintain ER homeostasis. Several physiological to pathological stimuli such as depletion of cellular calcium, nutrient deprivation, hypoxia, altered glycosylation, DNA damage, redox imbalance can cause ER dysfunction (89). This results in the accumulation of misfolded proteins in the ER, resulting in a condition which is commonly referred to as ER Stress. To ensure protein folding fidelity the ER has a complex circuitry of transmembrane stress sensors that are capable of detecting misfolded proteins in the ER lumen and relay the information to the nucleus via a signaling cascade involving transcription factors to up regulate chaperones and ERAD genes to restore ER homeostasis. This signaling cascade is collectively termed the Unfolded Protein Response (UPR) (90).

The extent and duration of ER stress determines cell fate. There is mounting evidence to suggest that basal level ER stress is essential for normal physiological processes of embryonic development and cellular differentiation. Specialized cell types, especially ones that actively secrete enzymes, antibodies and extracellular matrix proteins are known to have to cope with excessive ER load. With the increase in stress levels, this normal feedback regulation to maintain homeostasis becomes ineffective resulting in pathological ER stress. Pathological ER stress results in reprogramming of cellular differentiation as an adaptive strategy (91,92). But if the stress remains unresolved, it interferes with normal cellular proliferation and physiology and the cells become dysfunctional. ER stress has been implicated with pathology of several diseases including diabetes, obesity, colitis and cancer. Severe unmitigated ER stress leads to the activation of cell-death programs which are closely intertwined with the ER stress pathways. Such an apoptotic trigger involving ROS production and MOMP becomes dominant and causes

cells to succumb (93-95). The wide range of physiological consequences of ER stress makes it a necessary evil.

A. ER stress adaptation: Unfolded Protein Response

The ER has three trans-membrane stress sensors namely; Activating Transcription Factor 6 (ATF6), Inositol Requiring Enzyme 1 alpha (IRE1α) and Protein kinase R (PKR)-like Endoplasmic Reticulum Kinase (PERK). These three arms work in parallel using distinct mechanisms of action to relay ER stress from the ER lumen to the cytosol which eventually activates transcription factors that alter gene expression to relieve the existing stress. The luminal domains of the three transmembrane sensors binds to an ER-resident chaperone protein GRP78/BiP. When stress levels are elevated, BiP is recruited to aid folding of unfolded and misfolded proteins and releases the repression on the stress sensors. Thus, BiP is termed as the master regulator of ER stress (96).

ATF6 protein has two isoforms ATF6α and ATF6β, which is activated when ER stress liberates the protein from the negative regulation by BiP. It then undergoes translocation to the Golgi where a serial intramembrane proteolysis by site-1 protease and site-2 protease releases the N-terminal domain which has a nuclear localization sequence and basic leucine zipper (b-ZIP) domain for DNA binding (97,98). While ATF6α has been recognized as a strong transcriptional activator, the β form may serve as a negative regulator (99). The target genes of ATF6 include chaperone proteins BiP, protein disulphide isomerase (PDI) and glucose regulated protein 94 (GRP94).

IRE1 is the most well-conserved signaling arm of the ER stress pathway, and is the only known stress sensor in yeast. It is a unique molecule which is a bifunctional serine-threonine kinase/endo-ribonuclease. It is thought to be activated by dimerization, trans-auto phosphorylation which in turn activates the endo-ribonuclease function (100,101). IRE1 splices XBP1 via an unconventional splicing event which alters the translational reading frame of the protein fusing the N-terminal DNA binding domain to a C-terminal transactivation domain (102). The IRE1-XBP arm of the UPR is involved in lipid biosynthesis, ER homeostasis and ER-associated degradation (ERAD).

PERK, the third member of the UPR is also activated by ER stress and undergoes dimerization induced activation of the kinase function. Activated PERK phosphorylates translation initial factor eIF2a and causes translational attenuation. This translational pausing allows for existing proteins to be folded properly prior to the synthesis of new proteins (103). During such translational repression, selective genes such as ATF4 are continue to be translated using alternate open reading frames and cap-independent translation mechanisms (104,105). ATF4 is a member of the bZIP family of transcription factor which regulates several genes involved in amino acid biosynthesis and transport, oxidative stress response and ER-stress induced apoptosis (106). Another, phosphorylation target of PERK is nuclear factor erythroid2 related factor-2 (Nrf2) which increases gene expression of enzymes involved in ROS quenching.

There is a considerable degree of cross-talk between the three arms of the UPR, and the transcription factors downstream of each arm co-operatively regulation the expression of several genes resulting in functional redundancy. For example, ATF6 transcribes the un-spliced form of XBP-1(XBP1u) which is subsequently processed by the endonuclease activity of IRE1 (102) and ATF6 and PERK synergistically regulate the expression of CHOP (107).

B. UPR and cancer

During tumorigenesis, the high proliferation rate of cancer requires the efficient functioning of ER to meet the demands of metabolically overactive cells. Thus there is an increased load on the ER for folding, assembly and secretion of proteins, all of which make these cells exposed to elevated level of physiological stress. Thus cancer cells have to develop ways to synergize the adaptive UPR to facilitate their growth. Furthermore, growing tumor cells that are away from infiltrating blood vessels are prone to the exposure of nutrient deprivation and hypoxia, both of which are known to induce ER stress. Indeed, there has been accumulating evidence over the last few years that suggested heightened ER stress response in cancer cells and mechanisms by which cancer cells fine tune the UPR to co-operate with tumor progression (108,109).

It is well documented that GRP78/BiP protein level is elevated in a variety of cancer cell lines, tumor biopsies, in solid tumors as well as hematological malignancies (110,111). BiP has been reported to have anti apoptotic functions by direct interaction with the apoptosis machinery

localizing on the ER and blocking caspase activation (112). The expression of stress markers GRP78/BiP and XBP1 was also found to be elevated in breast cancer patients (113), further emphasizing the clinical relevance of ER stress in cancer. BiP expression was found to be associated with malignancy and metastasis and correlated with resistance to apoptosis as well as decreased chemotherapeutic efficacy (114).

The role of ATF6α has been appreciated in the ability of cells to cope with chronic ER stress and turn on the adaptive response to promote cell survival (115-117). The transcriptional targets of ATF6α which include GRP78/BiP and XBP-1 are also elevated in a number of cancer types and correlate with poor response to therapy (118).

The IRE1/XBP pathway is an important axis in a variety of tumor types including solid tumors such as breast and colorectal adenomas as well as hematological malignancies of multiple myeloma and lymphomas (119-122). It promotes cell survival in response to stress and hypoxia and enables the secretion of tumor promoting cytokines, IL-6 and VEGF in macrophages (123). In fact XBP-1 is an essential mediator of plasma cell differentiation leading to multiple myeloma and recently a small molecule inhibitor designed against the endonuclease function of IRE1 was tested and found to inhibit the splicing of XBP-1 both in-vitro and in-vivo (124). Recent evident implicated a role for XBP-1 in the tumorigenicity and progression of triple-negative sub-type of breast cancers, providing evidence for the direct interaction between XBP-1 with HIF-1α to regulated hypoxic stress response (125).

The PERK pathway has been implicated in different stages of cancer progression from cell survival, adaptation to hypoxia and induction of angiogenesis (126). However, there are reports for opposing roles for PERK/ATF4 in experimental scenarios (127). The animal model wherein MMTV-neu mice were bred to PERK -/- mice showed a delayed onset of tumors possibly due to loss of ROS buffering action providing evidence to support its positive role in tumorigenesis (128). Additionally PERK was also shown to be critical for Myc mediated tumorigenesis by activated ER-stress mediated autophagy (129).

New observations indicate that the adaptive ER stress response can initiate an inflammatory response and these pathways are coupled in health and disease (130). Concurrently, all the three arms of the UPR, ATF6, IRE1 and PERK arms have been linked to the NF-κB pathway which is central to the inflammatory response, including cytokine

transcription and regulation of genes required for immunomodulatory functions (131-133). However the precise mechanisms are still unclear.

While there exists ample evidence linking ER-stress induced pro-inflammatory response to tumor progression, a new line of thought suggests that this ER stress is in fact "transmissible" to the cells in the tumor microenvironment. For example macrophages cultured in the conditioned media of tumor cells exposed to ER stress were capable of sensing stress and amplifying the response themselves. This phenomenon is now termed TERS (transmissible ER stress). This suggests the ER stress response in the various cell types comprising of tumor cells as well as inflammatory cells aids cancer progression (134).

Several oncogenic insults induce mild chronic ER stress and thus adaptation and tolerance to such stress is critical for tumor cell survival (93). Heightened ER stress provides an attractive setting for the use of proteotoxic drugs such as proteasome inhibitors and HSP-90 inhibitors for enhanced tumor-cell specific cytotoxic activity (109,135).

II. Results

1. SCCA1 induces an epithelial to mesenchymal transition

During a previous study which aimed to understand the role of SCCA1 in response to a variety of stress inducing agents, SCCA1 was ectopically expressed in baby mouse kidney (BMK) cells (37). While the wild type BMK cells are epithelial in origin (136), the expression of SCCA1 led to a rounded up cell morphology (Fig. 1A and 1B). A detailed transcriptional profiling comparing BMK vector cells and SCCA1 expressing cells using an Affymetrix mouse cDNA array revealed the down-modulation of a set of cell-cell adhesion genes such as Epithelial cadherin (E-cadherin, gene name Cdh1), Epithelial cell adhesion molecule (EpCam); genes involved in tight junction complex formation like Claudin4 (Cldn4) and Occludin (Ocln) and epithelial specific intermediate filaments, cytokeratins 8 and 18 (Krt 8, Krt18). In addition, among the up-regulated genes were the transcriptional repressor of E-cadherin, Zinc-finger E-box binding homeobox 1 (Zeb1), a gene that controls cytoskeletal remodeling RhoB and the proto-oncogene Myc (Fig. 1C). These results suggest that SCCA1 can promote an EMT-like cellular reprogramming in BMK cells.

While these results gave us an important lead to the function of SCCA1 in cellular differentiation, we decided to explore a physiologically relevant cell system in which study the implications of such a phenotype. Having identified that SCCA is elevated in advanced breast cancers, we decided to use the immortalized but non-transformed human mammary epithelial cell line MCF10A.

Next, we retro-virally expressed SCCA1 in MCF10A cells to generate stable cell lines (Fig. 2A). Upon continued passage of these cell in culture, the cobble-stone epithelial morphology gradually transformed to spindle shaped cells resembling mesenchymal cells (Fig. 2B). To validate if the EMT-like changes observed in the mouse cDNA array also held good in the human system, transcript changes in the MCF10A SCCA1 cells were verified using q-RT PCR for several epithelial and mesenchymal cell type specific genes (Fig. 2C). Further, the loss of epithelial phenotype and a gain of mesenchymal status was confirmed also at the protein level by examining the changes in the markers, E-cadherin and vimentin respectively, using western blotting as well as immunofluorescence based staining (Fig. 2A and 2D).

It is important to note that the transition to mesenchymal appearance occurred not instantaneously but over the course of seven passages, passaged once every four days. In order to follow the chronological changes associated with the cell transformation, a passage wise analysis was performed and changes in the EMT marker profile was examined by western blotting. E-cadherin expression gradually dropped with increasing passage number in the SCCA1 expressing cells which was accompanied by a reciprocal increase in the mesenchymal cytoskeletal protein, vimentin (Fig. 2E). At passage 8, the SCCA1 expressing cells exhibited spindle shaped fibroblast-like appearance.

2. SCCA1 induced EMT is dependent on its protease inhibitory function

In order to determine if the protease inhibitory function of SCCA1 is required for its ability to cause EMT in MCF10A cells, catalytically deficient mutants, SCCA1 F352A, a point mutant which impairs proteinase inhibitor activity (137) and SCCA1Δ340-345, mutant with a deletion of the hinge region which renders the reactive site loop inflexible (138), were also expressed and followed up to 8 passages. Western blotting for E-cadherin and Vimentin revealed that only wild type SCCA1 but not the mutants was able to induce EMT (Fig. 3A and 3B). This data suggest that anti-protease activity of SCCA1 is necessary for cellular transformation.

3. SCCA1 induces oncogenic transformation of MCF10A cells

The activation of the EMT program is believed to be a critical step for acquisition of malignant properties. Epithelial-Mesenchymal transition is associated with the loss of cell-cell adhesion which enables cells to migrate away from the site of the primary tumor and enter the vasculature. SCCA1 expressing cells displayed enhanced migratory potential when compared to vector control cells as assessed by a "scratch closure assay" (Fig. 4A). When cells were plated under low adherent conditions, epithelial cells undergo a form of cell death known as anoikis (139). Interestingly, SCCA1 cells were capable of survival in suspension and were able to proliferate to form spheres suggesting that the EMT changes do indeed confer anoikis resistance (Fig. 4B). Anchorage independent growth was tested in soft agar which demonstrated that the SCCA1 transformed cells were capable of colony formation while the non-transformed vector

control were unable to do so further confirming the ability of SCCA1 to transform MCF10A cells in culture (Fig 4C).

To test the tumorigenic potential *in vivo* we used an orthotopic mouse model wherein, vector control or SCCA1 cells expressing GFP were transplanted into the mammary fat-pad of immune compromised XID mice. While MCF10A cells were non-tumorigenic, nine out of nine mice implanted with SCCA1-expressing cells developed tumors as shown by GFP imaging (Fig. 5A and 5B). The ectopic expression of SCCA1 was confirmed by immunohistochemistry (Fig. 5C).

4. SCCA1 renders MCF10A cells growth factor independent

While the growth of normal cells is tightly regulated by the action of growth factors and hormones present in the interstitial fluids, malignant cells lack such dependence on exogenous factors. In fact, they achieve autonomous control over their proliferation by synthesizing such growth-promoting peptides (140). MCF10A cells are dependent on a set of growth factors and hormones, namely EGF, insulin, hydrocortisone and cholera toxin in order to maintain its state of proliferation and differentiation. In order to test for the growth factor dependence of the transformed cells, we first depleted EGF from the media and found that MCF10A SCCA1 expressing cells were capable of growth even in the absence of this growth factor while the vector control cells exhibited growth arrest (Fig. 6A and 6B). This prompted us to evaluate signaling pathways that contribute to proliferation downstream of the EGF-Receptor, namely the ERK and AKT pathways. Interestingly, while vector control cells showed a decrease in the phosphorylation status of ERK and AKT upon EGF withdrawal, SCCA1 cells had elevated basal level of activation of these pathways which was sustained up to a time course of 8 hours following EGF depletion (Fig 6C). This result suggests that SCCA1 transformed cells have bypassed the requirement of EGF signaling axis for growth.

Subsequently, the cells were also treated with basal media lacking the four growth supplements previously mentioned. Again, while the vector control cells ceased to divide, the SCCA1 cells were still able to proliferate, albeit at a slower rate indicating that the other three components apart from EGF are still essential for these cells to retain their proliferation rate (Fig.

6D). These results indicate that SCCA1 might induce autocrine growth promoting pathways to sustain growth in the absence of exogenously supplied growth factors and hormones.

5. EGF independence of MCF10A-SCCA1 cells is not due to sustained activation of the EGFR pathway

Since SCCA1 was reported to interfere with protein degradation pathways, we hypothesized that sustained activation of EGFR, due to its impaired degradation might explain EGF independence of the transformed cells. A ligand induced EGFR degradation assay in MCF10A cells prior to transformation revealed that SCCA1 does not inhibit EGFR degradation in cells prior to cellular transformation (Fig. 7A). On the contrary, EGFR was down-regulated both at the transcript as well as the protein level, in SCCA transformed cells suggesting that these cells have bypassed the requirement of EGFR upon mesenchymal transition (Fig.7B and 7C). Next, we sought to determine if transcriptional up regulation of EGF family ligands, previously reported to play a critical role in tumor development (141), was elevated in SCCA expressing cells. q-RT PCR for *EGF*, transforming growth factor alpha (*TGFA)*, heparin-binding EGF-like growth factor (*HBEGF*), amphiregulin (*AREG*), betacellulin (*BTC*), and epiregulin (*EPR*) displayed no apparent changes between the vector control and the transformed cells (Fig. 7D). Collectively, these results suggest that SCCA1 transformed cells do not exhibit sustained EGFR pathway activation.

6. SCCA1 promotes EMT through the up regulation of Interleukin-6

In the literature, the activation of cytokine networks has been reported to drive EMT and transformation of human breast cell lines (65,80,81). Upon examination of several such cytokines, namely transforming growth factor beta (*TGFβ*), interleukin-6 (*IL6*), *IL-8*, *IL1β*, and (C-X-C motif) ligand 1 (*CXCL1*), it was identified that IL-6 showed a dramatic increase in the transcript levels of IL-6 in the SCCA transformed cells (Fig. 8A). Next, an enzyme linked immunosorbent assay (ELISA) was performed using the conditioned media from vector control cells and SCCA1 transformed cells which confirmed that IL-6 protein was indeed secreted to the extracellular milieu (Fig. 8B). To check for the activity of this secreted IL-6, conditioned media

collected from vector control cells and SCCA1 cells was used to stimulate EGF-deprived MCF10A cells and robust activation of phosphorylated Signal Transducer and Activator of Transcription (STAT3) was noticed in the media from SCCA1 cells (Fig. 8C). Further, pre-incubation of the conditioned media with a neutralizing antibody to IL-6 abrogated STAT3 activation, suggesting that IL-6 indeed is responsible for this downstream signaling event (Fig. 8D).

In order to determine if IL-6 production is a cause or consequence of SCCA1 induced transformation, a passage-wise analysis of IL-6 transcript was performed using a quantitative PCR approach. It was observed that IL-6 levels were elevated ten-fold as early as passage 1 in the SCCA1 cells compared to the vector control cells, which continued to rise steadily in the subsequent passages with about a 100-fold increase recorded in the transformed state (Fig. 8E).

Silencing of IL-6 in the MCF10A SCCA1 cells prior to cellular transformation, resulted in a delayed transformation phenotype when compared to non-targeting hairpin control. This was associated with a robust increase in the transcript and protein levels of E-cadherin suggesting a rescue of the epithelial phenotype (Fig. 9A-E). In summary, these results suggest that IL-6 is elevated upon SCCA1 expression in MCF10A cells and plays a critical role in driving the EMT of these cells.

7. Canonical NF-κB drives the expression of IL-6 in MCF10A SCCA1 cells

Analysis of the promoter region of IL-6 gene has led to the finding that it is predominantly regulated by NF-κB. A luciferase reporter assay for the IL-6 binding site of NF-κB suggested a significant increase in relative luminescence in SCCA expressing cells (Fig. 10A), which was accompanied by a marked increase in nuclear localized RelA/p65 sub-unit of NF-κB (Fig. 10B). The increased expression of IL-6 in SCCA1-expressing cells was abrogated by the pharmacological inhibitor of NF-κB, BAY11-7082 (Fig. 10C), at a concentration tolerated by the parental MCF10A cells (74). Further, IL-6 transcription was also diminished by the ectopic expression of the IκBαM (S32A, S36A) super-repressor mutant (142-144) (Fig. 10D). Accordingly, treatment with BAY11-7082 resulted in the abrogation of anchorage-independent sphere formation (Fig. 10E) and increased apoptosis (Fig. 10F) in SCCA1-transformed cells.

