Supplemental Materials and Methods

Cell culture

MCF10A cells were 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. BMK and HEK293T 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. MDA-MB-231 cells were 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. HMLE cells were cultured in DMEM supplemented with 10% FBS, EGF 10 ng/ml, insulin 10 μg/ml, hydrocortisone 0.5 mg/ml, 100 units/ml penicillin and 100 μg/ml streptomycin. SKBR3 cells were cultured in McCoy 5a medium supplemented with 10% FBS.

Plasmids and 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 SCCA1 F352A mutant was generated by site directed mutagenesis. The region 340-345aa was

deleted to generate the hinge deleted mutant of SCCA1. 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).

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 for WB), 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), 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 (R&D Systems MAB206, 1:1,000 for neutralization), Myc (Invitrogen 9E10, 1:500 for WB).

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 semi-Q PCR. PerfecTa SYBR Green Super mix (Quanta Bioscience 95055) was used for Q-PCR on the StepOnePlus (Applied Biosystems).

The following human primers were used for QPCR: E-cadherin 5'-TGCCACCCTGGCTTTGACGC-3'and 5'-AACGGAGGCCTGATGGGGCG-3'; EpCam 5'-GACCCCCTCGTCGCTGTCCT-3' and 5'-TTGGCAGCCAGCTTTGAGCA-3' ; Cldn4 5'-TTCATCGGCAGCAACATTGTCACC-3' and 5' AGTCGTACACCTTGCACTGCATCT-3'; Zeb1 5'-CCGCGGCGCAATAACGTTACAA-3' and 5'- GTGCCCTGCCGCTGGTCTTC-3'; RhoB 5' TTCTCATGTGCTTCTCGGTGGACA-3' and 5' TGATGGGCACATTGGGACAGAAGT-3'; Vimentin 5' GAGAACTTTGCCGTTGAAGC 3' and 5' GCTTCCTGTAGGTGGCAATC 3'; FN 5' CAGTGGGAGACCTCGAGAAG 3' and 5' TCCCTCGGAACATCAGAAAC 3'; EGF 5'-GGCCAGGCAGCAGATGGGTC–3' and 5'-GGGGCTTCTCGACACCCCCT-3'; TGFβ1 5'-GGCGATACCTCAGCAACCGGC- 3' and 5' GCGGCCGGTAGTGAACCCGTT-3'; IL-6 5' TCCACAAGCGCCTTCGGTCCA-3' and 5'-

AGGGCTGAGATGCCGTCGAGGA-3’ ; IL-8 5’-AAGGAAAACTGGGTGCAGAG-3’ and 5’-ATTGCATCTGGCAACCCTAC-3’; CXCL1: 5’-CACCCCAAGAACATCCAAAG-3’ and 5’-TAACTATGGGGGATGCAGGA-3’; TGFα 5’–ACGTCCCCGCTGAGTGCAGA–3’ and 5’-GCCAGGAGGTCCGCATGCTC–3’ ; HB-EGF 5’–CCGGGACCGGAAAGTCCGTG–3’ and 5’–CGGGTGGCAGATGCAGGAGG–3’ ; AREG 5’–CCCAAGCCTTCGAGAGCGGC–3’ and 5’–GCAGCATAATGGCCTGAGCCGA–3’; BTC 5’–CTGGCCCTTGCCCTGGGTCT–3’ and 5’–GCCACCACGAAGCGGCATCT–3’ ; EREG 5’-ACAGCCAACGTGGGGTCCCT-3’ and 5’ –TGGAAACCCAGGCAGAGCAGC– 3’; SCCA 5’ GGTCAGGAAATGTTCATCACCAG-3’ and 5’GATTGCGTTCACAAGAACC3’; ATF6α 5’ GCACCCACTAAAGGCCAGACGG -3’ and 5’- TGGGCTATTCGCTGAAGGGGCT -3’, GRP78/BiP 5’-ACTCCTGCGTCGGCGTGTTC-3’ and 5’- ACGGGTCATTCCACGTGCGG-3’ ; GAPDH 5’-AAGGTCGGAGTCAACGGATTTG-3’ and 5’-CCATGGGTGGAATCATATTGGAA-3’.

The following mouse primers were used: IL-6 5’ – CCTCTCTGCAAGAGACTTCCATCCA -3’ and 5’- AGCCTCCGACTTGTGAAGTGGT-3’; β-actin 5’ CTGTCGAGTCGCGTCCA 3’and 5’ CATCACACCCTGGTGCCTA 3’

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.

