Methods

cDNA cloning, expression and transfection. The human *SCCRO* cDNA was generated by RT-PCR of total RNA isolated from head and neck cancer cell line MDA1386 using sequential 5' primers to identify the full length cDNA (P1- 5'-TGGGGGAAAGAATGGATGAAC-3', P2- 5'-CTTGGTACAGCGCTGGGCGCT-3', and P3- 5'-CTGGAGGACACCAACATGAA-3' at the 5' end and P4- 5'-CCAGCCAGCAGAAATTGACT-3' at the 3' end). Primer pair P3 and P4 resulted in a product of 876 bp. The PCR products were sequenced to confirm the identity. The full length *SCCRO* cDNA was subcloned into pGEM-T vector (Promega, Madison, WI). *SCCRO* expression constructs were prepared by ligation of the cDNA (876 bp) at the *Not*1 site of the mammalian expression vector pUSEamp (Upstate Biotechnology, Lake Placid, NY). HA-tagged and GST tagged *SCCRO* constructs were made by ligation of the *SCCRO* open reading frame (780 bp) at the *Msc*1 and *Xho*1 sites of pCMV-HA (BD Bioscience, San Jose, CA) and BamH1 and Xho1 sites of pGEX-4T-3 (GE Healthcare Life Sciences, Piscataway, NJ) respectively. Regulated expression of *SCCRO* in the Tet-Off MEF-3T3 system was established according to manufacturer's protocols (BD Biosciences Clontech, Palo Alto, CA). Transfections were performed by lipofection using the LipofectAMINE PLUS^{TM} Reagents (Life Technologies, Carlsbad, CA) according to manufacturer's protocols. Stable transfectants, were isolated in the presence of 600 μg/ml G418 in the media (GIBCO, Carlsbad, CA) selection.

Genomic sequence analysis and prediction. Genomic annotation was performed using the GENSCAN (http://gene.mit.edu/genscan.html) and Genie: Gene Finder Based on

Generalized Hidden Markov Models software (http://www.fruitfly.org/seq_tools/genie.html) prediction programs. PredictProtein (http://www.EMBL-Heidelberg.DE/Services/sander/predictprotein), Jped[2] (http://jura.ebi.ac.uk:8888/) and PSORT II programs (http://psort.nibb.ac.jp/) were utilized for computational analysis. DNA and protein sequence homology searches were performed using the BLAST and FASTA service of the NCBI and the SAMT99 program (http://www.cse.ucsc.edu/research/compbio/HMM-apps/T99-query.html).

Cell lines, tumor tissue and chemical reagents. The derivation and growth conditions for head and neck cancer cell lines (584, MDA886, MDA1186, and MDA1386) were as described previously (1). Cell lines derived from lung carcinomas (H157, H322, H522, H1299, H549, H2030), NIH-3T3 cells (mouse fibroblasts), and HeLa cells were purchased from the American Type Culture Collection. HaCaT cells (spontaneously immortalized human skin keratinocytes with defective *p53*) were a gift from Dr. Jonathan Garlick (State University of New York, Stonybrook, NY). A *SCCRO*-T1 line was derived from culturing xenograft resulting from the injection of NIH-3T3 cells stably transfected with *SCCRO* into the flank of a BALB/C athymic nude mouse. Human adult multi-tissue Northern blot (BD Bioscience Clontech, Palo Alto, CA) and fetal and adult cDNA were obtained from a commercial source (Promega, Madison, WI).

5' rapid amplification of cDNA ends. The sequences of the 5'-end of cDNA were derived using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) according to manufacturer's protocols. cDNA was generated by RT-PCR from human squamous cell carcinoma (MDA886) total RNA. The oligonucleotide

primers used for 5' RACE included P-1 (5'-CATGCAATAATCAACACACTAATGCTG-3') for first PCR amplification and NP-1 (5'-ATCGAGTGCCAGGTCATCACAGA-3') for nested PCR and P-2 (5'-TACACTCTCTCGTATATAAAGTTCAGG- 3') for sequencing the nested PCR product, which were designed based on the sequence of *SCCRO* (AF 456425).

Soft agar assay. Cells were plated in 60-mm dish followed by incubation for 2-3 weeks and staining with crystal violet (Sigma-Aldrich, St. Louis, MO). Soft agar forced suspension culture assay was performed on HaCaT cells transiently transfected with either *SCCRO* construct or vector alone.