These results indicate that activation of NF-κB plays an essential role in SCCA1-mediated IL-6 production and cell transformation.

8. SCCA1 induces pro-inflammatory signaling by activating a low level chronic UPR

An earlier study from our group has reported that expression of SCCA1, through its protease inhibitory activity, leads to impairment in lysosomal and proteasomal protein turnover (37). This impaired protein degradation caused by elevated SCCA1 expression is non-lethal and may lead to UPR that has been implicated in pro-inflammatory response in numerous human diseases including cancer (83). Hence, we speculated that SCCA1 could promote NF-κB activation and subsequently IL-6 production by inducing a non-lethal yet prolonged UPR.

The activation of the ATF6 arm was apparent in the SCCA1-expressing cells as observed by the ATF6α-p50 (Fig. 11A). Activation of the PERK arm of UPR was detected as SCCA1-expressing cells displayed a slight increase in ATF4 expression, which was further enhanced upon treatment with the ER stress inducer tunicamycin (Fig. 11A). In addition, CHOP, a synergistic transcriptional target of ATF4 and ATF6 (107), was more robustly induced by tunicamycin in SCCA1-expressing cells (Fig. 11A). Both ATF6α-p50 and ATF6α-p36 translocate to the peri-nuclear and nuclear regions where p50 is believed to function as a transcription factor (97) while the function of p36 remains unclear (145). An increased nuclear and peri-nuclear ATF6α was detected by subcellular fractionation (Fig. 11B) and immunofluorescence (Fig. 11C) in SCCA1-expressing cells. Furthermore, the elevated ATF6α transcriptional activity was revealed by the induction of its transcriptional target the unspliced form of XBP1 (XBP1u) (Fig. 11D), which was distinguished from the spliced form XBP1s by restriction digestion with PstI that cuts XBP1u but not XBP1s (146).

With regard to the IRE1 arm, SCCA1-expressing cells exhibited a decreased basal level of spliced XBP1s (Fig. 11D and 11E). This supports our notion that SCCA1 induces a prolonged low level UPR, which has been shown to suppress the IRE1α signaling (147). Moreover, upon tunicamycin treatment, the SCCA1-expressing cells were able to convert this increased amount of XBP1u to XBP1s (Fig. 11E), indicating that while the steady-state level of XBP1 splicing (as an indicator of IRE1α activity) is low, the signaling machinery is still intact in these cells.

The UPR-promoting function of SCCA1 is dependent on its protease inhibitory activity as the SCCA1 F352A and SCCA1Δ340-345 inactive mutants did not alter the levels of ATF6α-p50 (Fig. 12A and 12B).

We next looked for the activation of UPR along the passages leading up to the mesenchymal transformation of MCF10A cells. It was observed that ATF6α-p50 was elevated at early passages even prior to the appearance of the mesenchymal cytoskeletal marker vimentin. (Fig 13). This data suggests that induction of UPR is a relatively early event accompanying the progressive loss of E-cadherin and gain of vimentin.

Collectively, the data presented indicate that increased SCCA1 expression leads to a chronic UPR with the activation of the PERK and ATF6 arms and slight inhibition of the IRE1 arm.

9. Silencing the activated stress sensors decreases SCCA1 induced IL-6 and transformation

To determine whether activation of PERK and ATF6α are critical for SCCA1-mediated IL-6 expression and cell transformation, we silenced PERK or ATF6α in SCCA1 cells. Silencing either PERK or ATF6α led to down-regulation of *IL-6*, impaired cell proliferation, and decreased anchorage-independent sphere formation in SCCA1-expressing MCF10A cells (Fig. 14A-F). In contrast, silencing XBP1 led to an increase of *IL-6* and no effects on cell proliferation or sphere formation (Fig 14G-I).

Similarly, silencing PERK and ATF6α in MDA-MB-231 cells with the endogenous level of SCCA1 also led to decreased *IL-6* expression and reduced cell proliferation (Fig. 15A-D). This result confirms that ER stress arms play a critical role in the tumorigenic properties of breast cancer cells that were tested above.

10. Loss of function of SCCA1 decreases UPR, IL-6 expression and cell-growth

The specific role of SCCA1 in mediating UPR, IL-6 production, and transformation was further tested by silencing the over-expressed SCCA1 in MCF10A cells (Fig. 16 A-C) and endogenous SCCA1 in MDA-MB-231 and MDA-MB 468 cells (Fig. 17 A-F), which also

resulted in decreased expression of ATF4 and IL-6, and cell proliferation. This set of data highlights the role of SCCA in regulating basal ER stress levels. While it confirms that SCCA expression is required for the continued production IL-6 in the MCF10A-SCCA1 cell line, it further substantiates the regulation of IL-6 gene expression by endogenously expressed SCCA in aggressive breast cancer cell-lines.

11. SCCA1 induces EMT like phenotype, IL-6 and UPR in multiple cell lines.

To test the generality of the finding that SCCA1 expression can indeed result in EMT like phenotype with elevated IL-6 and UPR, we used a range of epithelial cell lines including BMK, HMLE, and SKBR-3.

Expression of SCCA1 in the BMK mouse cell line, resulted in dramatic change in morphology with the loss of epithelial cell-cell adhesion (Fig. 1A-C). This observation was supported by the loss of epithelial marker E-cadherin both at the protein and RNA level. (Fig. 18A and 18B). The results from the microarray (Fig. 1C) were validated using quantitative PCR which further confirmed the loss other cell-cell adhesion molecules Epcam and Claudin4 (Fig. 18B). On the other hand, the mesenchymal marker vimentin was not up regulated in these cells indicating partial EMT (Fig 18A). Interestingly, we noted an increase IL-6 transcript levels as observed in MCF10A cells (Fig. 18B and 18C). Further, treating these cells with the ER stress inducer Tunicamycin revealed an exaggerated UPR as observed by ATF6α and ATF4 (Fig. 18D). These results indicate that SCCA1 induced EMT, IL-6 and UPR hold true across the human and mouse cell systems.

The HMLE cells comprise of a primary human mammary epithelial cell system generated by the sequential retroviral expression of the catalytic subunit of telomerase (hTERT), SV40 large and small T antigens which inactivate the tumor suppressor pathways (148). Retroviral infection was employed to generate stable HMLE cells expressing SCCA1 (Fig. 19A). Elevated expression of IL-6 was observed when cells were harvested at the first passage post infection and antibiotic selection. Examination of EMT markers revealed that while that no decrease in the expression of epithelial specific genes, E-cadherin, claudin4 and Epcam, there was a significant increase in mesenchymal specific genes, vimentin and fibronectin1 (Fig. 19B).

Further, SCCA1 was also stably expressed in the HER2 positive breast cancer cell line which has a distinct epithelial morphology (Fig. 19C). As in the other cell lines previously described, IL-6 transcript level was elevated when cells were harvested at the first passage post infection and puromycin selection. Additionally, mesenchymal markers, Vimentin and Zeb1 also showed a significant up-regulation (Fig 19D), while epithelial markers did not show the expected decrease.

This observation of acquisition of mesenchymal traits with the retention of epithelial features is consistent with previous reports of partial EMT proceeding with intermediate transitory states (149). In fact a recent publication reported that circulating breast tumors cells simultaneously expressed both epithelial and mesenchymal markers suggesting a dynamic inter-conversion between the two states (59).

12. SCCA1 alters UPR and inflammation in a transgenic mouse tumor model

To further study the role of SCCA1 in affecting UPR and tumorigenesis *in vivo*, we generated a conditional SCCA1 transgenic mouse strain with a 129Ola/C57Bl/6 mixed background. The human SCCA1 open reading frame cDNA was cloned into the housekeeping *Hprt* gene located on the X-chromosome. A stop cassette flanked by LoxP sites was placed upstream of SCCA1 to allow for Cre-specific tissue expression of SCCA1 (LSL-SCCA1; Fig. 20A). By breeding to the MMTV-Cre mice that specifically express Cre in mammary epithelium, SCCA1 was expressed in mammary glands confirmed by immunoblotting and IHC (Fig. 20B and 20C). The SCCA1-expressing mice were fertile, sustained pregnancies to term, produced viable offspring, and displayed no apparent defects in ductal morphogenesis, indicating that elevated expression of SCCA1 is not sufficient to drive mammary tumorigenesis by itself.

Separately, as our above previous results also showed that SCCA1 can promote chronic UPR and IL-6 production in cell lines, we bred the LSL-SCCA1 mice to the MMTV-neu mice (in an FVB genetic background; (150)) to determine whether expression of SCCA1 can modulate tumor microenvironment and accelerate tumorigenesis. A total of 8 neu^+; $SCCA^+$ mice and 10 neu^+ litter mate control female mice were generated. Four mice from each group developed palpable tumors. We were able to collect 8 tumors for histology and 4 tumors for western blot (2 tumors from each group). Strikingly, when compared with neu^+ only tumors, all neu^+; $SCCA1^+$

tumor cells breached the basement membrane to invade into the stromal tissue (Fig. 21A). The $SCCA^+$ tumors also displayed elevated UPR indicated by increased expression of ATF4 and ATF6α-p50 (Fig. 21B), as well as increased intratumoral IL-6 expression and the infiltration of F4/80-positive cells (Fig. 21C). These results indicate that while SCCA1 is not able to accelerate tumor development in the MMTV-neu background, it can clearly alter the tumor microenvironment and increase the potential of tumor invasiveness.

13. Oncogenic Ras-induced SCCA expression regulates UPR, IL-6 and tumorigenesis

The previously described results have clearly demonstrated that SCCA1 regulates IL-6 levels and UPR upon ectopic expression in breast epithelial cells MCF10A, HMLE and SKBR-3 cells and the knock down of endogenous SCCA in breast cancer cell-lines MDA-MB 231 and MDA-MB-468 cells, we wanted to address how SCCA1 gene expression is induced in the context of breast cancer. We tested this hypothesis by over-expressing c-Myc and oncogenic Ras (K-RasV12) in MCF10A cells. Interestingly, while Myc expression had no effect on SCCA gene expression, oncogenic Ras displayed a marked increase in SCCA levels at both the gene expression as well as the protein levels (Fig. 22A and 22B). The expression of Ras in MCF10A cells resulted in an EMT-like transition as reported previously (77,151). This further supports our idea that SCCA is critical for this type of morphological stage further confirming its role in tumor cell de-differentiation and transformation. Additionally, Ras transformed cells show a distinct increase in cytokine production, with IL-6 being a critical mediator of Ras-induced tumorigenesis (152). In this system we decided to test if SCCA expression is critical for Ras induced tumorigenic phenotypes. We knocked down Ras-induced SCCA expression using a short hairpin and demonstrated a decreased UPR as observed by activated p-50 ATF6α and the ablation of IL-6 expression (Fig. 22C and 22D). This data suggests that Ras activation can induce SCCA expression in breast cancer.

III. Figures

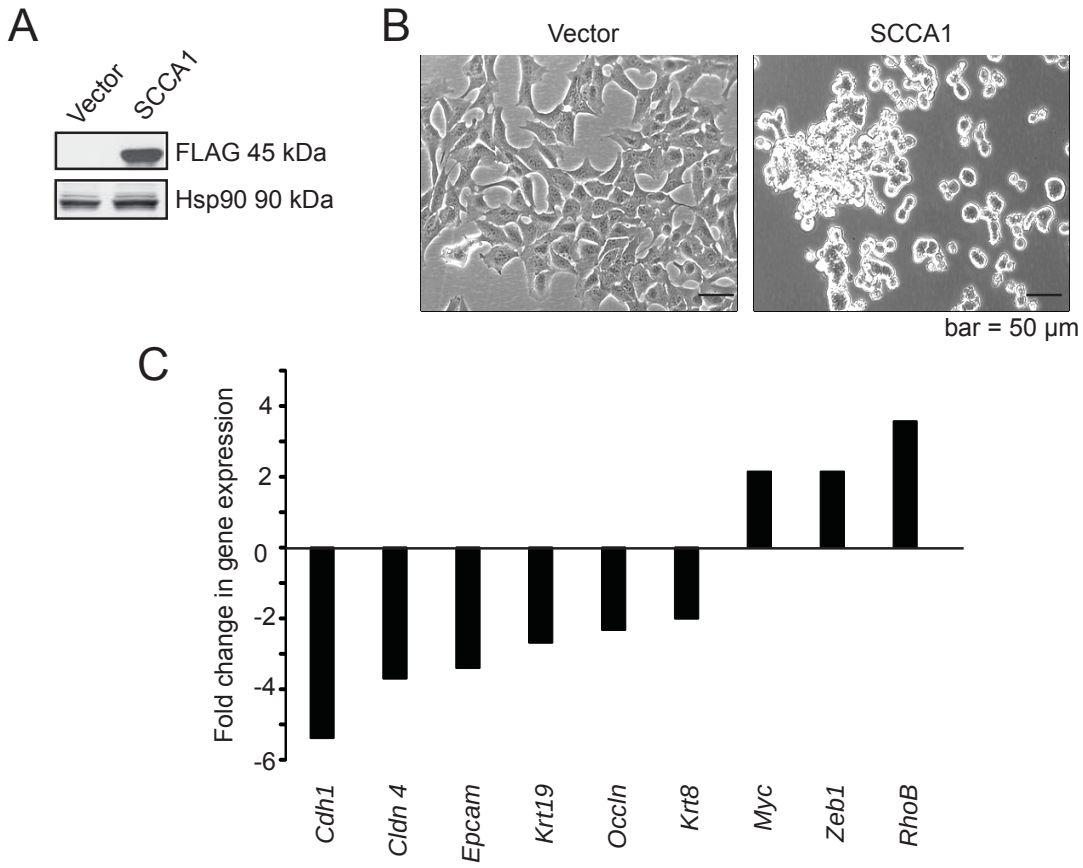

Figure 1. Ectopic expression of SCCA1 leads to EMT-like phenotype in baby mouse kidney epithelial cells.

Baby mouse kidney cells were retrovirally infected with empty vector or FLAG-tagged SCCA1. Stable cell lines were established by antibiotic selection. (A) Whole cell lysates were probed for indicated proteins by immunoblotting. (B) Phase contrast photomicrographs were taken at 20X magnification. (C) Cells were subjected to cDNA microarray using an Affymetrix mouse cDNA array chip. The fold change of the expression level of selected EMT-related genes is shown.

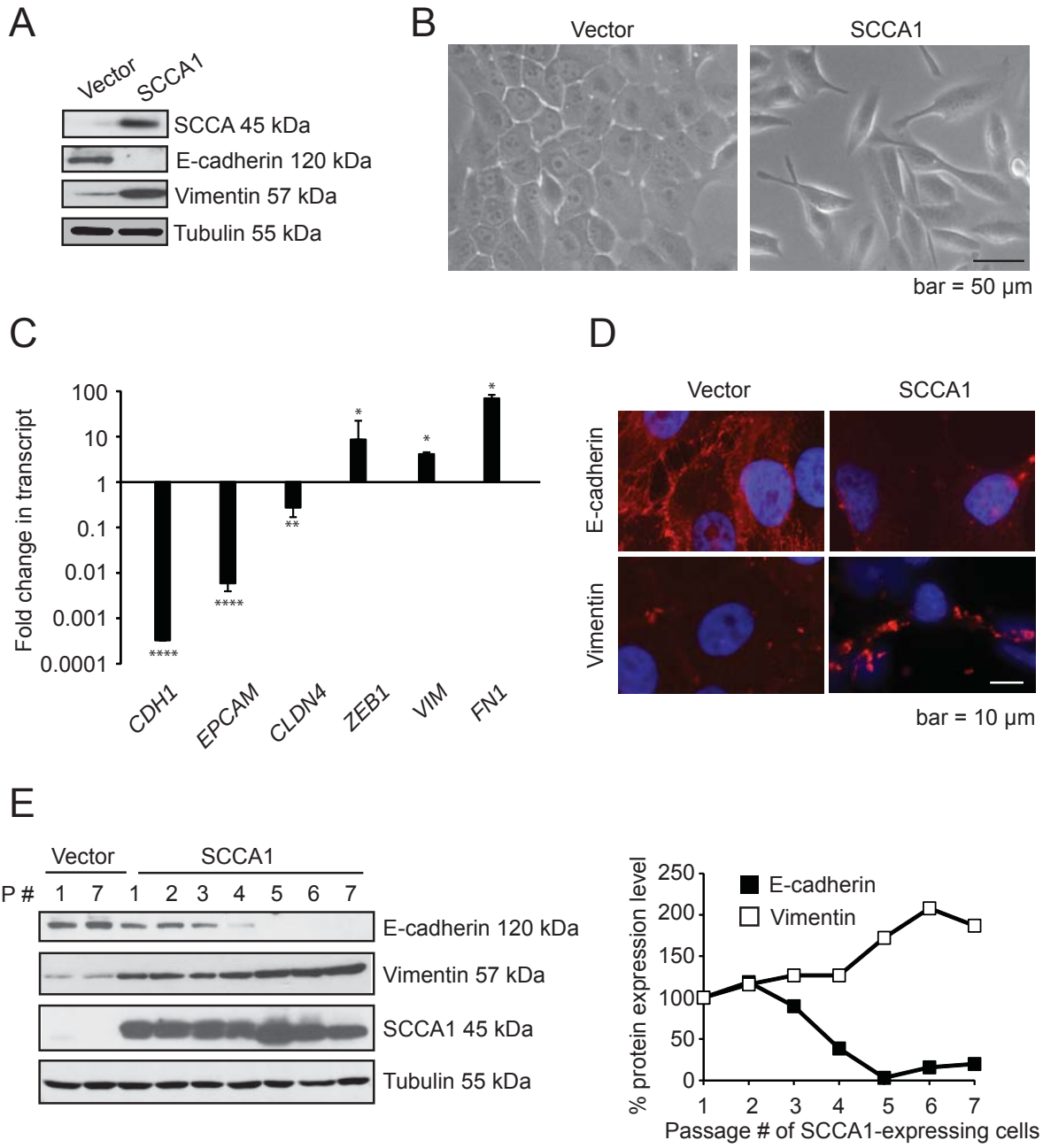

Figure 2. SCCA1 expression in MCF10A cells leads to Epithelial- Mesenchymal Transition. (A-E) MCF10A cells were retrovirally infected with vector control of Flag-SCCA1, and were continuously passaged. At passage #8, (A) whole cell lysates were prepared and western blotting was performed for the indicated proteins, (B) phase contrast images were taken 20x. (C) Total RNA analyzed for the transcript level of indicated genes via qRT-PCR which was normalized against that in vector control cells. Data shown are the mean fold change + SEM of two independent experiments performed in triplicates. *$p<0.05$, **$p < 0.01$, ****$p < 0.0001$. (D) Immunofluorescence was performed using antibodies against E-cadherin and vimentin, and counter stained with DAPI. Images were taken by a deconvolution fluorescence microscope 63x. Note the loss of cell-surface E-cadherin and increase in vimentin expression in SCCA1-expressing cells. (E) Whole cell lysates were harvested at indicated passage numbers and analyzed for indicated proteins by immunoblotting. The graph on the right indicated the trend in the change in the markers for EMT.