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). Protein quantification was carried out using BCA assay. Detection of proteins after western blotting was carried out using ECL or an Odyssey Imager (LI-COR). For phospho-proteins, phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 μM PMSF) were also included in the lysis buffer. For the 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). Immunoblotting was performed multiple times for all immunoblots, and representative blots are shown.

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) and processed for brightness and contrast in Adobe Photoshop.

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.

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.

Luciferase reporter assay

The dual-luciferase activity kit (Promega E1910) was employed to examine the luciferase activity. The luciferase assay is performed following the common protocols. The NF-κB binding site of IL-6 promoter was fused to a luciferase reporter gene. Briefly, 250 ng NF-κB-luciferase vector and 100 ng pCMV-RL (renilla luciferase) were transfected into $5x10^4$ cells in a 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.

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 using a FACs caliber. For anchorage-independent sphere formation, cells were infected with the indicated lentiviral short-hairpins and 1×10^4 cells were plated 4 days post infection on non-attachment dishes in triplicates. The culture was imaged 10 d post-seeding.

Measurement of cell proliferation

3.5×10^4 cells were seeded in each well of 6-well plate 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.

Orthotopic mouse tumor experiment

Female beige nude XID mice (Hsd:NIHS-*Lyst*$^{bg\text{-}J}$*Foxn1*^{nu}*Btk*^{xid}), age 6–8 wk, 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 d for the duration of the experiment. Fluorescence imaging of GFP-expressing tumors was performed using the Maestro *in vivo* fluorescence imager.

SCCA1 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.

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.

Supplemental Figure Legends

Figure S1. SCCA1-expressing MCF10A cells become independent of the hormonal factors. (A) Vector control and SCCA1-expressing MCF10A cells were cultured in complete medium or the basal medium that was deprived of the four hormonal factors (EGF, insulin, hydrocortisone, and cholera toxin). Relative cell growth was determined by crystal violet staining. Data shown is the mean +/- S.D. of triplicate experiments. The difference between the samples in complete media is not statistically significant, while the difference between Vector and SCCA1 cells in basal media and that between Vector cells in complete and basal media as well as SCCA1 in complete and basal media are significant. **$p<0.01$, ***$p<0.001$.

Figure S2. Expression of SCCA1 leads to IL-6 production. (A) 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. Data shown are the mean +/- S.D. of triplicate measurements of a representative graph of three independent experiments. Significance was judged as *$p<0.05$, **$p<0.01$, ***$p<0.001$

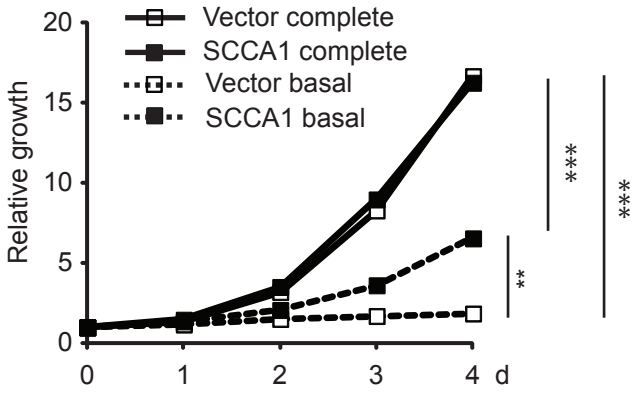
Figure S3. The induction of UPR by SCCA1 is dependent on its protease inhibition function. MCF10A cells stably expressing the vector, point mutant SCCA1-F352A (A) or the hinge deletion mutant SCCA1 Δ340-345 (B) were treated with 5 μg/ml tunicamycin for indicated periods of time. Total cell lysates were probed for indicated proteins by immunoblotting.

Figure S4. 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 measure as in Figures S5A and S5B.

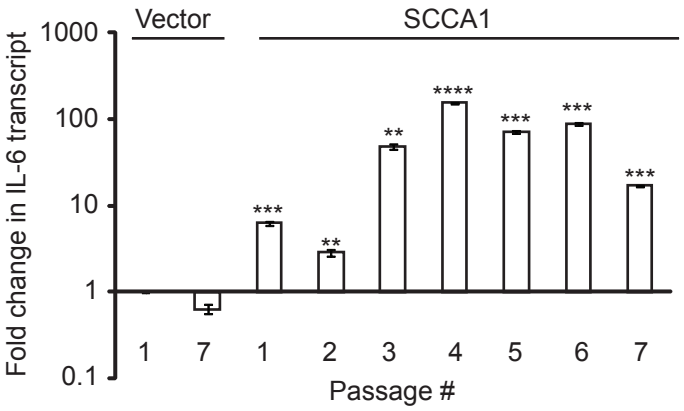
Figure S5. Silencing of SCCA results in decreased UPR, IL-6 production, and cell growth. SCCA1-expressing MCF10A cells (A-C), MDA-MB-231 cells (D-F), or MDA-MB-468 cells (G-I) were infected with control (shNTC) or SCCA (shSCCA) short hairpin RNA. (A, D, G) Cells were harvested 4 days post infection and analyzed by immunoblotting. (B, E, H) 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, I) 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. ***$p<0.001$.