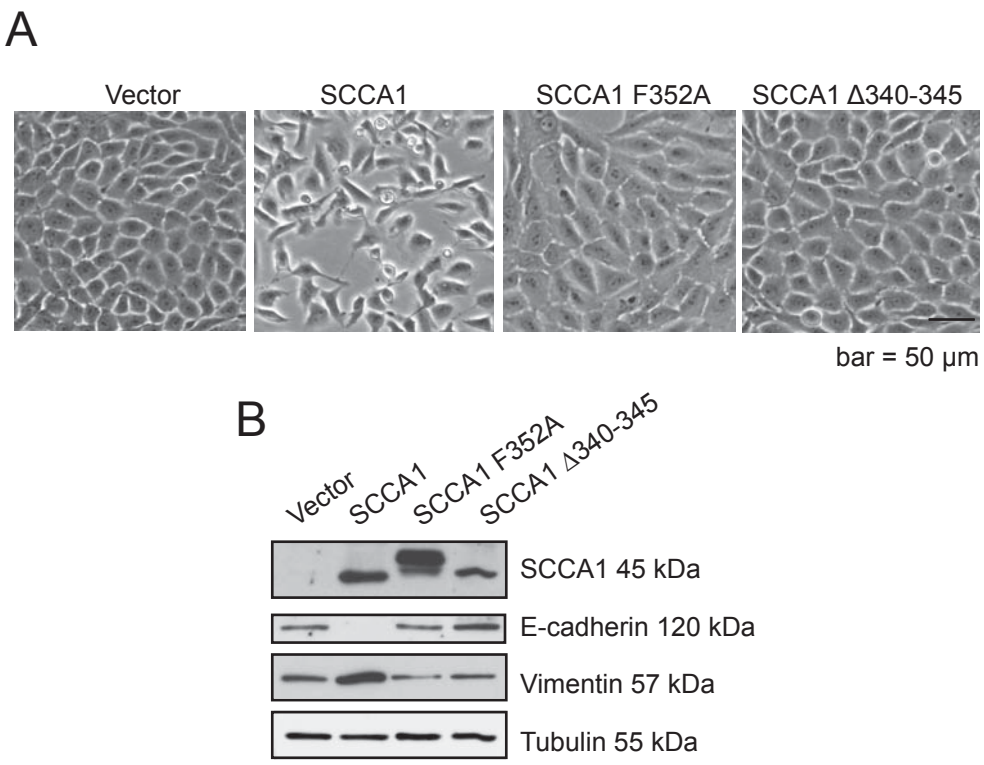

Figure 3. SCCA1 induced EMT is dependent on its protease inhibitory activity.
MCF10A cells stably expressing vector control, Flag-SCCA1, Flag-SCCA1 F352A and Flag-SCCA1Δ340-345 were generated by retroviral transduction and selected by antibiotic selection. Cells were continuously passaged 8 times. (A) Phase contrast images were taken at 20x. (B) Whole cell lysates were analyzed for indicated proteins by immunoblotting.

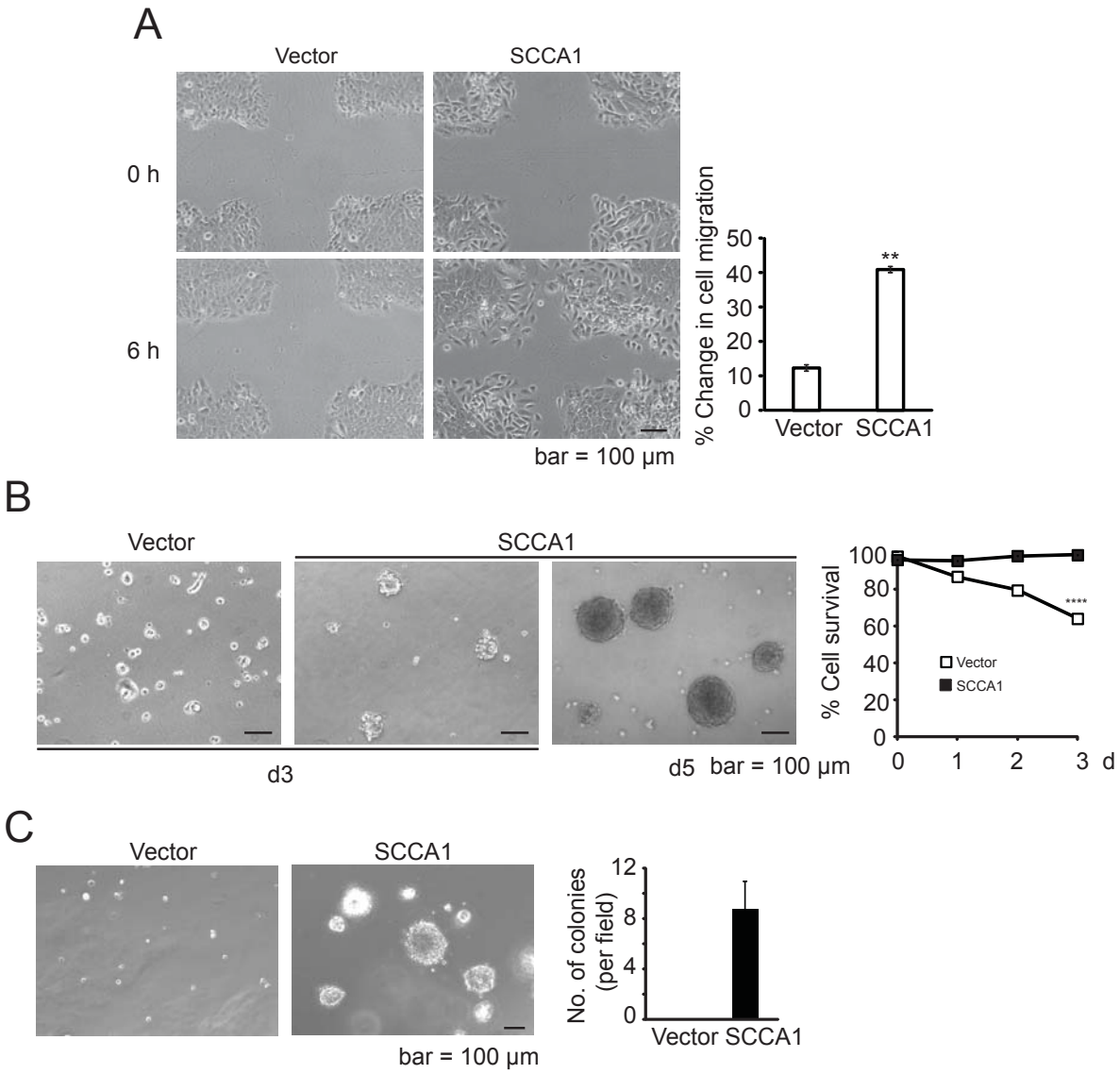

Figure 4. Ectopic expression of SCCA1 in MCF10A cells leads to oncogenic transformation.

(A) Vector and SCCA1-expressing MCF10A cells were assessed by the wound healing assay 6 h after the wound scratch on cell culture monolayer. Representative images are shown. Wound closure was quantified by the percent change in cell migration. Data shown are the mean of two independent experiments performed in triplicates +/- SEM. **$p < 0.002$.

(B) Indicated cells were cultured in non-adherence culture dishes for indicated periods of time. Phase contrast images were taken at 10x. Cell viability was mesured by PI exclusion. Data shown are the mean +/- S.D. of an experiment performed in triplicate. Error bars are too small to be visible compared to the physical size of the symbol. ****$p < 0.0001$. (C) Indicated cells were trypinised to obtain single cell suspensions and cultured in soft agar for three weeks. Images of cell colonies were taken. The quantification shown is the average number of colony spheres ≥ 100 µm per field from triplicate experiment + S.D. Five fields were counted for each replicate.

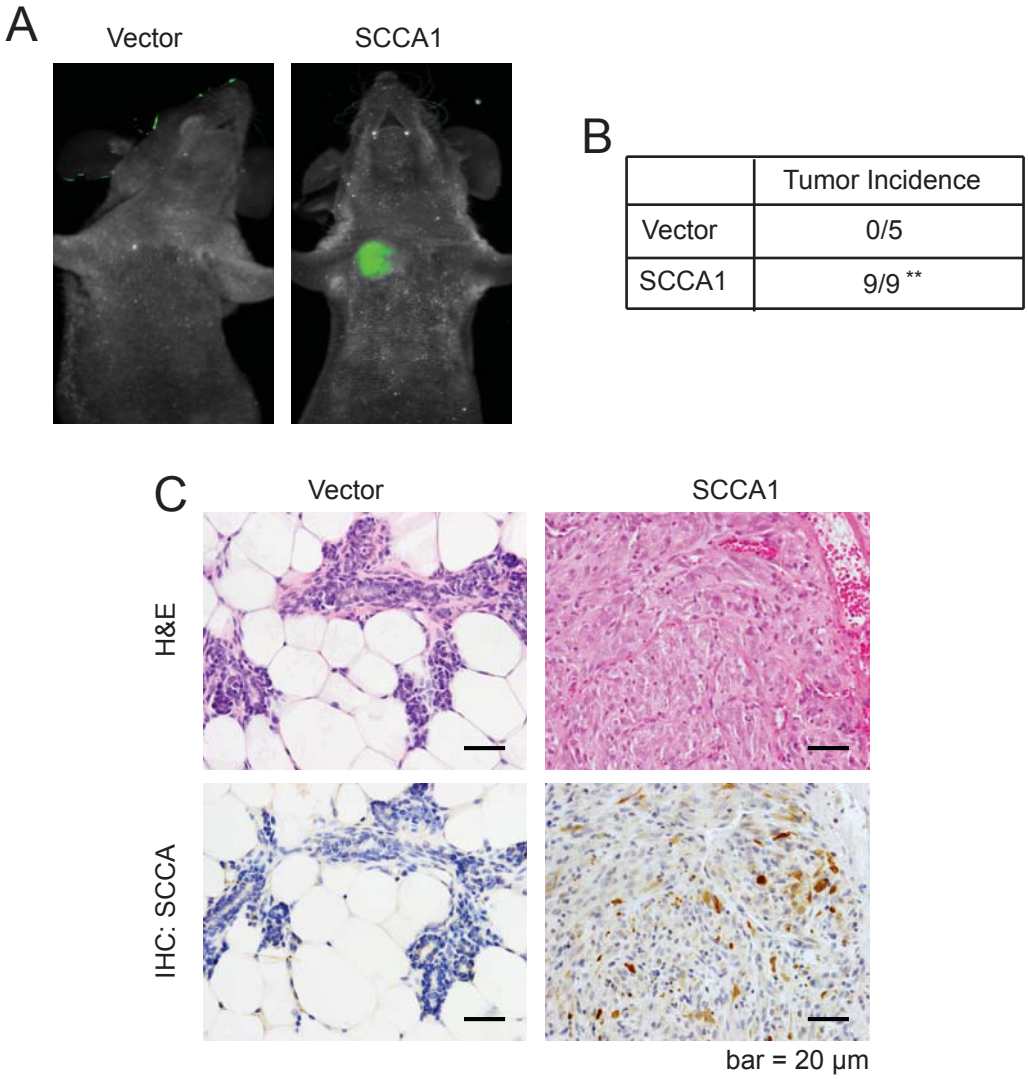

Figure 5. MCF10A-SCCA1 cells form tumors in an orthotopic model in immune compromised mice.
7.5 x 10^5 MCF10A cells expressing either GFP alone (n=5) or GFP plus SCCA1 (n=9) were implanted into the mammary fat pad of XID mice. (A) Mice were imaged for tumor formation 60 days post implantation using a fluorescence small animal imager. (B) The formation of palpable tumors from the vector control and SCCA1-expressing cells are summarized. Significance was judged based on Fischer's exact test. **$p < 0.01$ (C) Tumors were sectioned and stained using H&E (top panels) or by IHC with the SCCA antibody (bottom panels).

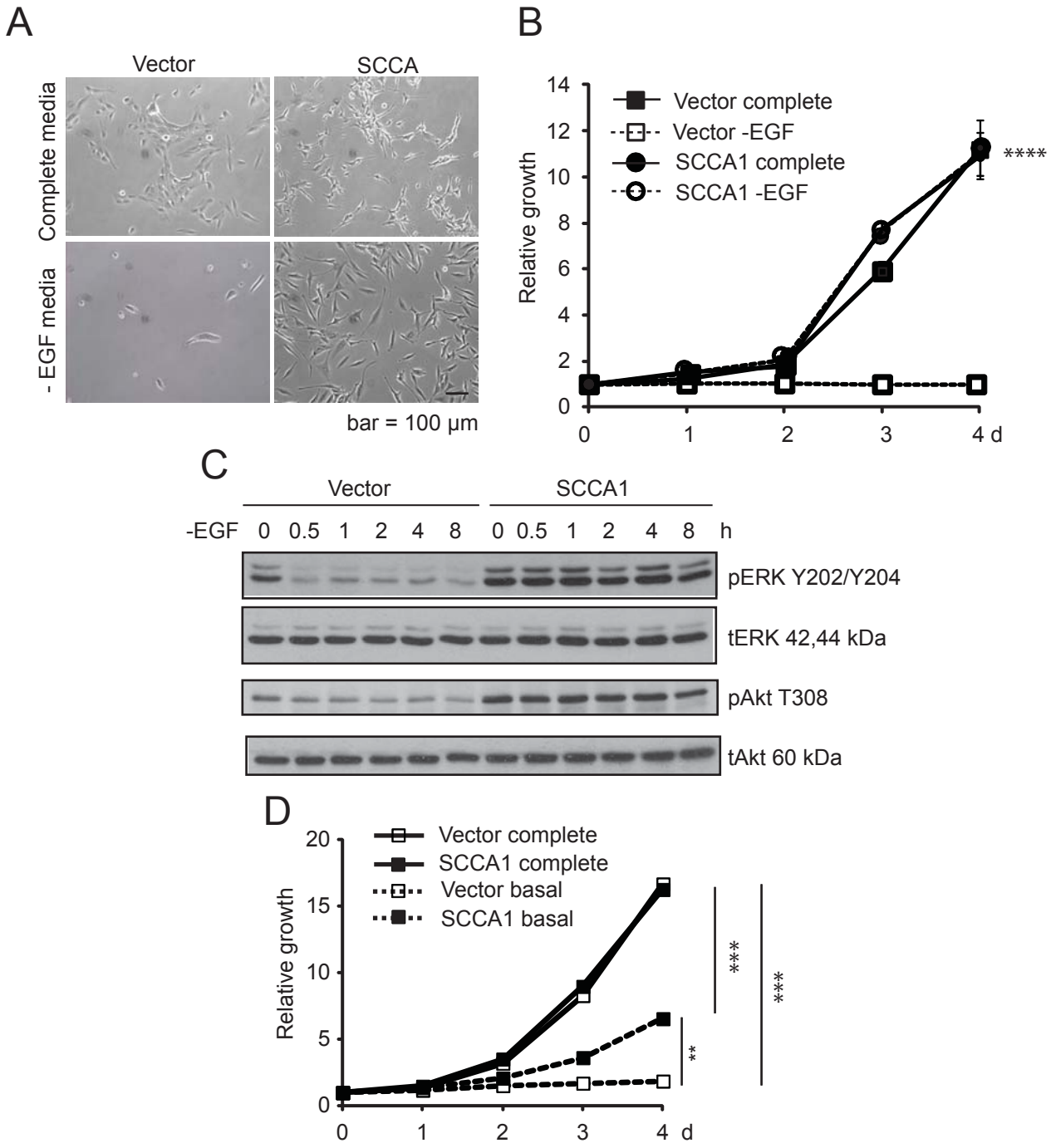

Figure 6. SCCA1 expression renders MCF10A cells independent of EGF.
(A) Indicated cells were cultured in complete or EGF-deprived medium. Phase contrast images were taken at 10x on day 4 post culturing in the above mentioned conditions. (B) Relative cell growth was measured by crystal violet staining normalized to the O.D. reading of the cells at time zero. Data shown are the mean +/- S.D. of two experiment performed in triplicate. ***$p < 0.001$. (C) Cells were cultured in EGF-deprived medium for indicated times and analyzed for indicated proteins by immunoblotting. (D) Cells were cultured in either complete medium or basal medium (lacking growth supplements EGF, insulin, hydrocortisone and cholera toxin) and relative growth was measured by crystal violet staining. The difference between the sample in complete media is not statistically significant (ns), the difference between Vector and SCCA1 in basal media is significant **$p<0.01$, the difference between Vector in complete and basal media as well as SCCA1 in complete and basal media is highly significant ***$p<0.001$

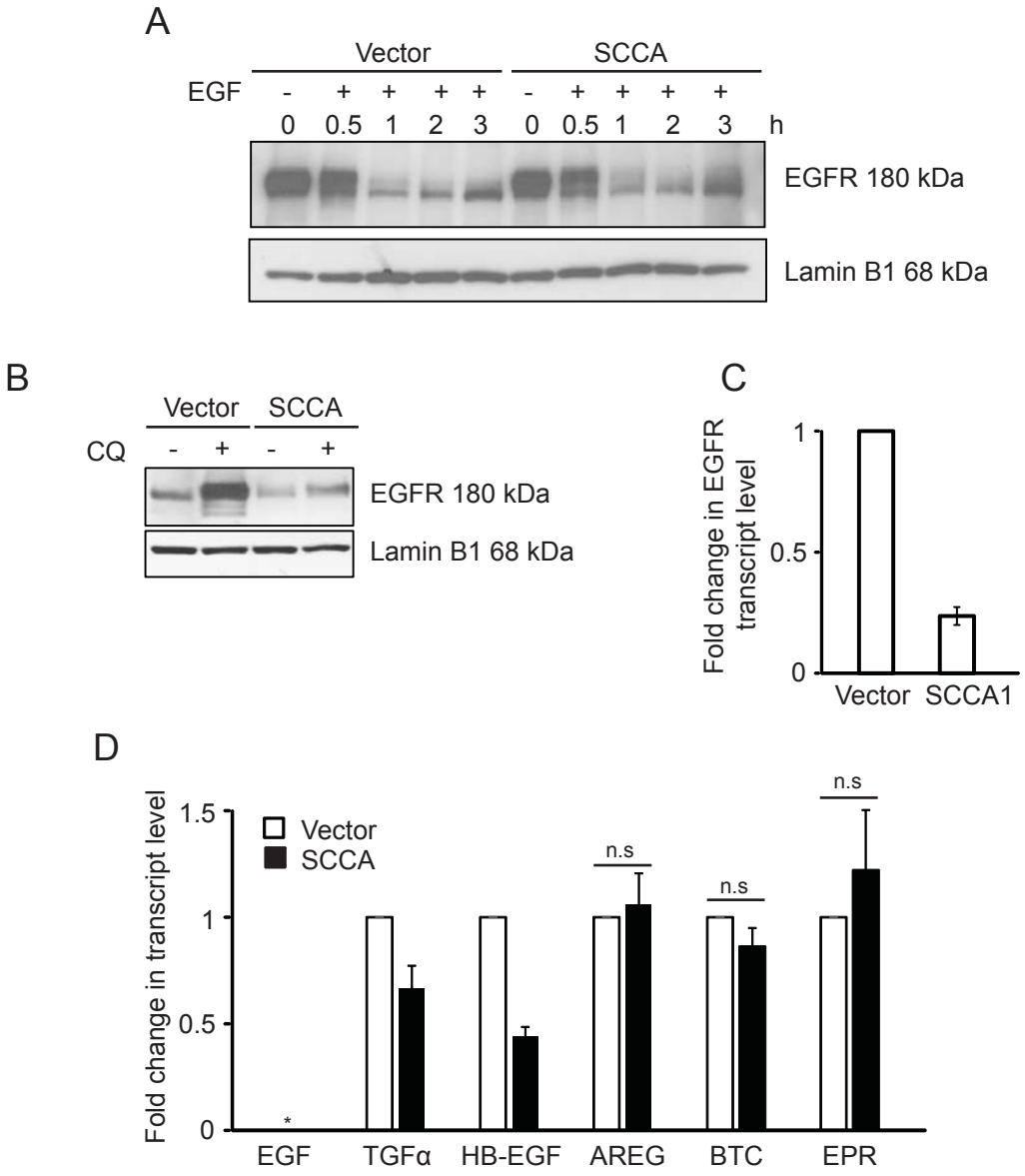

Figure 7. EGF independence of MCF10A-SCCA1 cells does not result in sustained activation EGFR pathway.

(A) Ligand induced EGFR degradation was examined in Vector control and SCCA1 cells by western blotting. The indicated cells were starved of EGF overnight and stimulated with 100ng/ml of EGF. Cells were lysed the indicated time points and and analysed for EGFR after immunoblotting. LaminB1 was used as loading control. (B) Cells were treated with 20μM of chloroquine for 24 hours to block lysosomal protein turnover and then harvested. Total EGFR as well as steady state protein level is reflected with or without treatment with chloroquine, which was measured by immunoblotting using the indicated antibodies. (C) q-RT PCR analysis of EGFR transcript level in vector control and SCCA1 transformed cells. Gene expression was normalized to the endogenous control GAPDH and fold change was plotted (D) q-RT PCR analysis of EGF-family ligands in vector and SCCA transformed cells. Gene expression was normalized to 1 in the vector control cells after using an endogenous baseline of GAPDH.* indicated undetected transcript levels.

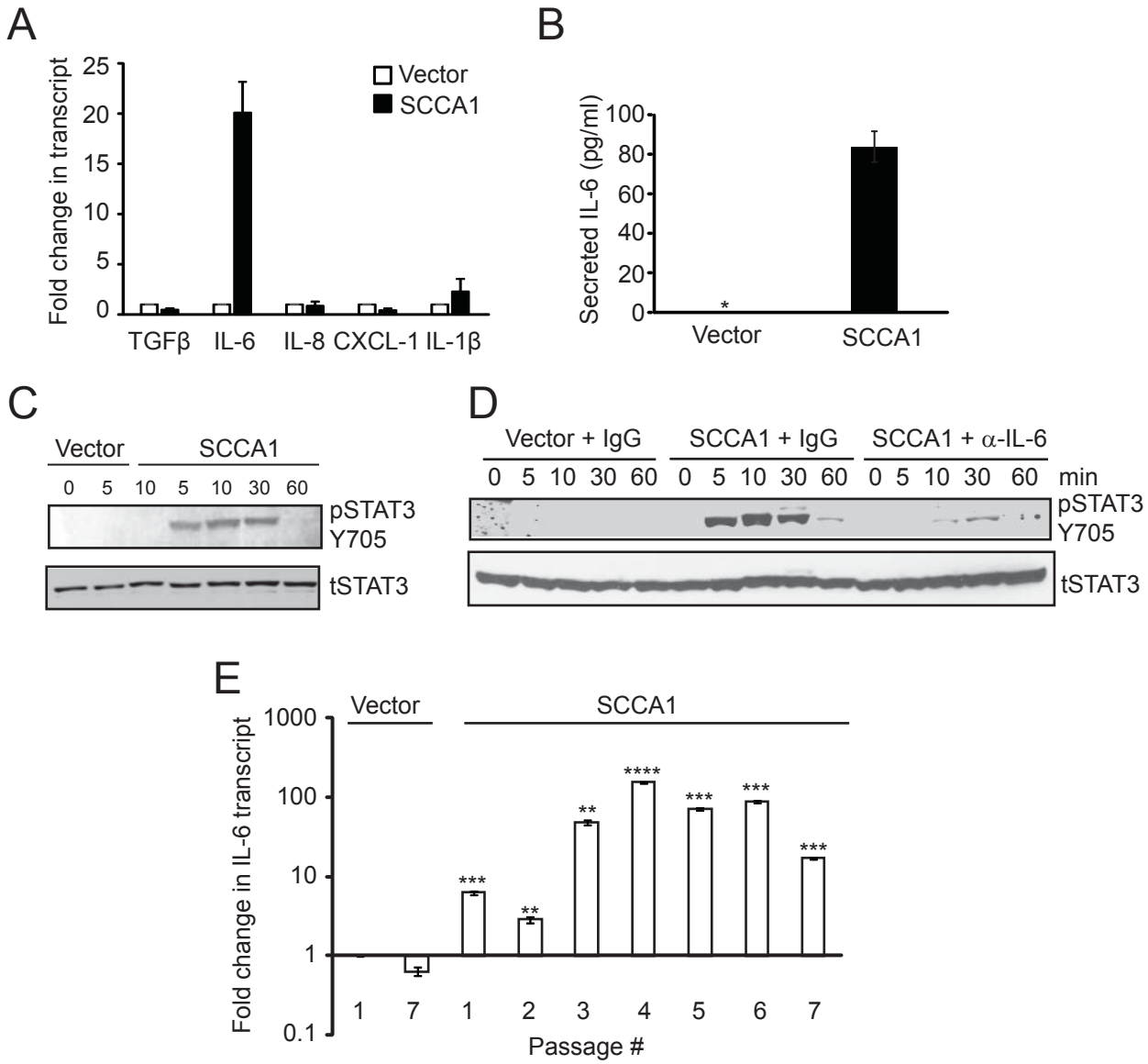

Figure 8. SCCA1 promotes transformation through the up-regulation of Interleukin-6. (A) Total RNA isolated from vector control or SCCA1-expressing MCF10A cells were analyzed for the expression of indicated cytokines by qRT-PCR. (B) Cell culture medium of vector control and SCCA1 expressing cells from overnight cultures was collected and measured for the level of secreted IL-6 by ELISA. *the absolute concentration of IL-6 in the control cells was below the sensitivity of the kit (3.25 pg/ml). Data shown are the mean + S.D. of two independent experiments performed in triplicates. (C, D) **V**ector control or SCCA1 expressing cells were cultured in EGF-free medium for 24 h. These media were added to EGF-starved MCF10A cells. (C) The medium from vector control cells was incubated with control IgG, and that from SCCA1 cells was incubated with IgG or an IL-6 neutralizing antibody **(**D). Whole cell lysates were collected at indicated times and probed for phospho-Stat3 (Y705) and total Stat3. (E) MCF10A cells were retrovirally infected with vector control or SCCA1, selected with antibiotics, and continuously passaged. Total RNA was isolated at indicated passage numbers. The IL-6 transcript level was measured by qRT-PCR, and normalized to that of the vector control cells at passage #1. One-sample t-test was used to judge significance. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

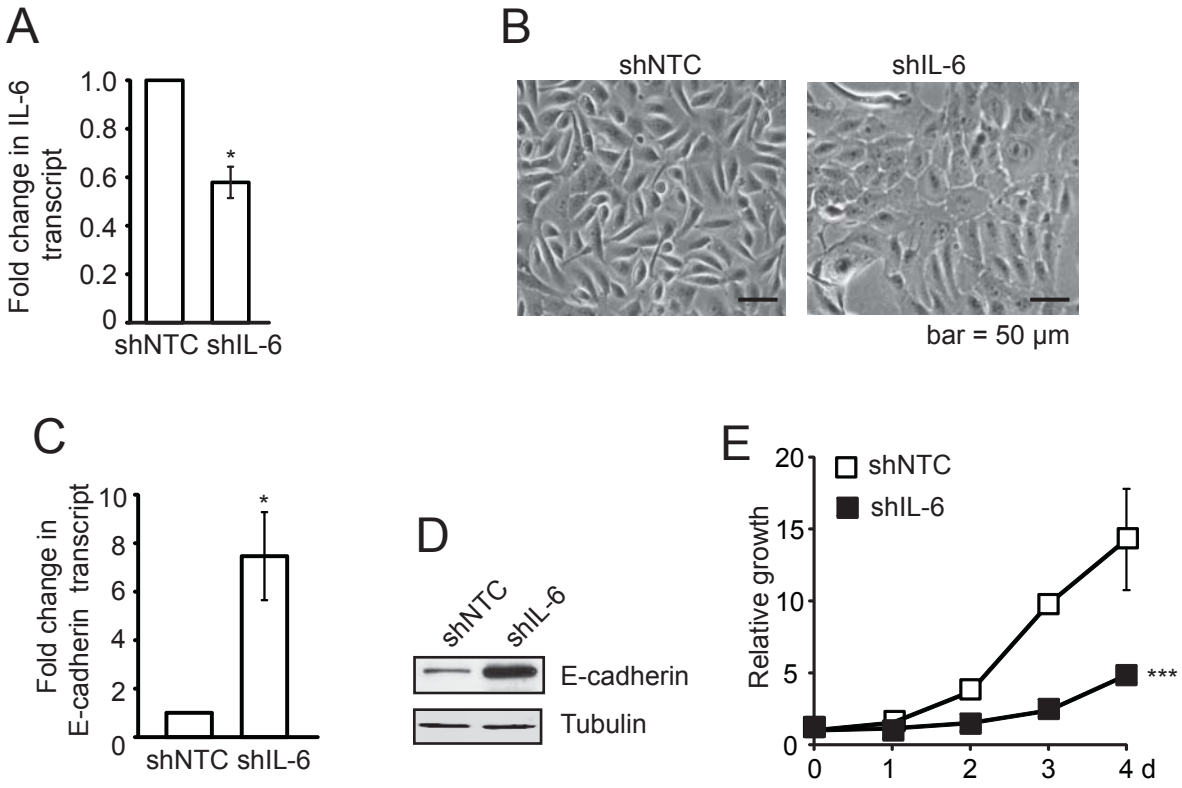

Figure 9. Silencing IL-6 in early passage SCCA1 cells restores epithelial phenotype. SCCA1-expressing MCF10A cells at early passage (passage #3) were infected with control (shNTC) and IL-6 (shIL-6) shRNA for 4 days. (A and C) Total RNA was analyzed by qRT-PCR for IL-6 (A) and E-cadherin (C) transcript levels. Data shown is the average +/- SEM of three independent experiments performed in triplicate. *$p<0.05$. (B) Phase contrast images of the cell culture was taken. (D) Whole cell lysates were analyzed by immunoblotting. (E) Relative cell proliferation was determined by crystal violet staining. Data shown is the mean +/- S.D. of duplicate measurements from three independent experiments. ***$p<0.001$.

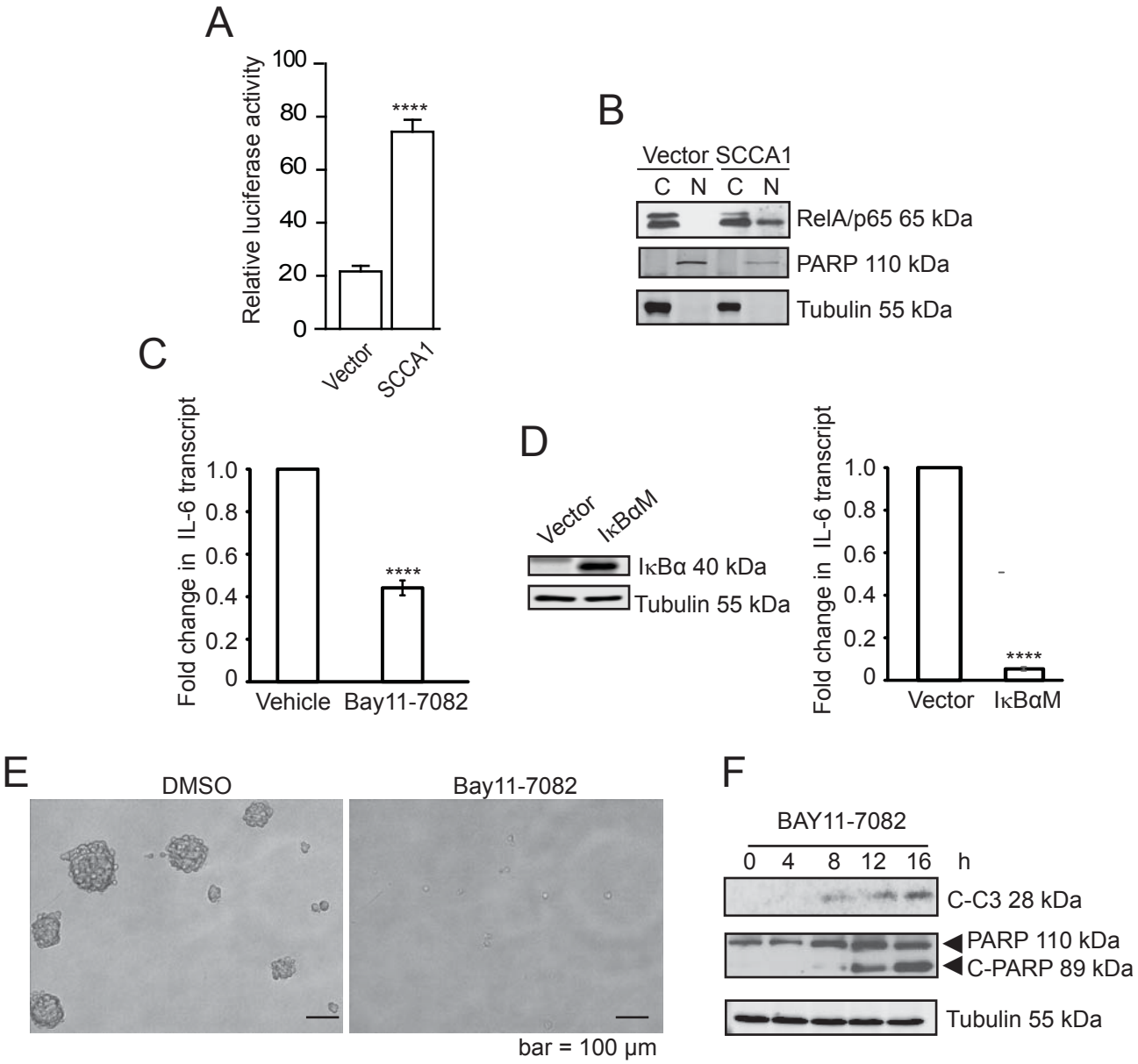

Figure 10. Canonical NF-κB drives the expression of IL-6 in MCF10A SCCA1 cells.
(A) Indicated cells were transfected with the NF-κB luciferase reporter and a renilla luciferase construct. 24 h post-transfection, NF-κB luciferase activity was calculated by normalizing against the renilla luciferase activity. Data shown is a representative graph of three independent experiments performed in triplicates, showing the mean + S.D. ****$p<0.0001$. (B) Indicated cells were subjected to subcellular fractionation. The cytosol (C) and nuclear (N) fractions were analyzed by immunoblotting. (C)Cells were treated with either DMSO or 5 μM BAY-117082 for 4 h . (D)SCCA1-expressing cells were retrovirally infected with vector control or IκBαM (S32A, S36A) for 4 d. Expression of IκBαM was verified by immunoblotting. (C,E) IL-6 transcript level was analyzed by qRT-PCR. Data shown are the mean +/- SEM of three independent experiments performed in triplicate. ****$p<0.0001$. (E,F) SCCA1-expressing MCF10A cells cultured in non-adherent culture dish, in the presence of DMSO or 5 μM BAY-117082, for 4 d. Phase contrast image of the cell culture was taken (E) and indicated proteins were probed by immunoblotting (F).

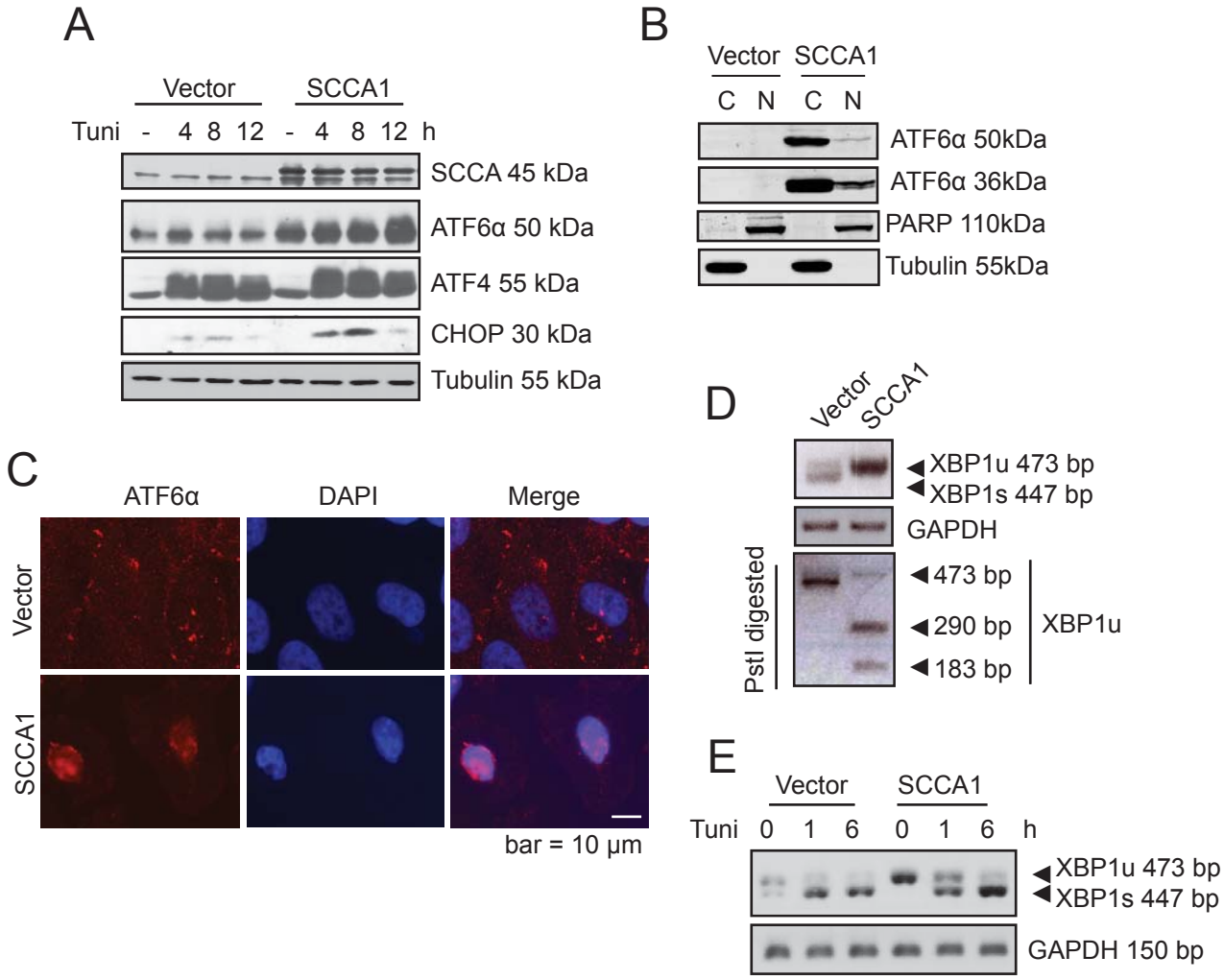

Figure 11. SCCA1 activates a low level chronic UPR
(A) Vector control or SCCA1-expressing MCF10A cells were treated with 5 μM tunicamycin and analyzed by immunoblotting. (B) Cells were subjected to subcellular fractionation. The cytosol (C) and nuclear (N) fractions were analyzed by immunoblotting. (C) Cells were probed for ATF6α by immunofluorescence, and counter stained with DAPI. Images were taken by a deconvolution fluorescence microscope. (D) Primers across the splice junction of XBP1 were used to amplify XBP1 by semi-Q PCR. The PCR product was divided into two halves. One was subjected to PstI digestion and the other was not, and then resolved on an agarose gel. PCR of GAPDH was used as a control for equal amplification. Note that SCCA1 cells had increased amount of XBP1u and decreased XBP1 splicing. (E) Vector and SCCA1-expressing cells were treated with 5 μg/ml tunicamycin for indicated times. XBP1 transcript was detected by semi-Q PCR using primers specific for unspliced and spliced form.