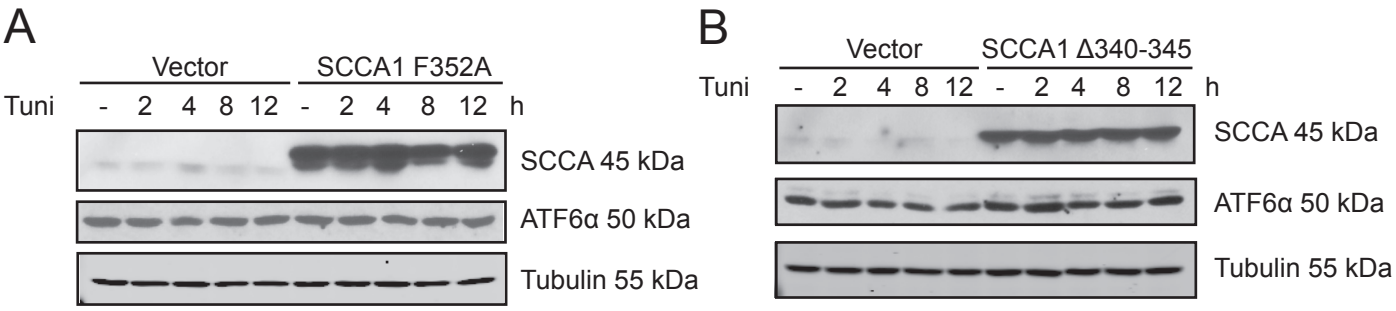
Figure S6. Elevated SCCA1 expression leads to UPR and IL-6 production in various cell lines. (A-D) Baby mouse kidney cells were stably infected with empty retroviral vector or FLAG-tagged SCCA1. Whole cell lysates were probed for indicated proteins by immunoblotting (A). Total RNA was isolated from the indicated cells and semi-quantitative RT-PCR (B) and qRT-PCR (C) for IL-6 expression were performed. The data shown are the mean +/- SEM of three independent experiments performed in triplicates after normalization to the vector control. **$p<0.01$. (D) Vector control or SCCA1 cells were treated with DMSO or tunicamycin for the indicated time course. Whole cell lysates were immunoblotted for the indicated proteins. Note that SCCA1 expression leads to increased IL-6 production and UPR. (E-H) HMLE cells (E and F) or SKBR3 cells (G and H) were stably infected with vector control or SCCA1-expressing retrovirus. Whole cell lysates were probed for indicated proteins (E and G). Total RNA was isolated and qRT-PCR was carried out for the indicated genes (F and H). Data shown are the mean +/- SEM of three independent experiments performed in triplicates after normalization to the vector control sample. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Sheshadri et al. Suppl. Fig S1