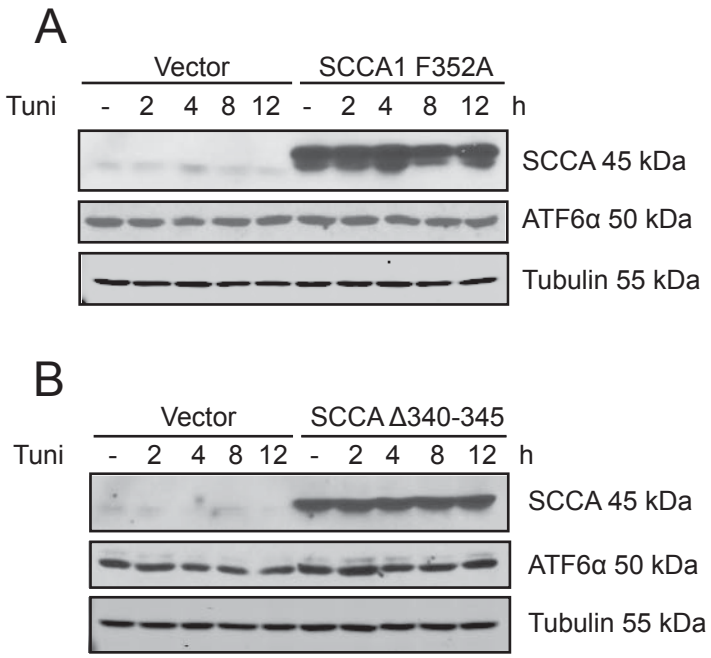

Figure 12. The induction of UPR by SCCA1 is dependent on its protease inhibition function. MCF10A cells stably expressing the vector, point mutant SCCA1-F352A (A) or the hinge deletion mutant SCCA1 Δ340-345 (B) were generated. Cells were treated with 5 μg/ml tunicamycin for indicated periods of time. Total cell lysates were probed for indicated proteins by immunoblotting.

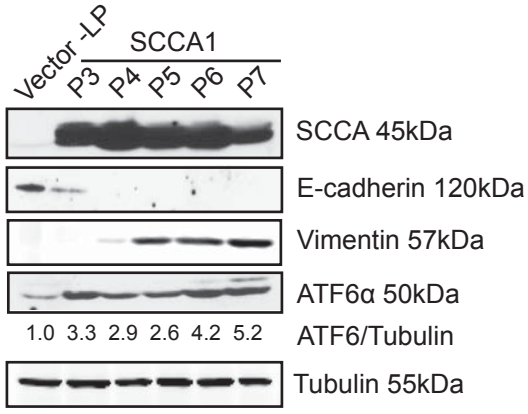

Figure 13. UPR induction is an early event in MCF10A transformation by SCCA1.
Whole cell lysates from sequential passages of SCCA1 expressing cells were used to probe for markers of EMT and UPR induction. Late passage (LP) vector control cells were used as a control. The levels of ATF6α-p50 was quantified using Image J and normalized to the intensity of tubulin.

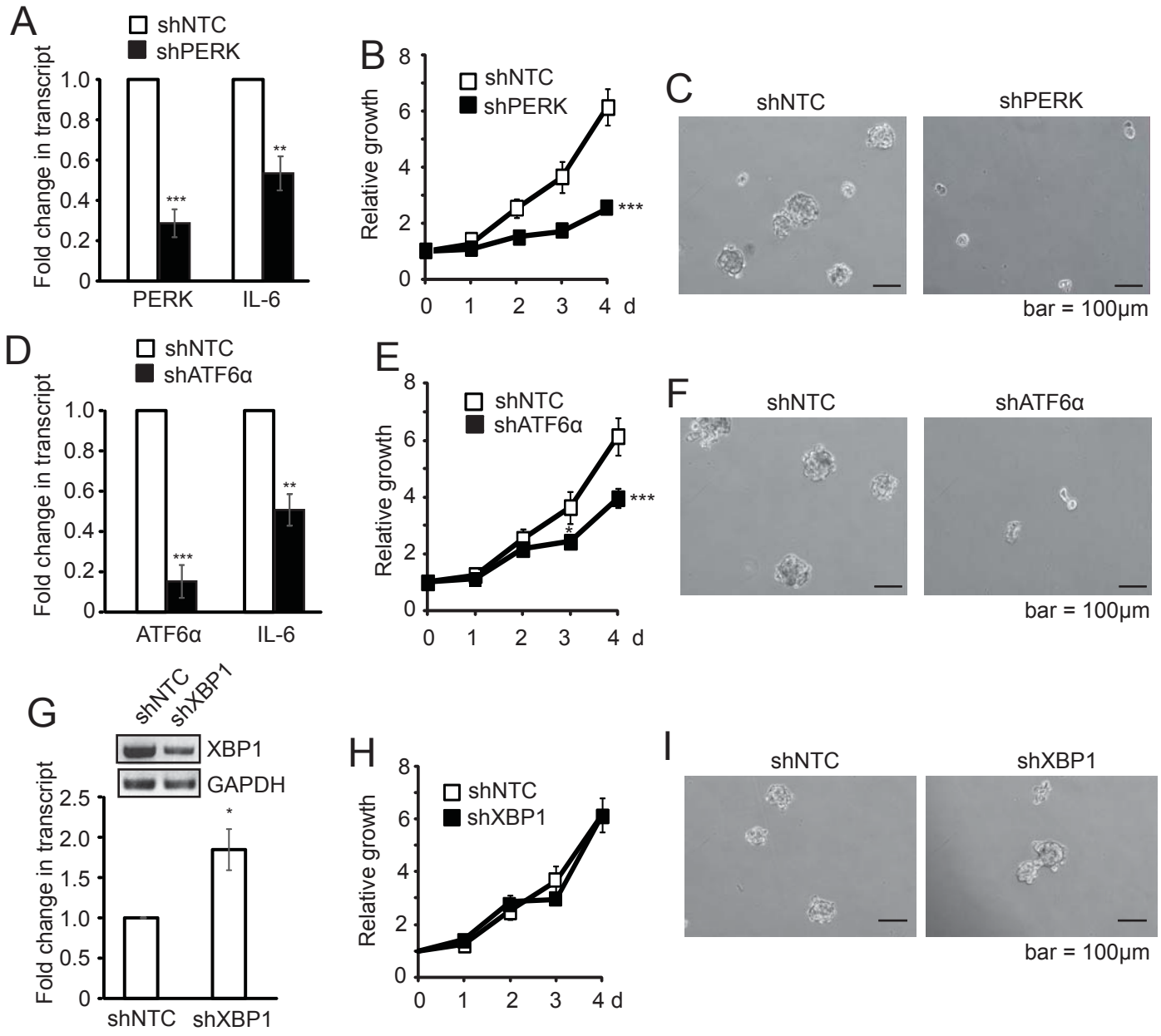

Figure 14. Silencing the activated stress sensors decreases SCCA1 induced IL-6 and transformation
(A-I) SCCA1-expressing MCF10A cells were lentivirally infected with short hairpins of control (shNTC) and PERK (A-C), ATF6α (D-F), or XBP1 (G-I). Cells were harvested 4 days later and qRT-PCR was performed for the transcript level PERK, ATF6α, IL-6, and immunoblotting for XBP1. Data shown are the mean +/- SEM of three independent experiments performed in triplicate. Relative cell growth was determined by crystal violet staining normalized to the reading of cells at Day 1 (B, E, and H). Significance judged by longitudinal data analysis was ***$p<0.001$ for C and F, and ns for I. Cells were cultured in suspension for 10 days. Phase-contrast images of cell spheres were taken (C, F, and I).

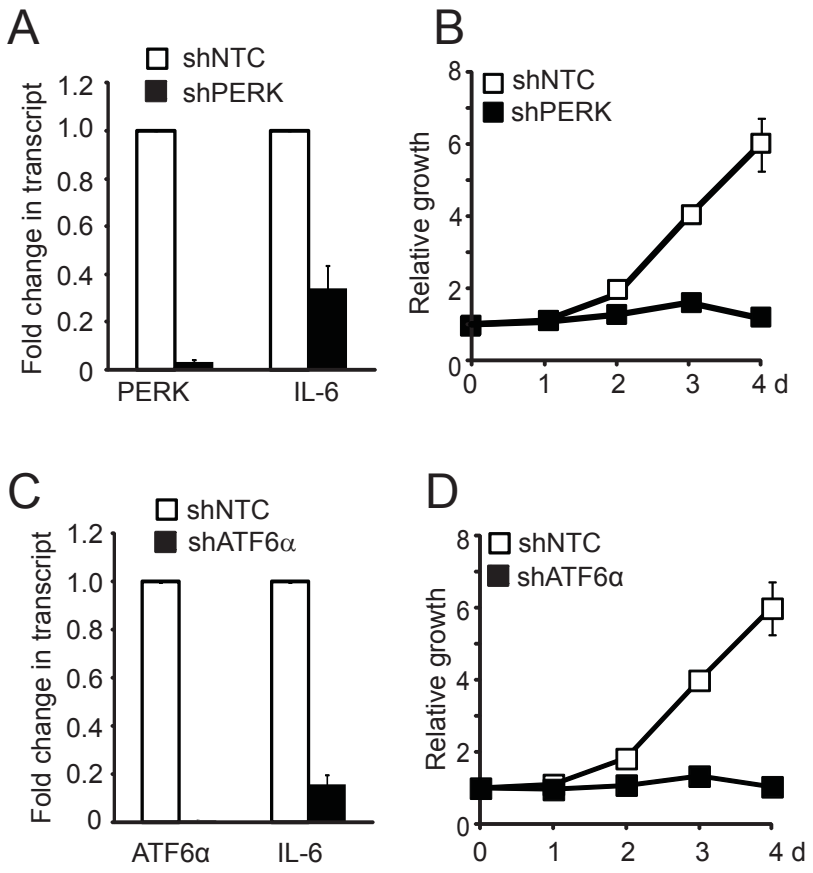

Figure 15. Silencing of ATF6a and PERK leads to decreased IL-6 production and cell proliferation in MDA-MB-231 cells. (A and B) MDA-MB 231 cells were lentivirally infected with shNTC and shPERK, and selected with puromycin for 48 h. (A) Cells were harvested 6 days post infection. Total RNA was analyzed by qRT-PCR for the expression levels of PERK and IL-6. The expression level was normalized to that of shNTC cells. Data shown are the mean + S.D. of triplicate experiments. (B) Relative cell growth was measured by crystal violet staining. Data shown are the mean +/- S.D. of a triplicate experiment. (C and D) MDA-MB 231 cells were lentivirally infected with shNTC and shATF6α, and selected with puromycin for 48 h. The expression levels of ATF6α and IL-6, as well as relative cell growth were measured by crystal violet staining.

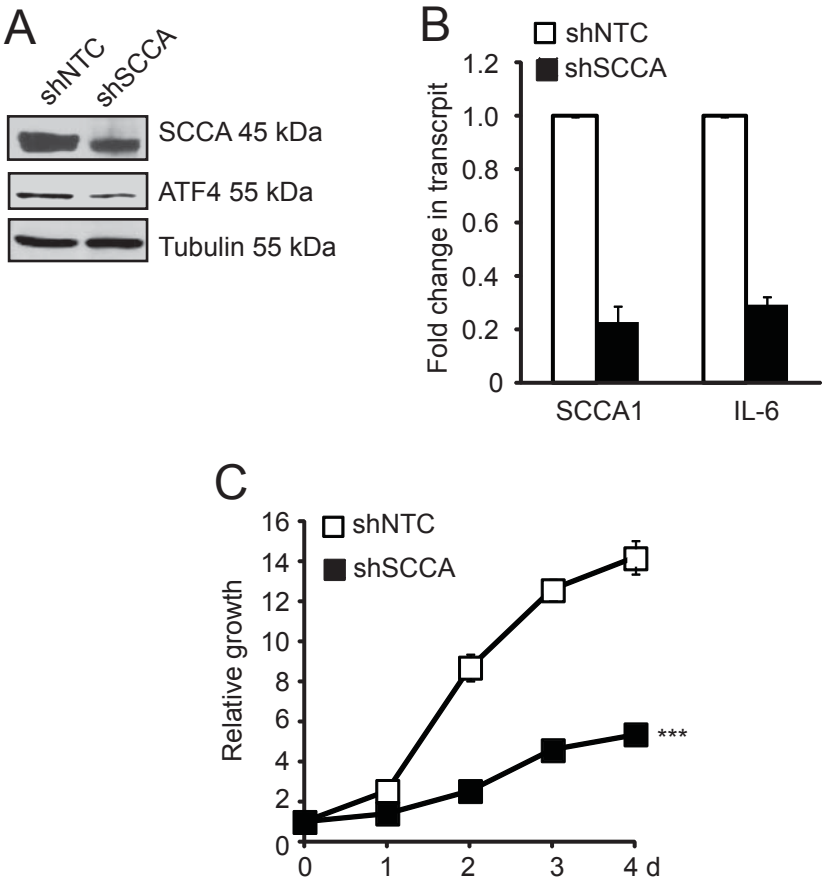

Figure 16. Silencing of SCCA results in decreased UPR, IL-6 production, and cell growth. (A-C) SCCA1-expressing MCF10A cells were infected with control (shNTC) or SCCA (shSCCA) short hairpin RNA. (A) Cells were harvested 4 days post infection and analyzed by immunoblotting. (B) Total RNA was analyzed by qRT-PCR for the transcript level of indicated genes, and was normalized to that of the shNTC cells. Data shown are the mean + S.D. of triplicate experiments. (C) Relative cell growth was measured by crystal violet staining. Data shown are the mean +/- S.D. of triplicate experiments. Some error bars are too small to be visible on the plots. ***$p<0.001$

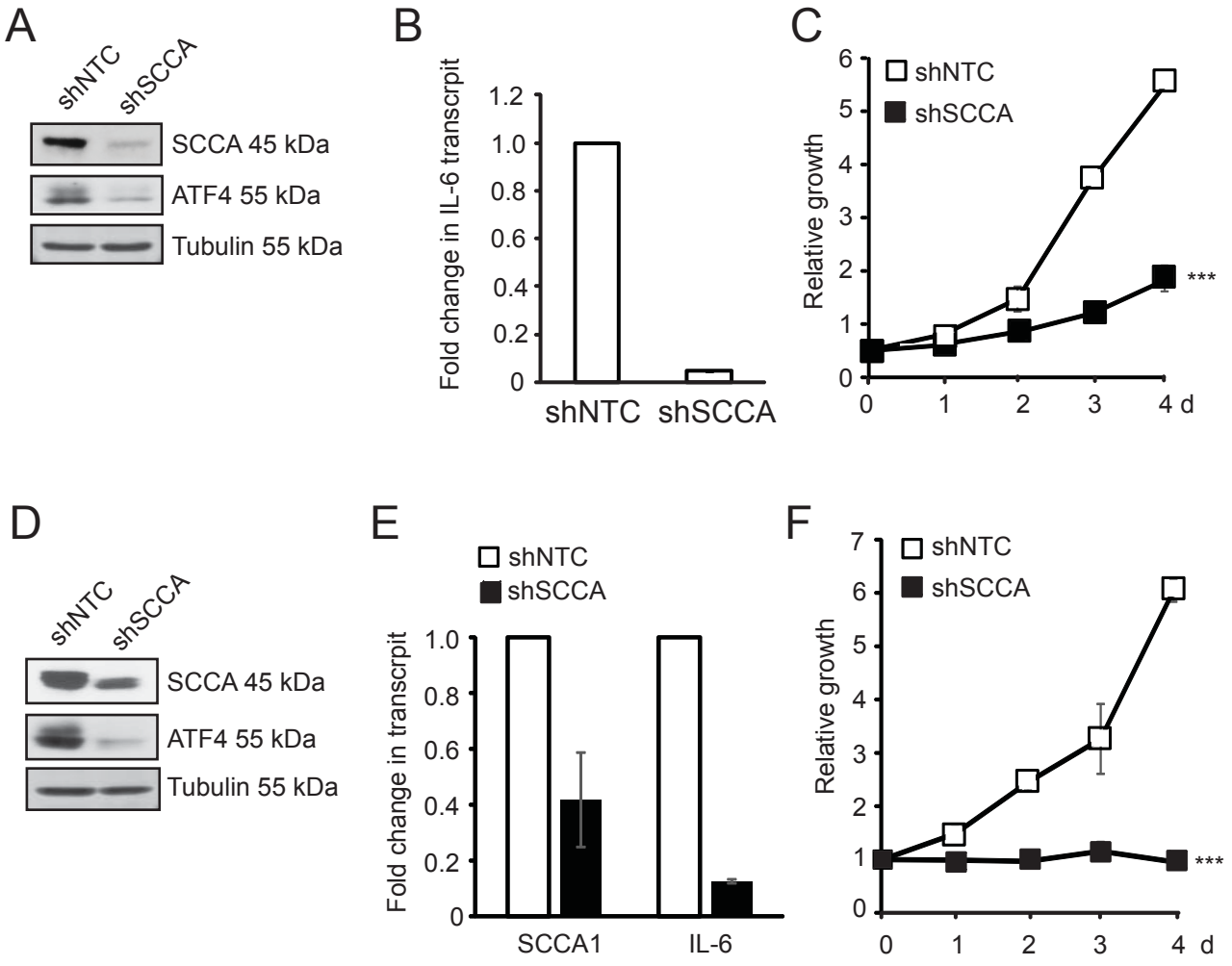

Figure 17. Silencing of endogenous SCCA in breast cancer cell lines MDA-MB 231 and MDA-MB 468 also results in decreased UPR, IL-6 production, and cell growth.
(A-F) MDA-MB 231(A-C) or MDA-MB 468(D-F) cells were infected with control (shNTC) or SCCA (shSCCA) short hairpin RNA. (A,D) Cells were harvested 4 days post infection and analyzed by immunoblotting. (B,E) Total RNA was analyzed by qRT-PCR for the transcript level of indicated genes, and was normalized to that of the shNTC cells. Data shown are the mean + S.D. of triplicate experiments. (C,F) Relative cell growth was measured by crystal violet staining. Data shown are the mean +/- S.D. of triplicate experiments. Some error bars are too small to be visible on the plots. ***$p<0.001$

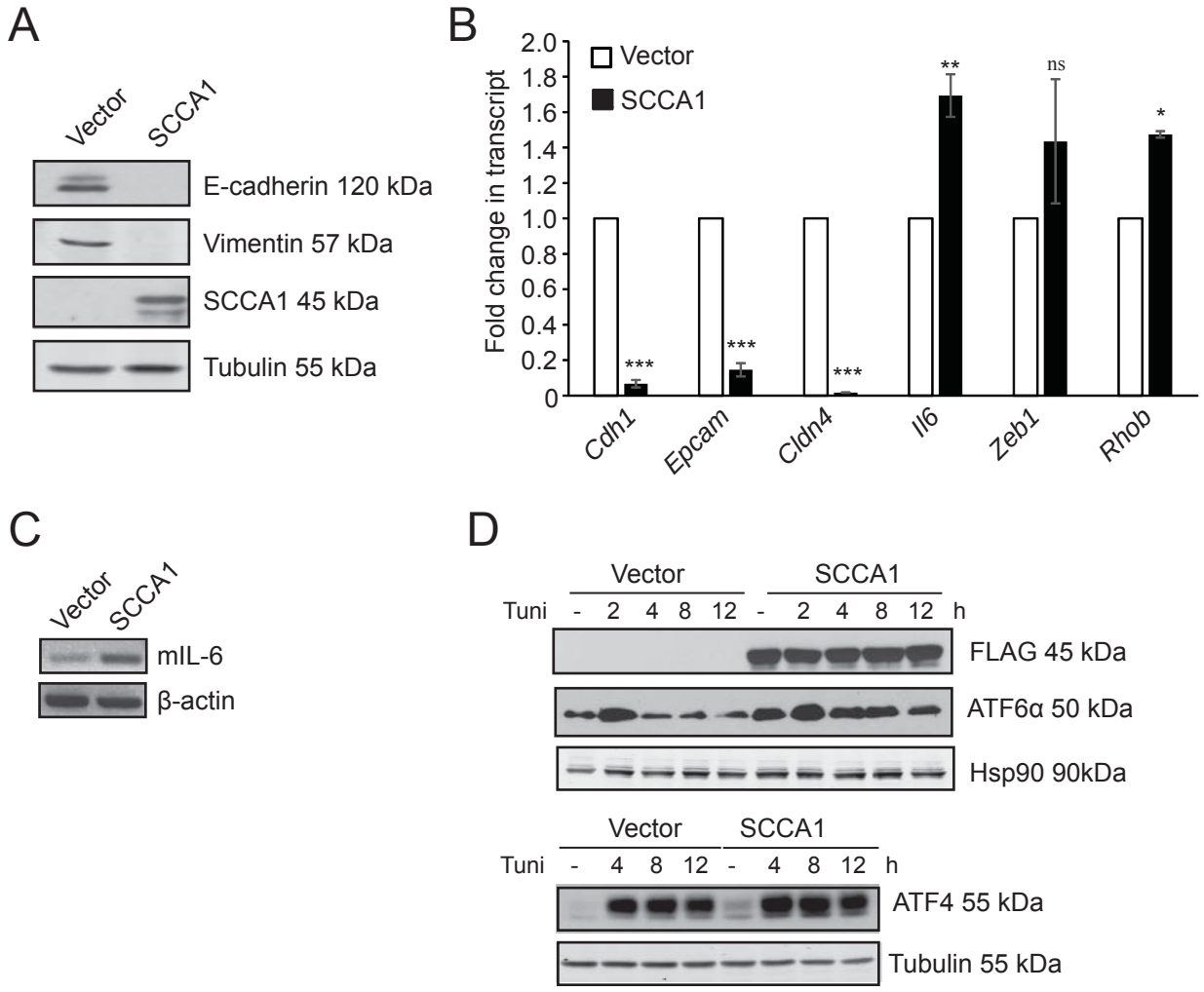

Figure 18. Elevated SCCA1 expression leads to partial EMT, UPR and IL-6 production in baby mouse kidney epithelial cells.

(A-D) SCCA1 was expressed in baby mouse kidney (BMK) cells as in Fig. 1. (A) Whole cell lysates were immunoblotted using the indicated antibodies. (B) Total RNA was isolated from the indicated cells and qRT-PCR was performed. The data shown are the mean +/- SEM of three independent experiments performed in triplicates after normalization to the vector control. ns not significant *p<0.05, **p<0.01, ***p<0.001. (C) Total RNA was used to perform semi-quantitative RT-PCR for mIL-6 (D) Vector control or SCCA1 cells were treated with DMSO or tunicamycin (5μg/ml) for the indicated time course. Whole cell lysates were immunoblotted for the indicated proteins.

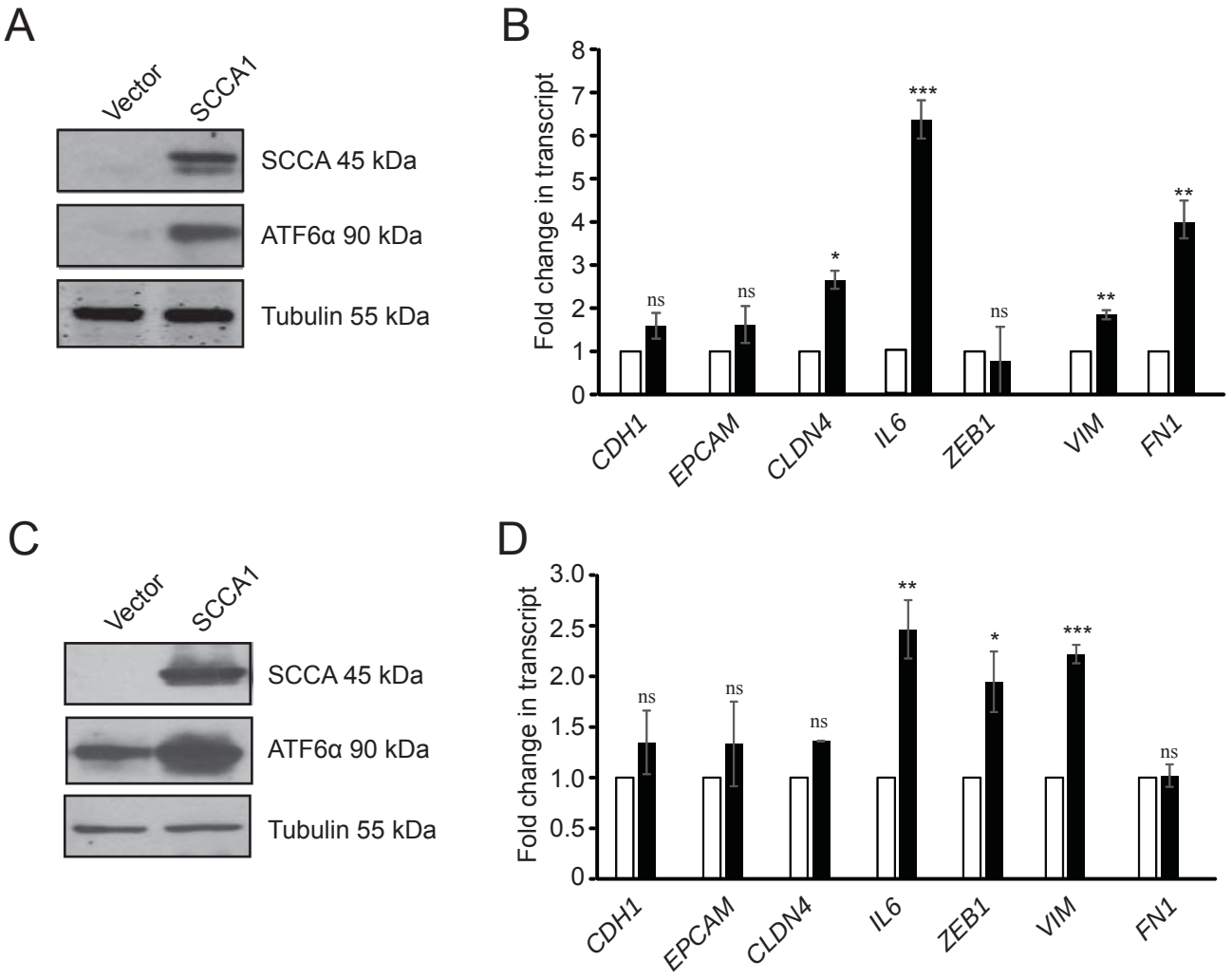

Figure 19. SCCA1 expression induces EMT-like changes, IL-6 production and UPR in mammary cell lines HMLE and SKBR3 cells.

(A-D) HMLE cells (A and B) or SKBR3 cells (C and D) were stably infected with vector control or SCCA1-expressing retrovirus and selected using 1μg/ml puromycin. Whole cell lysates were probed for the indicated proteins (A and C). Total RNA was isolated and qRT-PCR was carried out for the indicated genes (B and D). Data shown are the mean +/- SEM of three independent experiments performed in triplicates after normalization to the vector control sample. ns not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

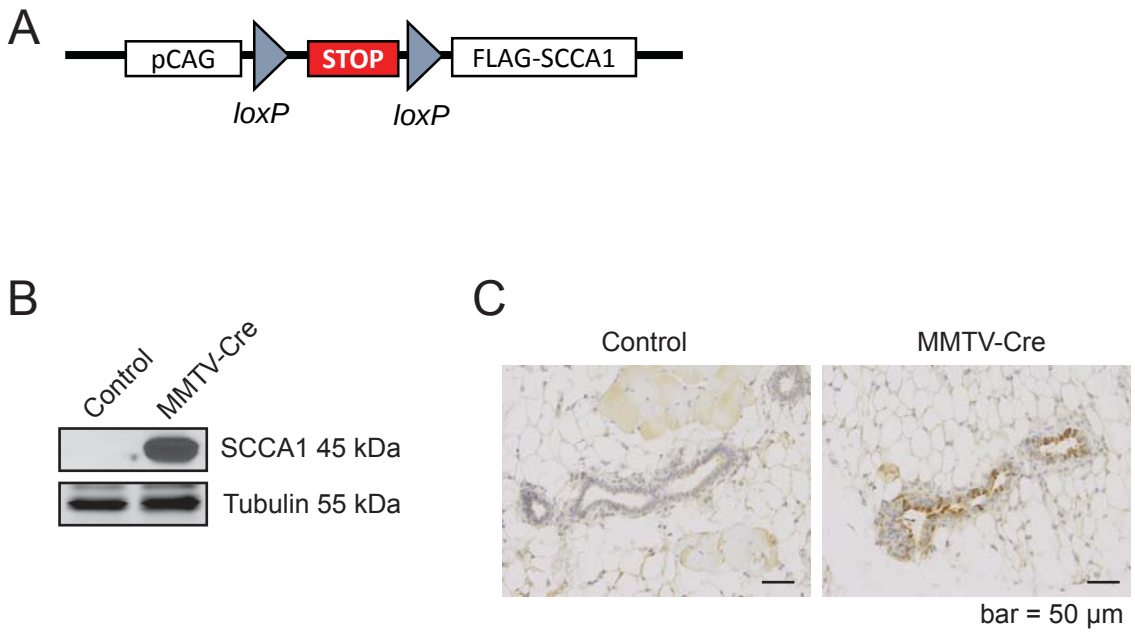

Figure 20. Generation of mammary specific SCCA1 transgenic mouse.
(A) Schematic representation of the LoxP-Stop-LoxP-Flag-SCCA1 (LSL-SCCA1) conditional knock-in strategy. (B and C) LSL-SCCA1 mice were bred to MMTV-Cre mice to achieve mammary specific expression of SCCA1. SCCA1 expression in mammary gland epithelium of control (LSL-SCCA1; Cre-) or LSL-SCCA1; Cre+ mice was detected by western blot (B) and IHC (C).

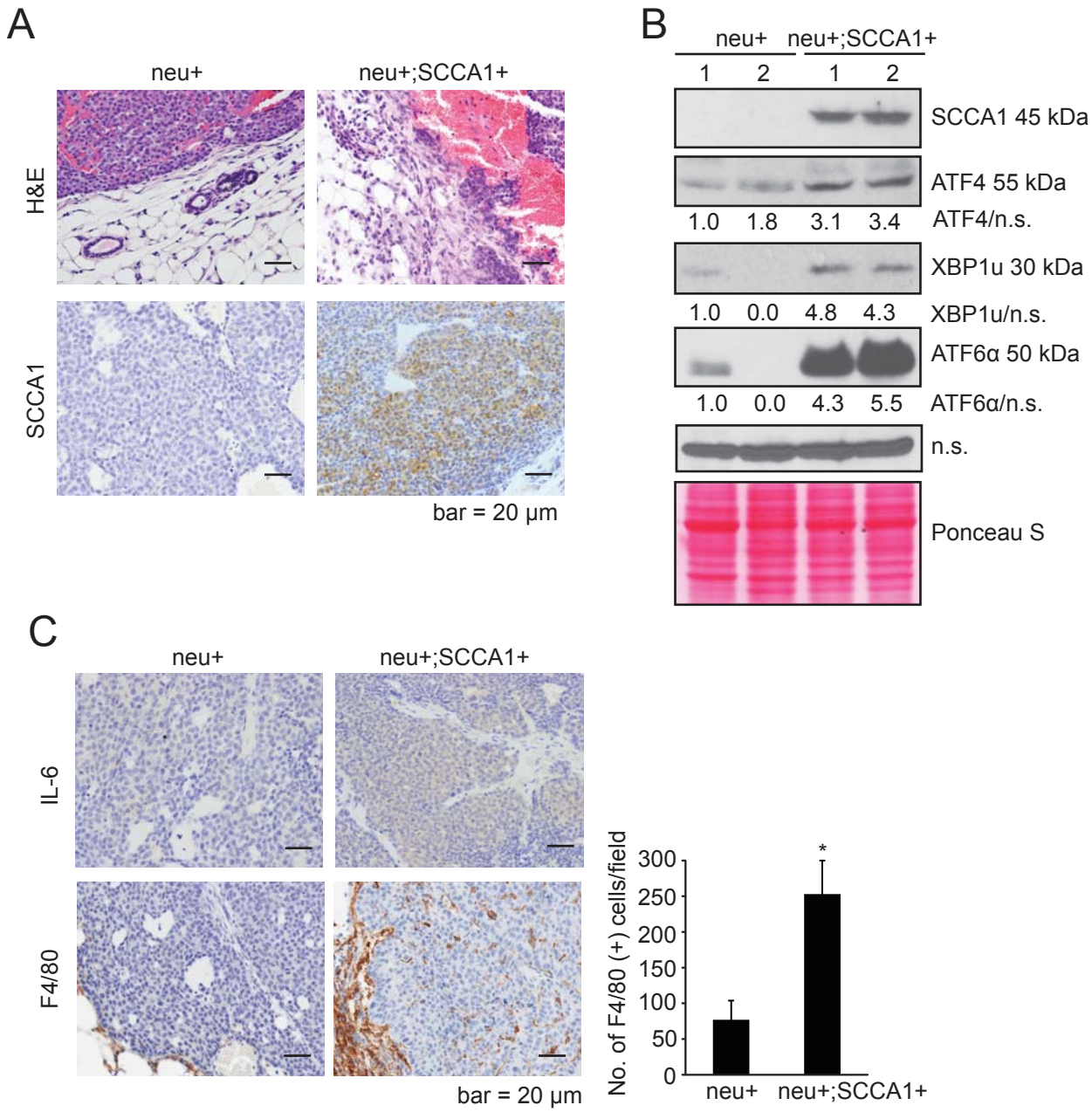

Figure 21. SCCA1 induces UPR and inflammatory response in MMTV-neu mice.
(A-C) LSL-SCCA1 mice were bred to MMTV-Cre, then to MMTV-neu mice. Tumors were isolated from neu+ only (n = 4) or neu+; SCCA1+ (n = 4) littermates at end points were compared. (A) Representative images of H&E and SCCA1 IHC are shown. H&E staining shows the junction areas of tumor and normal tissues. Note that in neu+;SCCA1+ tumors, tumor cells had invaded the basement membrane into surrounding stroma. (B) Total tumor lysates were made and indicated protein levels were probed by western blots. A non-specific (n.s.) band and Ponseau S staining were used as loading controls. Densitometric ratio of respective proteins to loading control is normalized to that of neu+ mouse #1. (C) IHC staining for IL-6 and F4/80 in neu+ only (n =4) and neu+; SCCA1+ (n =4) tumors. For the quantification of F4/80 staining, 3 or 4 most intensive areas (x200 magnification) from each tumor of all tumors were photographed. The number of positive cells from each picture was analyzed using Image J software. Shown are representative images. The graph shows the average number of positive cells from each tumor section + SEM. *p<0.05.

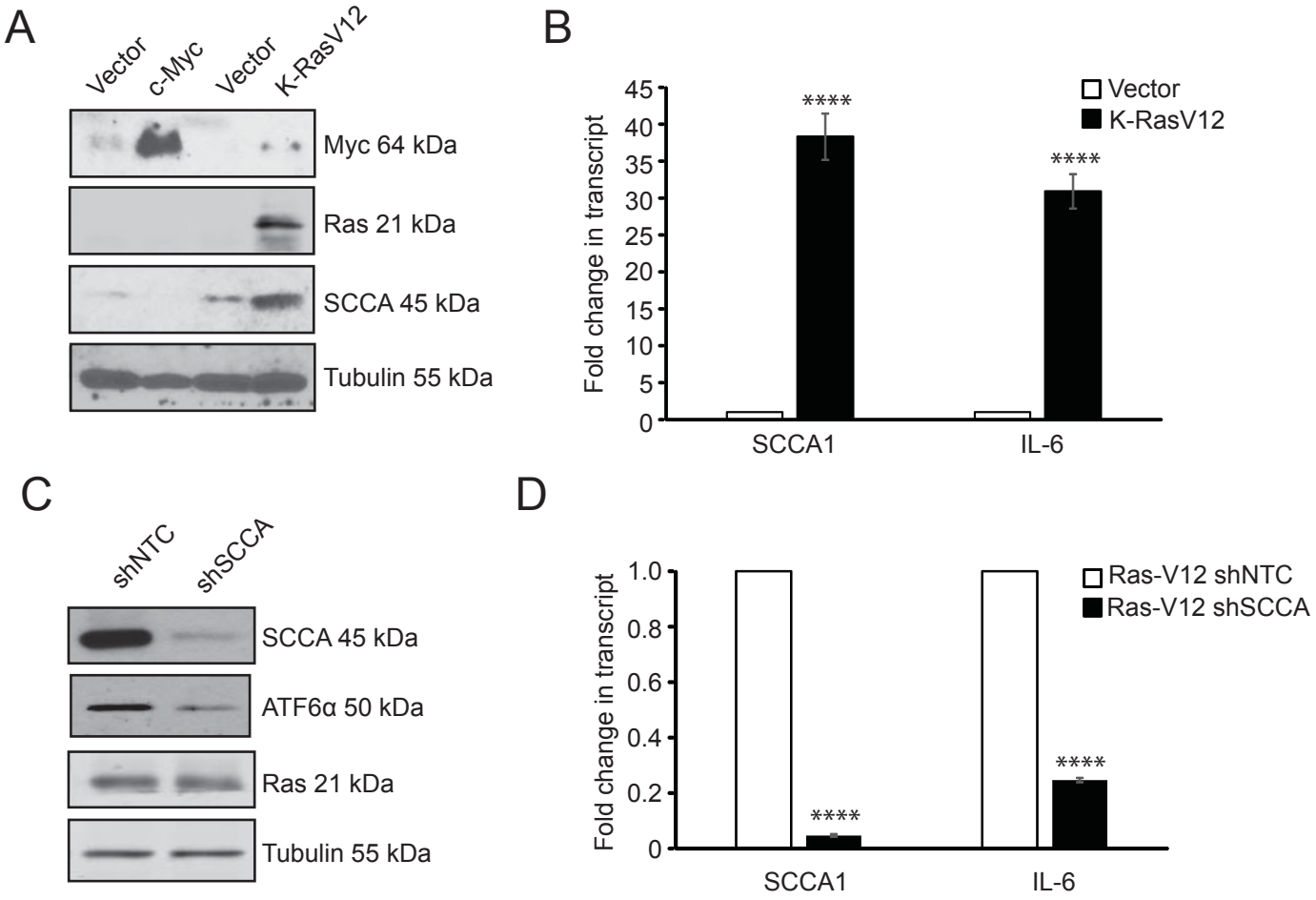

Figure 22. Oncogenic Ras induced SCCA expression regulates IL-6 production and UPR in MCF10A cells.