Sheshadri et al. Suppl. Fig S2

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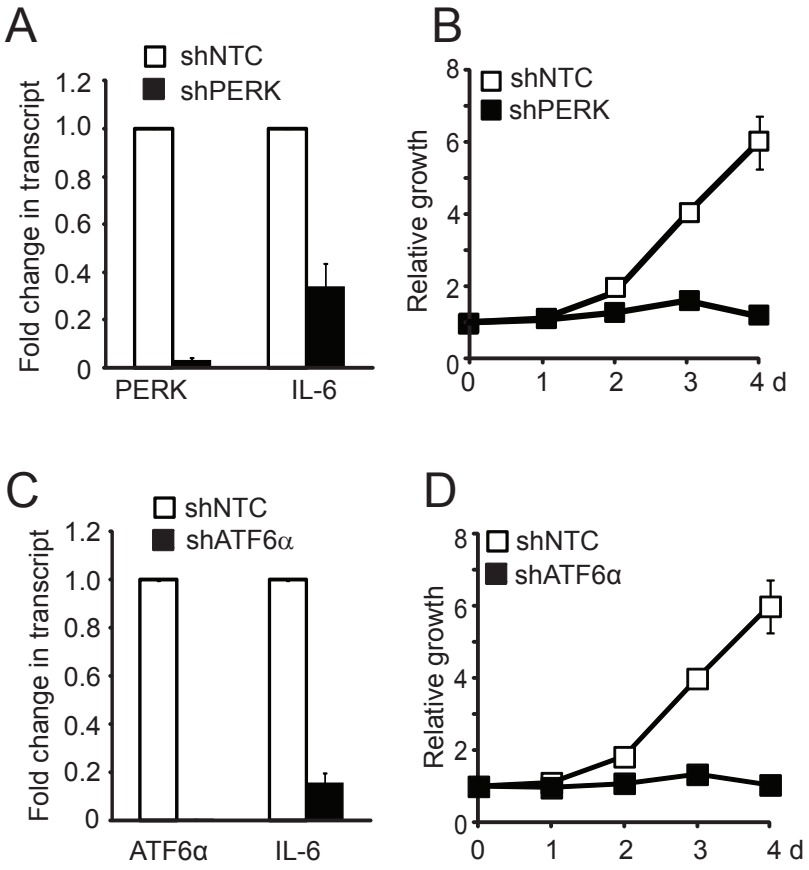
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