(A) MCF10A cells were retrovirally infected with c-Myc or oncogenic KRasV12. Stable cells were generated after antibiotic selection. Whole cell lysates were probed for indicated proteins by immunoblotting. (B) Total RNA was used to perform qRT-PCR for the indicated genes. The data shown is +/- SEM of three independent experiments executed in triplicates after normalization to the vector control. ****$p<0.0001$ (C-D) MCF10A KRasV12 transformed cells were lentivirally infected with shNTC or shSCCA and cells were harvested 4 days post infection. (C) Whole cell lysates from these cells were immunoblotted using the mentioned antibodies. (D) Total RNA was used to perform qRT-PCR for the indicated genes. The data shown is +/- SEM of three independent experiments executed in triplicates after normalization to the vector control. ****$p<0.0001$

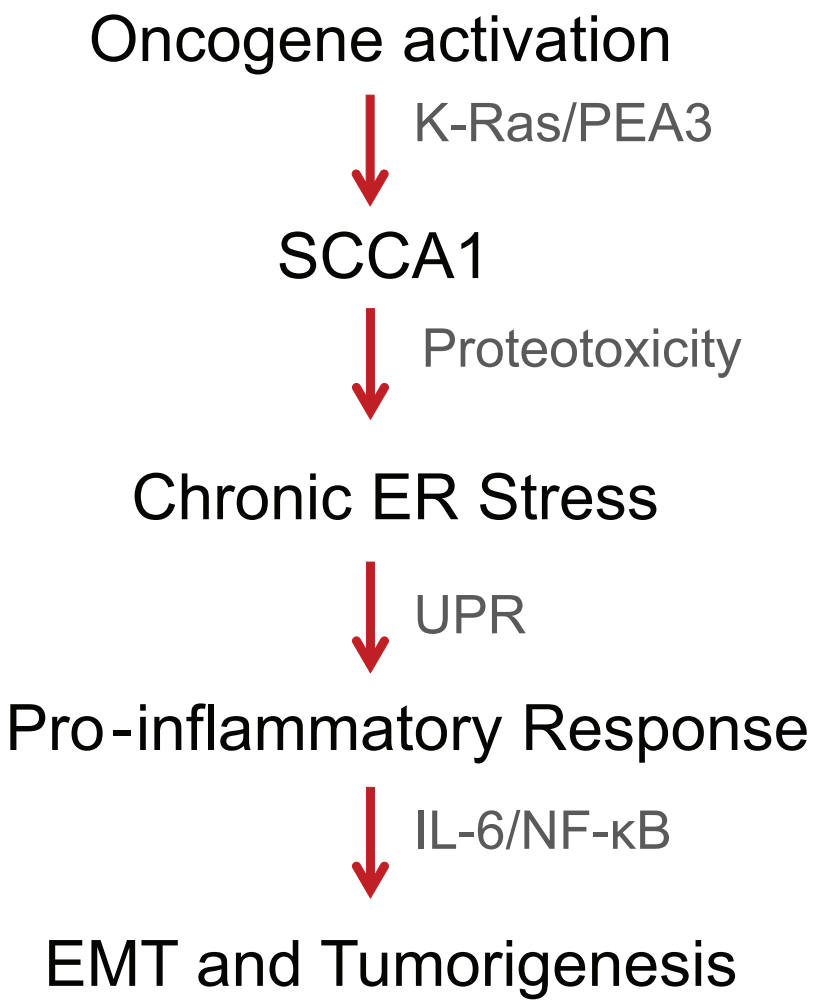

Figure 23. Proposed model for SCCA1 induced EMT and Tumorigenesis.
The activation of oncogenes, for instance K-RasV12, results in the transcriptional activation of SCCA1 in a variety of cell systems. Elevated levels of SCCA induces a proteotoxic stress owing to its ability to impair protein turnover via the lysosomal as well as proteasomal pathway. The accumulation of proteins that are meant for degradation results in a build-up of protein aggregates and misfolded proteins which disrupts Endoplasmic Reticulum (ER) protein homeostasis. The ER then activates a homeostatic response known as the Unfolded Protein Reponse (UPR) to help alleviate the stress. This includes the activation of the pro-survival NF-κB pathway as an adaptive response. The activation of the NF-κB pathway leads to the transcription of inflammatory cytokines, such as IL-6, which plays a critical role in an autocrine as well as paracrine fashion to promote tumorigenesis and aid tumor progression via induction of EMT.

IV. Discussion

1. SCCA1 has a novel tumor promoting function

Since the identification of SCCA in patients with cervical cancer in the late 1970s, it has been widely recognized as a biomarker for the diagnosis and prognosis of squamous cell carcinomas (1,2). Over the last decade, SCCA has been well-appreciated as an anti-apoptotic molecule with the ability to promote the survival of cells that have accumulated mutations upon exposure to UV, DNA damaging agents and therefore potentially contribute to tumorigenesis (10,20).

Accumulating clinical data provide a clear correlation of SCCA expression with poorly differentiated, aggressive forms of breast and pancreatic adenocarcinoma, in addition to hepatoblastoma, an embryonal liver malignancy (13,153,154) suggesting a more direct role for SCCA in tumor progression. Here we report that ectopic expression of SCCA1 in the non-tumorigenic mammary epithelial cell line MCF10A leads to Epithelial-Mesenchymal transition and cellular transformation (Fig. 1 and Fig. 4). Further, in an animal model of mammary tumors, MMTV-neu, it promoted invasion of the tumor cells into the surrounding stromal tissue (Fig. 21). This is the first report implicating a tumor promoting function to SCCA in breast cancer. The EMT promoting function of SCCA has been independently confirmed in the hepatocellular carcinoma cell line, HepG2 (155).

Silencing endogenously expressed SCCA resulted in attenuated growth in multiple breast cancer cell lines including the aggressive triple negative cell lines, MDA-MB 231and MDA-MB 468 (Fig. 17). This data provided experimental evidence for the clinical association of SCCA with poorly differentiated breast cancers.

Further, we note that SCCA1 expression is regulated by oncogenic Ras in MCF10A cells (Fig 22). We confirmed that mutant Ras was capable of up-regulating SCCA expression in a MAPK-ERK-PEA3 dependent manner in multiple cell types. Apart from this we have provided consolidated evidence that SCCA is a critical mediator of Ras induced tumorigenic features in pancreatic and colon cancer cell lines as well as xenograft tumor models (153). This establishes its co-operative role with powerful oncogenes such as Ras.

2. SCCA induces a delayed EMT which displays dynamic properties

It is important to note that SCCA1 expression in MCF10A did not result in mesenchymal transformation instantly but over seven passages in our case, suggesting that it is a weak inducer of EMT unlike Ras or Twist (151,156). There was a gradual loss of E-cadherin and a subsequent gain of vimentin (Fig. 1E), with a prominent intermediate metastable phase. This is consistent with previous reports on the transient metastable cell type reported in development as well as cancer (157,158), suggesting the dynamic inter-conversion between the epithelial and mesenchymal states (60,159). Evidence for the causative factor, IL-6 as early as the first passage which steadily rises to a hundred fold up regulation at the time of transformation indicates that the delay may be explained by the effective dose of the EMT inducing factor required for the cells to overcome the threshold for the transition (Fig. 8E).

Moreover, silencing IL-6 in the early passages after SCCA1 expression resulted in a dramatic re-expression of E-cadherin suggesting reversal to epithelial phenotype (Fig. 9). This is supported by the theory that the transition from the metastable state back to epithelial state has been proposed to be energetically more favorable (149).

However, knocking down SCCA after transformation resulted in decrease in IL-6, UPR and cell growth (Fig. 16) but did not reverse the EMT phenotype, indication the acquisition of a stable mesenchymal state. This can possibly be explained the previous observations of an "epigenetically fixed" stage of mesenchymal differentiation indicating an irreversibility of this state (157). When cells progress into the mesenchymal state, there have been reports indicating a dramatic re-programming of the epigenetic landscape of cells (149). However, there have been some reports suggesting that long term treatment of "fixed mesenchymal" cells with epigenetic modifiers, DNA de-methylating agents and HDAC inhibitors can revert it to an epithelial phenotype (160).

The expression of SCCA1 in BMK cells resulted in an obvious loss of epithelial phenotype, whereas its expression in HMLE and SKBR-3 breast cell-lines led to the acquisition of mesenchymal markers along with the retention of epithelial characteristics. This observation supports the view of the dynamic nature of EMT which was recently confirmed clinically, when isolated circulating tumors cells were found to simultaneously express epithelial and mesenchymal markers (59).

3. SCCA1 regulates pro-tumorigenic cytokine production

A q-RT PCR based screen for EMT inducing factors revealed that SCCA1 expression results in a marked increase in the expression of IL-6. Silencing the expression of IL-6 prior to transformation rescued the epithelial phenotype of MCF10A-SCCA1 cells suggesting a causative role for IL-6 in the process of cellular transformation. The increased transcription of IL-6 upon SCCA1 expression was also observed in other epithelial cell lines, BMK, HMLE and SKBR-3.

While we report that IL-6 is a key player in the tumorigenic process downstream of SCCA, several other cell intrinsic changes might be involved in channelizing IL-6 receptor activation towards a malignant phenotype. It has been appreciated that IL-6 has growth inhibitory properties in pre-malignant cells, but in malignant cells, it can act as a switch to aid metastatic progression (161).

In the context of Ras-induced tumors, SCCA mediated the expression of several cytokines apart from IL-6 including IL-8, GM-CSF, G-CSF and CXCL-1 further establishing its contribution to inflammation induced tumorigenesis. Our observation of the role of SCCA in inflammation is supported by previous observations which links SCCA expression to non-squamous epithelia undergoing squamous metaplastic changes in response to inflammation or infection in the lung, submandibular glands and the prostate (9,11). Clinical samples from patients with idiopathic pulmonary fibrosis (IPF) showed strong staining of SCCA in metaplastic alveolar cells (162), suggesting its involvement in inflammation and de-differentiation.

Additionally, SCCA expression is associated with several inflammatory disorders (11). Elevated levels of both serum and tissue expression of SCCA was noted in conditions of inflammatory cutaneous conditions such as psoriasis and atopic dermatitis (163,164). There are reports suggesting that SCCA is also associated with autoimmunity in systemic lupus erythematosus associated nephritic syndrome and systemic scleroderma (20).

Collectively, SCCA1 is known to activate an inflammatory response in several pathological conditions, including cancer.

4. SCCA induces of chronic misfolded protein stress to promote inflammation and EMT

In a previous study, we reported that SCCA1 expression impaired protein turnover via autophago-lysosomal pathway and proteasomal pathway indicating a possible increase in misfolded protein stress (37). The expression of SCCA resulted in elevated UPR in multiple cells lines including MCF10A, BMK, HMLE and SKBR-3. Importantly, the expression of reactive site mutant, SCCA1-F352A and hinge deletion mutant, SCCA1 Δ340-345 did not result in elevated UPR. Knocking down endogenous SCCA in breast cancer cell lines MDA-MB 231and MDA-MB 468 resulted in decreased UPR suggesting that SCCA regulates basal ER stress in multiple cell types.

Chronic ER stress has been implicated in the activation of the immune response as an adaptive strategy (93). Here we report that knocking down ER stress sensors in SCCA transformed cells resulted in decreased IL-6 production, cell growth and sphere formation in suspension culture. This provides a novel molecular connection between SCCA1-induced proteotoxic stress and tumorigenesis. Our work is strongly complemented by a parallel study which implicates a role for UPR mediators in the molecular signature of the aggressive triple negative subtype of breast cancer (125).

Although the precise mechanism underlying this SCCA1-mediated blockade of protein turnover remains to be determined, our finding is in line with a recent report that the cathepsin L-deficient cells display impaired lysosomal turnover, accumulation of ubiquitinated proteins and protein aggregates, and disrupted ER homeostasis (165).

Adaptive ER stress has previously been implicated in dynamic cellular re-programming and de-differentiation in pancreatic cells, thyroid cells, chondrocytes as well as alveolar epithelial cells (166-170). This supports our claim that chronic UPR can indeed promote EMT-like phenotype of MCF10A cells. More recent lines of evidence suggest that the EMT transition is associated with elevated ER stress (171,172). However, the precise sequence of events during tumor progression calls for further investigation.

The current study provides a molecular rationale to support the idea that elevated basal ER stress renders SCCA1 expressing cells sensitive to treatment with proteotoxic therapeutic

agents. This finding offers a novel therapeutic opportunity to treat cancers with elevated SCCA levels.

5. Modeling the tumorigenic function of SCCA1 in vivo

The human *SERPINB3* gene is represented by four orthologous genes in mice *SerpinB3a, SerpinB3b, SerpinB3c* and *SerpinB3d.* However, the mouse serpin which functions phenocopy the human counterpart is yet to be identified. In the meanwhile, we generated mice that express LSL-SCCA1 and bred them to mice with MMTV-Cre to ensure SCCA1 expression in the mammary epithelial cell compartment of the mouse. The SCCA1-expressing mice were fertile, sustained pregnancies to term, produced viable offspring, and displayed no apparent defects in ductal morphogenesis, indicating that elevated expression of SCCA1 is not sufficient to drive mammary tumorigenesis as a single hit. Since our earlier results showed that SCCA1 can promote orthotopic tumor development in MCF10A cell line, which harbors loss of p19Arf tumor suppressor gene, we are currently investigating the role of SCCA1 in tumorigenesis in spontaneous mouse tumor models with loss of tumor suppressor genes such as the p19Arf flox/flox model (173).

Nevertheless we continued to breed the LSL-SCCA1 mice to the MMTV-neu mice to study the potential role of SCCA in tumor development and/or progression. It involves the mammary specific expression of the unactivated neu oncogene under the MMTV promoter. This model allows focal tumor formation with a relatively long tumor latency of 7-14 months with 50% incidence (150). In this model, we did not observe a difference in tumor latency, burden or multiplicity. The average latency of tumor formation was 407 days and 551 days for neu^+; $SCCA^+$ and neu^+ only mice, respectively ($p = 0.24$). The prolonged tumor latency is reminiscent of that of the F1 virgin mice of the FVB and B6 crossing (174). Metastatic tumors were not observed in these mice at endpoint (tumor size 300-500 mm^3) which could possibly be a result of the mixed background. However, a marked increase UPR and inflammatory changes were consistent with the findings in the MCF10A cells. These results indicate that while SCCA1 is not able to accelerate tumor development in the MMTV-neu background, it can clearly alter the tumor microenvironment and increase the potential of tumor invasiveness. Further

characterization of the role of SCCA1 in tumorigenesis in tumor models with other genetic lesions is under way.

V. Current and future perspectives

The current study illustrates a molecular connection linking SCCA to protein homeostasis, inflammatory response and cancer progression. While this is an important sequence of events also relevant in the context of previously described oncogenes such as Ras, the precise biochemical mechanism of SCCA1 mediated proteotoxicity remains to be dissected. The proteomic analysis of interacting partners of SCCA1 will be used as to strategy to address this proposition. The transient interaction of SCCA with its target proteins makes this a challenging approach to study.

In several states of pathogenesis associated with inflammation and cancer, SCCA has demonstrated a strong nuclear localization (36). However, the role of SCCA in nucleus is yet to be determined. One can speculate its possible role in regulation of gene expression or turnover of transcription factors via inhibition of the nuclear ubiquitin-proteasome.

With clinical data supporting the co-relation of elevated SCCA levels with advanced cancers, SCCA could be potentially used as a drug target. The reactive site loop could be used to develop small molecule inhibitors. These could prove useful in making cancers with high levels of SCCA reverse their observed resistance to apoptosis in response to chemotherapeutic agents.

Thus far, UPR has largely been studied in the laboratory using pharmacological agents such the use of the N-glycosylation inhibitor, Tunicamycin or by disruption of ER calcium homeostasis using Thapsigargin. Genetic ablation of key molecules of the ER stress pathway have indicated their crucial roles in tumor development. However, physiological triggers of the UPR and studying its role in pathophysiological context would be necessary for completely appreciating the contribution of UPR to tumorigenesis.

While chronic ER stress is known to activate the inflammatory response, the precise mechanistic association remains to be identified. This research would facilitate the targeting of UPR induced inflammation in multiple disease pathologies including diabetes, neurodegenerative disease, obesity and cancer.

VI. Materials and methods

1. Cell lines and culture conditions

MCF10A cells were obtained from ATCC and cultured in DMEM/F12 Ham's Mixture supplemented with 5% Equine Serum (Gemini Bio), EGF 20 ng/ml(Sigma), insulin 10μg/ml (Sigma), hydrocortisone 0.5 mg/ml (Sigma), cholera toxin 100 ng/ml (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were culture as per protocols from Brugge Lab.

HMLE cells were obtained from Dr. Robert Weinberg's lab, a kind gift from Dr. Jian Cao, School of Medicine, Stony Brook University. It was cultured in DMEM/F12 Ham's mixture supplemented with 10% FBS (Gibco), EGF 10 ng/ml (Sigma), insulin 10μg/ml (Sigma), hydrocortisone 0.5 mg/ml (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin.

MDA-MB 231 and MDA-MB 468 were also obtained from ATCC and cultured in Minimum essential medium (MEM) supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acid mixture, 1% vitamin solution, 1% L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

SKBR-3 cells obtained from ATCC were cultured in McCoy's 5a media supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

HEK 293T cells (ATCC) and Baby Mouse Kidney (BMK) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. BMK cells were generated as previously described (136).

All cell-lines were cultured at 37°C with 5.5% CO_2.