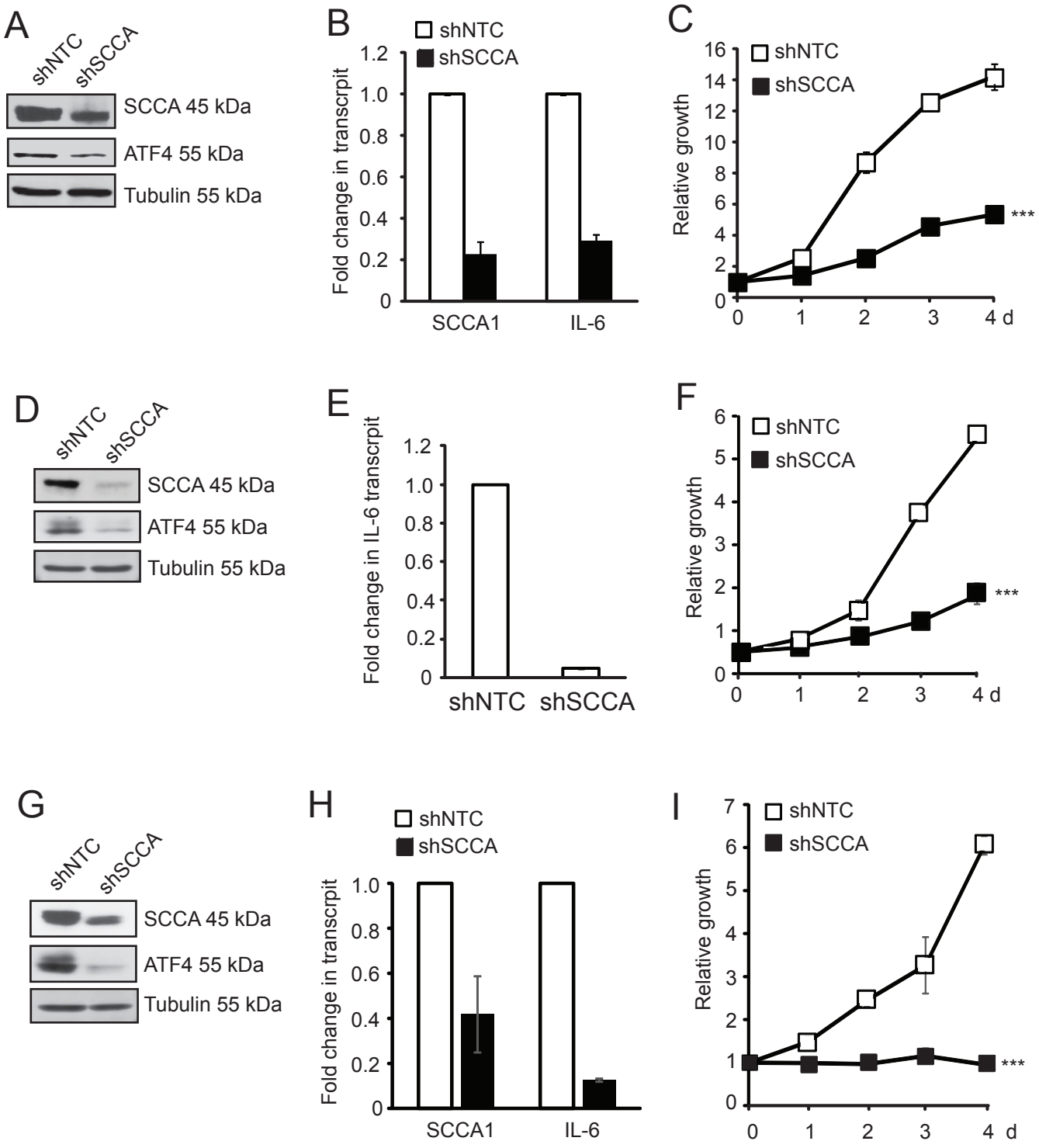
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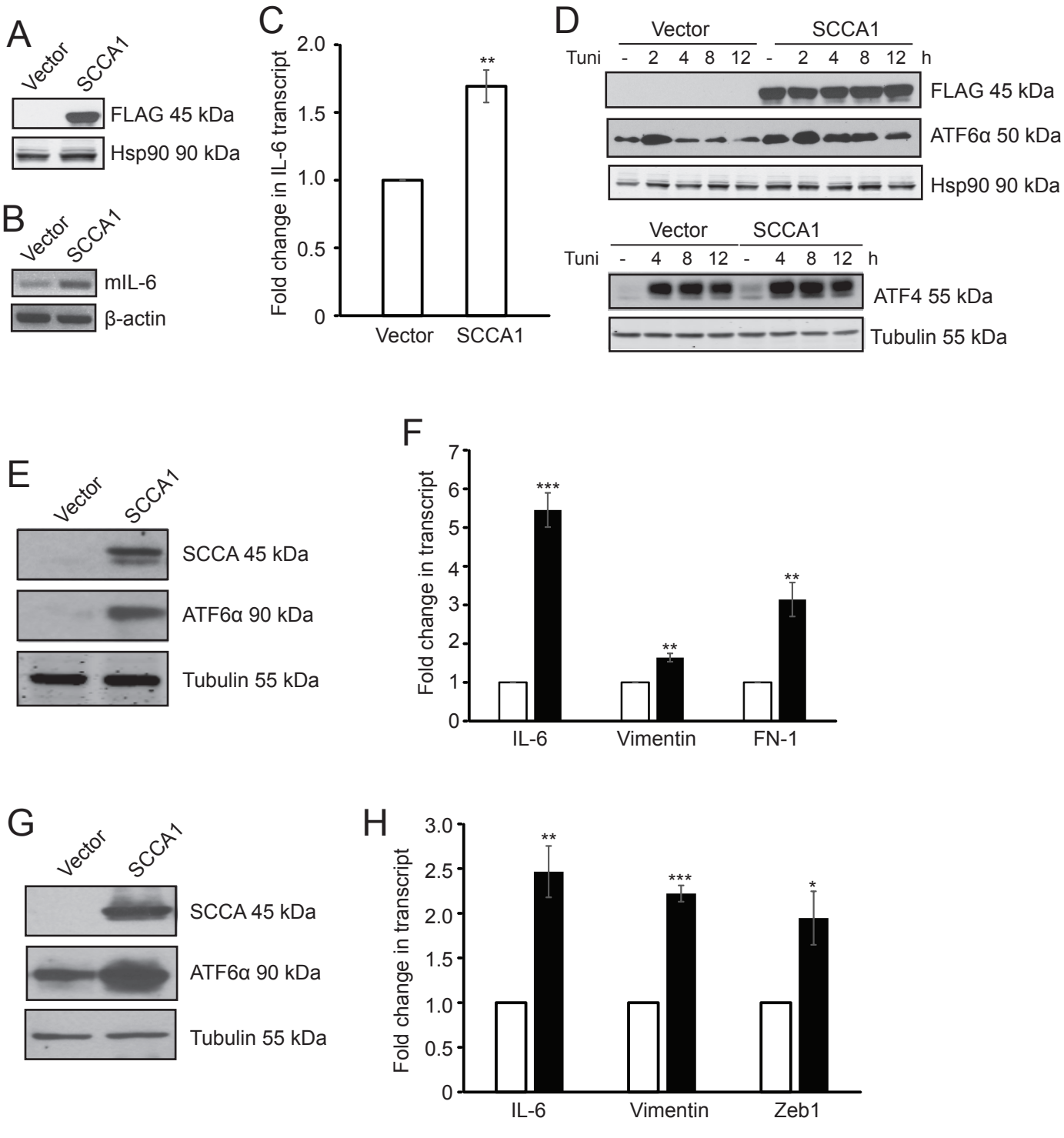
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