2. Plasmids and short hairpin constructs

SCCA1 construct was generated by PCR amplification from cDNA of MDA-MB468 cells using the primers 5'-CGGGATCCACCATGGACTACAAGGACGACGATGACAAGAATTCACTCAGTGAAGCCAAC3' and 5′-CCCTCGAGCATCTACGGGGATGAGAATCTGCCA-3′. The forward primer included BamHI site and FLAG tag at the N-terminus and XhoI site in the reverse primer. This was followed by restriction digestion and ligation into the retroviral backbone vector LPC. The SCCA2 construct was generated by PCR amplification from the cDNA of MCF10A cells using

the same set of primers as that of SCCA1. The SCCA1 F352A mutant was generated by site directed mutagenesis. The region 340-345aa was deleted to generate the hinge deleted mutant of SCCA1. The plasmids were verified by sequence analysis.

All shRNA lentiviral constructs were in the pLKO (Sigma) backbone. shRNA targeting sequences used: shGFP: 5'-TACAACAGCCACAACGTCT AT-3'; shScramble: 5'-CAACAAG ATGAAGAGCACCAA-3'; SCCAsh400: NM_006919.1-697s1c1; shIL-6: NM_000600.1-211s1c1; shATF6α: NM_007348.1-690s1c1, shPERK NM_004836.3-35251c1 (Mission shRNA, Sigma).

pBABE K-Rasv12 was a kind gift from Dr. Scot Lowe.

3. Transfection and Infection

HEK 293T cells were used as a packaging cell line to generate virus particles. $4x10^5$ cells were plated in a 6-well plate for transfection using Lipofectamine 2000 (Invitrogen). For retroviral infections, the three plasmid system consisting of gene of interest, helper virus and VSVG was used at the ratio of 4:3:1. Media was changed 4 hours post transfection.

The viral supernatant was harvested at 24, 36 and 48 hours post transfection and filtered using a 0.45μ filter. The supernatant was then applied to the target cells after the addition of 10 μg/ml polybrene (Sigma). Successfully infected cells were selected using puromycin at 1 μg/ml.

Lentiviral infections were carried out using the above mentioned protocol by replacing the helper virus with the ΔR8.91 plasmid. Knock down was evaluated four days post infection.

4. Affymetrix oligonucleotide array

Total cellular RNA was isolated with RNeasy kit from vector control and SCCA1-expressing BMK cells. Subsequent RNA processing procedures and Affymetrix Murine Genome Array U74Av2 application followed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) by the University DNA Microarray Facility (Stony Brook, NY). The data sheet for Affymetrix Murine Genome Array for the U74Av2 chips can be found at http://www.affymetrix.com/products/arrays/specific/mgu74.affx. Data were analyzed using Affymetrix Genechip and GeneSpring software. The analysis of the data was performed at the

University DNA Microarray Center (http://www.osa.sunysb.edu/udmf/) and with the help of the Bioinformatics Core Facility (http://www.osa.sunysb.edu/bioinformatics/index.html) at SUNY-Stony Brook. Software employed in the analyses included the GeneSuite software of Affymetrix and the GeneSpring software.

5. RNA extraction, cDNA synthesis, PCR

RNA was isolated using RNeasy kit (Qiagen), 2 μg RNA was used for cDNA synthesis using oligo dT primers using the SuperscriptIII First Strand Synthesis system for RT-PCR (Invitrogen).

The buffer, dNTP, Taq polymerase (Genscript) was used for PCR. Semi quantitative analysis was carried out at the following temperature condition for 25 cycles to prevent saturation: Denaturation 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds.

PerfecTa SYBR Green Super mix (Quanta Bioscience 95055) was used for Q-PCR using the StepOnePlus (Applied Biosystems). Primers were designed using Primer-BLAST from NCBI using the following criterion: across exon-exon junction, 150-250bp amplicon size and annealing temperature of 60°C. All reactions were executed in triplicates. GAPDH and β-actin were used to normalized gene expression in the human and mouse samples respectively. The data was analyzed using delta-delta Ct method to arrive at fold changes in gene expression.

Table 1: Mouse primers

Gene	Primers
Cdh1	5’ GCCGGAGAGGCACCTGGAG 3'
	5’ TTCAGAGGCAGGGTCGCGG 3'
Epcam	5' GCCGCGGCTCAGAGAGACTG 3'
	5' GTGCCGTTGCACTGCTTGGC 3'
Cldn4	5' ATGGTCATCAGCATCATCGTGGGT 3'
	5' TGTAGAAGTCGCGGATGACGTTGT 3'
Zeb1	5' CCGCGGCGCAATAACGTTACAA 3'
	5' GTGCCCTGCCGCTGGTCTTC 3'
Rhob	5' AAGCCTATGACTACCTCGAGTGCT 3'
	5' CAGTTGATGCAGCCATTCTGGGAT 3'
Il6	5’ CCTCTCTGCAAGAGACTTCCATCCA 3’
	5’ AGCCTCCGACTTGTGAAGTGGT 3’
βactin	5’ CTGTCGAGTCGCGTCCA 3’
	5’ CATCACACCCTGGTGCCTA 3’

Table 2: Human Primers

Gene	Primers
CDH1	5' TGCCACCCTGGCTTTGACGC 3'
	5' AACGGAGGCCTGATGGGGCG 3'
EPCAM	5' GTGCCGTTGCACTGCTTGGC 3'
	5' TTGGCAGCCAGCTTTGAGCA 3'
CLDN4	5' TTCATCGGCAGCAACATTGTCACC 3'
	5' AGTCGTACACCTTGCACTGCATCT 3'
ZEB1	5' GCCGCTGTTGCTGATGTGGC 3'
	5' CCTTTCCTGTGTCATCCTCCCAGC 3'
VIM	5’ GCTTCCTGTAGGTGGCAATC 3’
	5’ GCTTCCTGTAGGTGGCAATC 3’
FN1	5’ CAGTGGGAGACCTCGAGAAG 3’
	5’ TCCCTCGGAACATCAGAAAC 3’
EGF	5’ GGCCAGGCAGCAGATGGGTC 3’
	5’ GGGGCTTCTCGACACCCCCT 3’
TGFβ1	5’ GGCGATACCTCAGCAACCGGC 3’
	5’ GCGGCCGGTAGTGAACCCGTT 3’
IL-6	5’ TCCACAAGCGCCTTCGGTCCA 3’
	5’ AGGGCTGAGATGCCGTCGAGGA 3’
IL-8	5’ AAGGAAAACTGGGTGCAGAG 3’
	5’ ATTGCATCTGGCAACCCTAC 3’
CXCL	5’ CACCCCAAGAACATCCAAAG 3’
	5’ TAACTATGGGGGATGCAGGA 3’
TGFα	5’ ACGTCCCCGCTGAGTGCAGA 3’
	5’ GCCAGGAGGTCCGCATGCTC 3’
HB-EGF	5’ CCGGGACCGGAAAGTCCGTG 3’
	5’ CGGGTGGCAGATGCAGGAGG 3’
AREG	5’ CCGGGACCGGAAAGTCCGTG 3’
	5’ GCAGCATAATGGCCTGAGCCGA 3’
BTC	5’ CTGGCCCTTGCCCTGGGTCT 3’
	5’ GCCACCACGAAGCGGCATCT 3’
SCCA	5’ GGTCAGGAAATGTTCATCACCAG 3’
	5’ GATTGCGTTCACAAGAACC 3’
ATF6α	5’ TGGGCTATTCGCTGAAGGGGCT 3’
	5’ GCACCCACTAAAGGCCAGACGG 3’
GAPDH	5’ AAGGTCGGAGTCAACGGATTTG 3’
	5’ CCATGGGTGGAATCATATTGGAA 3’

6. Immunoblotting

Cells were lysed in RIPA lysis buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 0.01M Tris pH 8.0, 0.14 M NaCl) supplemented with protease inhibitor cocktail and EDTA (ProteCEASE plus EDTA, GBioscences). For phospho-proteins, phosphatase inhibitors (50mM NaF, 10mM sodium pyrophosphate, 100μM sodium orthovanadate, 100μM PMSF) were also included in the lysis buffer. For EGFR and Stat3 detection, equal number of cells were lysed in 2X Laemelli's buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl pH 6.8).

Protein quantification was carried out using BCA assay (Thermo Scientific-Pierce). Equal amount of protein was loaded onto polyacrylamide gels of the desired percentage and electrophoresed at 125V for 1.5 hours to obtain optimal resolution. The proteins were transferred to a nitrocellulose membrane (0.45μm pore size for proteins >20kDa and 0.2 μm pore size for proteins, 20kDa) at 110V for 1.5 hrs. The membrane was subsequently blocked using 5% non-fat dry milk or 5% bovine serum albumin (BSA) in PBST (0.05% Tween-20) for phospho-proteins for 1 hour at room temperature. The membrane was incubated with the primary antibody at the mentioned dilution in PBST over-night at 4°C. Blots were washed with PBST and incubated with the appropriate secondary antibodies with HRP conjugate (Rockland) or Alexafluor 680/800 (Molecular probes, Invitrogen) also in the blocking buffer. Detection of proteins after western blotting was carried out using ECL (Thermo Scientific- Pierce) for HRP conjugated antibodies or an Odyssey Imager (LI-COR) for fluorescent labelled secondary antibodies.

7. Reagents and antibodies

The following reagents were used: crystal violet (dissolved in 10% ethanol for the growth curve) 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma D9542, used at 1 μg/ml), propidium iodide (Invitrogen P3566, used at 1 μg/ml for the death assay), tunicamycin (Calbiochem, used at 5 μg/ml), hematoxylin, eosin, Bay 11-7082 (Sigma, used at 5 μg/ml), Triton X-100, paraformaldehyde (4% in PBS).

The following antibodies were used: SCCA1/2 (Santa Cruz FL390 sc-25741, 1:2,000 for WB), E-cadherin (HECD-1 Abcam ab1416 1:500 for WB and 1:100 for IF), vimentin (Alice B Fulton, University of Iowa AMF17b 1:5,000 for WB and 1:1,000 for IF), beta-tubulin (Sigma T4026, 1:10,000 for WB), phospho-STAT3 Y-705 (Santa Cruz sc-8059 1:1,000 for WB), total STAT3 (Santa Cruz sc-482 1:2,000 for WB), p65 (Santa Cruz sc-8008 1:500), PARP (Cell Signaling Technology #9542, 1:1,000 for WB), caspase-3 (Cell Signaling Technology #9661, 1:1,000 for WB), IκBα (Santa Cruz sc-371 1:1,000 for WB), GRP 78/BiP (BD Transduction Laboratories 610978, 1:5,000 for WB), ATF6α (Santa Cruz sc-166659 1:500 for WB and 1:200 for IF), XBP1 (Santa Cruz sc-7160 1:500 for WB), GADD153/CHOP (Santa Cruz sc-575 1:500 for WB), ATF4 (Santa Cruz sc-7351 1:500 for WB), lamin B1 (Santa Cruz sc-6217, 1:1,000 for WB), Hsp90 (Cell Signaling 1:1,000 for WB), FLAG (Sigma F1804 1:1,000 for WB), IL-6 (MAB 206, R&D Systems 1:1,000 for neutralization), Ras (Millipore, 1:5000), Myc (Santa cruz, sc-40 1:500 for WB)

8. Immunofluorescence

Cells were plated on glass coverslips. They were fixed with 4% PFA, permeabilized with 0.1% Triton X (0.5% for staining nuclear proteins), blocked in 5% goat serum, incubated with primary antibody in 5% goat serum in PBS overnight at 4°C, washed with PBST (0.1% Tween 20), incubated with fluorophore conjugated secondary antibody for 1 h, washed again, counter stained with DAPI and mounted onto glass slides. Imaging was carried out using an inverted deconvolution microscope (Axiovert 200M; Carl Zeiss Inc.) using the 63x oil immersion objective lens. Image analysis was carried out using Axiovision software (Carl Zeiss Inc).

9. IL-6 ELISA, conditioned medium, and neutralization experiment

The concentration of IL-6 secreted into the media was measured using IL-6 ELISA kit (R&D systems D6050), as per the manufacturer's instructions. Equal number of vector control and SCCA1 expressing cells were grown in EGF free media overnight, the conditioned media was collected next day and used for stimulation of EGF-deprived MCF10A cells.

For the neutralization experiment, IgG_1 control or monoclonal antibody to IL-6 (MAB 206 R&D Systems) was incubated with the conditioned media for 2 h at room temperature and used to stimulate EGF-deprived MCF10A cells. Cells were harvested to analyze downstream signaling by immune-blotting.

10. Subcellular fractionation

Subcellular fractionation was carried out using the subcellular proteome extraction kit (Calbiochem). The fractions were quantified using the BCA assay and equal amount of protein was then used for precipitation using four times the volume of ice cold acetone. The samples were incubated for 120 min at -20°C and centrifuged at 13,000 g for 15 min at 4°C. The pellets were air dried for 10 min and resuspended in 2X SDS sample buffer and boiled at 95°C for 5 min. The proteins in the cytosolic and nuclear fractions were then detected by western blotting.

11. Luciferase reporter assay

The dual-luciferase activity kit (Promega E1910) was employed to examine the luciferase activity. The NF-κB binding site of IL-6 promoter fused to a luciferase reporter gene was used for this assay. Briefly, 250ng NF-κB-luciferase vector and 100 ng pCMV-RL (renilla luciferase) were transfected into $5x10^4$ cells in 24 well plate. One day post transfection, cells were harvested in PLB lysis buffer provided in the kit and 20 μl of the lysate was used for luciferase activity assay. Luminescence was read on SpectraMax M5 Microplate Reader. The ratio of firefly luciferase versus renilla luciferase is used as relative luciferase activities.

12. Wound healing assay

Equal number of vector and SCCA1 expressing cells were plated and allowed to grow to sub-confluency. Two perpendicular wounds were made with the help of western loading tips across the culture dish, washed 3 times to remove cell debris, and imaged 6 hours later at the junction of the wound. The cell front was marked out and the area of the gap was measured using the NIS elements software (Nikon Instruments Inc.).

13. Soft agar assay

Cells were trypinized to obtain a single cell suspension. 5 x 10^4 cells were resuspended in 1.5 ml MCF10A complete media with soft agar, 0.5% Noble agarose and overlaid onto base agar, 1.5 ml complete medium with 0.7% agarose in each well of a 6-well plate in triplicates. Media was replenished every 3-4 days. After 3 weeks, colonies larger than 100 μm were images and counted.

14. Anoikis assay and anchorage independent sphere formation

1x10^4 vector control or SCCA1-expressing cells were seeded in non-attachment tissue culture dishes in triplicates for each time-point. Cells were harvested, washed with PBS and stained with propidium iodide at 1 μg/ml. Cell viability was determined by flow cytometry (BD FACSCalibur).

For anchorage-independent sphere formation, cells were infected with the indicated lentiviral short-hairpins and 1 x 10^4 cells were plated 4 days post infection on non-attachment dishes in triplicates. The culture was imaged 10 days post-seeding. Cells were harvest for preparing lysates for immunoblotting

15. Measurement of cell proliferation

3.5 x 10^4 cells were seeded in each well of 6-well plate in triplicates for a 5 day time course. On each day, cells were gently washed with PBS, fixed with 4% paraformaldehyde (PFA), rinsed with sterile water, stained with 0.1% crystal violet, rinsed with water 3 times to wash out excess unbound dye. The plates were allowed to dry completely and the bound crystal violet was extracted using 10% acetic acid. The absorbance was measured at 590 nm using the SpectraMax M5 Microplate reader. Relative growth was calculated after normalizing the absorbance value on day 0 to 1 and plotted as a graph.

16. XBP1 splicing assay

cDNA from vector control or SCCA1 cells were subject to PCR using the following primers. XBP1 5'-AAACAGAGTAGCAGCTCAGACTGC-3' and 5'-TCCTTCTGGGTAGACCTCTGGGAG-3' and GAPDH as a reference control. The PCR product was resolved in 2.5% agarose gel. Equal amount of the PCR product was digested using PstI restriction enzyme to clearly distinguish between the spliced and unspliced forms of XBP1 (146).

17. Orthotopic mouse tumor experiment

Female beige nude XID mice (Hsd: NIHS-*Lyst*$^{bg\text{-}J}$*Foxn1*^{nu}*Btk*^{xid}), age 6–8 week, were obtained from Harlan Laboratories. 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 7.5 x 10^5 vector-control or SCCA1-expressing MCF10A cells in 50 μl of PBS/matrigel mixture (1:1). Cells were injected into the thoracic mammary fat pads of anesthetized mice. Incisions were sutured close and animals were monitored and imaged every 4-5 days for the duration of the experiment. Fluorescence imaging of GFP-expressing tumors was performed using the Maestro *in vivo* fluorescence imager.

18. SCCA conditional transgenic mice

SCCA1 knock-in transgenic mice with 129Ola/C57Bl/6 mixed background were developed by Genoway. Briefly, human SCCA1 open frame cDNA was cloned into the housekeeping *Hprt* gene located on the X-chromosome. A stop cassette flanked by loxP sites was placed upstream of SCCA1 to allow for Cre-specific tissue expression. Once the stop cassette is excised, SCCA1 expression is driven by the pCAG promoter. Females homozygous for SCCA were bred with MMTV-Cre (003553; The Jackson Laboratory) and MMTV-neu mice (002376; The Jackson Laboratory). Mice were then bred to obtain neu$^+$ only and neu$^+$; SCCA1$^+$ mice. Females homozygous for *SCCA1* and Cre-positive are considered SCCA1-positive. Mice

were palpated weekly to detect the presence of mammary tumors. All mouse experiments were done in compliance with the Stony Brook University and University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

19. Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated and antigen retrieval obtained by heating in a microwave for 15 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide. After 15 min blocking with horse serum, the primary antibody SCCA at 1:250, XBP-1 at 1:50, IL-6 at 1:100, F4/80 at 1:100 or the control isotype IgG was applied and incubated overnight at 4°C. Slides were washed three times with phosphate-buffered saline for 5 min. The biotinylated secondary antibody and the streptavidin–biotin complex (Vector Laboratories, Burlingame, CA) were applied, each for 30 min at room temperature with interval washings. After rinsing with phosphate-buffered saline, the slides were immersed for 5 min in the coloring substrate 3, 3′-diaminobenzidine (Sigma) at 0.4 mg/ml with 0.003% hydrogen peroxide, rinsed with distilled water, counterstained with hematoxylin, dehydrated and a coverslip was applied.

Scoring for F4/80 expression

In all tumors, the expression of F4/80 was not even through the whole section. Therefore we photographed 3 or 4 most intensive areas (x200 magnification) from each tumor and the number of positive cells from each picture was analyzed using Image J software. Average of the number of positive cells from each tumor section was calculated in Excel. Statistical significance was analyzed using unpaired t-test in Excel.

20. Statistics

Student's t-test was performed on Graph Pad Prism used to evaluate significance between two groups. The longitudinal data analyses were performed to assess the growth curves under different treatments. The Fisher's exact test was used to test the difference between tumor incidence rates in two groups. The independent two-sample and one sample t-tests were used to make comparisons between groups and to evaluate whether fold changes are different from one,

respectively. The analyses were carried out using PROC MIXED, PROC FREQ, PROC MEANS and PROC TTEST in the SAS 9.4 (SAS institute, Cary, NC). Statistical significance level was set at ns $p>0.05$, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$. ns stands for not significant.

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