Athymic mouse tumorigenicity. Approximately $5x10^5$ pUSEamp-*SCCRO*-3T3 or pUSEamp-3T3 cells in log phase of growth were injected subcutaneously into each flank of BALB/C athymic nude mice. The mice were sacrificed twelve weeks after injection and the expression of *SCCRO* in all tumors was confirmed by real-time RT-PCR. Autopsy studies performed on all animals included whole organ evaluation of liver, spleen, lung, and axillary and inguinal lymph node tissues. All animal experiments were performed following institutional guidelines.

Southern and Northern blot analyses. Southern and Northern blot analyses were performed according to standard protocols as described earlier (2). Probes for these analyses were generated by PCR using the following primers: *SCCRO* 5'-CTGGAGGACACCAACATGAA-3' and 5'-CCAGCCAGCAGAAATTGACT–3'; *β-actin* 5'-TGGGACGACATGGAGAAAATC-3' and 5'-AGGGAGGAGCTGGAAGCAGC-3'.

Western blot analysis. Whole cell lysates or nuclear extracts were prepared following manufacturer's protocols (Pierce Biotechnology, Rockford, IL). A rabbit polyclonal antibody directed against the carboxy-terminal 16 amino acids of *SCCRO* (Antibody 1B-D125/I140-DDMSNYDEEGAWPVLI) was raised and affinity purified (Bethyl Laboratories, Montgomery, TX). The targeted amino acid sequence is identical in human and mouse genes. Western blots were performed using anti-*SCCRO* antibody at a concentration of 1:5000. The specificity and sensitivity of the *SCCRO* antibody was confirmed in several control experiments. An antibody against *Gli1* was obtained from Chemicon International (Temecula, CA) and utilized at a concentration of 1:5000.

Real time PCR. Two μg of total RNA was reverse transcribed with MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Conditions for all PCR reactions were optimized with regard to primers and various annealing temperatures (55-60^{o}C). Specificity of the RT-PCR amplification products were documented with melt curve analysis. A 4% agarose gel electrophoresis was done to check the quality of the PCR product. Optimized results were analyzed on the iCycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR green detection. Each real-time reaction was performed in triplicate in a total volume of 20μL, containing 10μL of 2x SYBR® Green PCR master mix (PE Applied Biosystems, Foster City, CA), 1μL each of forward and reverse primers (5μM stock), and 2μL of the reverse transcribed template. The following 3-step iCycler PCR protocol was used: denaturation program (95^{o}C for 10 minutes), amplification and

quantification program repeated 30-50 cycles (95^{o}C for 30 seconds, specific annealing temperature for 30 seconds, 72^{o}C for 30 seconds, and specific acquisition temperature for 15 seconds). Melt curve analysis was performed following amplification (3). The acquisition temperature was set 1^{o}C to 2^{o}C below the T_m of the specific PCR product. The relative quantification of a target gene in comparison to a reference gene (*18S r*RNA) was performed as described (4, 5). PCR primers and conditions are detailed in *Supplementary Data Table 2.*

Immunohistochemistry. Five micrometer sections from human squamous cell carcinoma cell lines, primary human lung squamous cell carcinoma tissue, and non-neoplastic lung tissue were fixed in buffered formalin and embedded in paraffin. The sections were de-paraffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were subjected to antigen retrieval by microwave treatment for 15 minutes in 0.01 M citrate buffer at pH 6.0. For *SCCRO* immunohistochemistry, sections were incubated in 10% normal goat serum for 30 minutes and then overnight at 4°C with polyclonal rabbit anti-human *SCCRO* antibody (antibody 1B) at a concentration of 0.18 µg/mL . Samples were then incubated with biotinylated anti-rabbit IgG at 1:1000 dilution for 30 minutes followed by avidin peroxidase at 1:25 dilution for 30 minutes (Vector Laboratories, Inc, Burlingame, CA). For Gli1 immunohistochemistry, slides were incubated in 10% normal rabbit serum for 30 minutes and then overnight at 4°C with polyclonal goat anti-human Gli1 antibody (sc-6153, Santa Cruz Biotechnology, Inc., CA) at a concentration of 4 mg/mL. Samples were incubated with biotinylated anti-goat IgG (Vector Laboratories, Inc, Burlingame, CA) at a 1:800 dilution for 30 minutes followed by avidin peroxidase at 1:25 dilution for 30

minutes. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. For *SCCRO* immunohistochemistry, parallel sections of the tumor were subjected to the same immunoperoxidase procedure. The primary anti-*SCCRO* antibody was pre-incubated with the synthetic blocking peptide DTW 1 (against which the antibody was raised) and used as a specificity control for the experiments. The ratio of blocking peptide to primary antibody was 4:1 (peptide 0.72 μg/mL: 0.18 μg/mL primary antibody).

Statistical analysis. All analyses were performed using JMP4 statistical software (SAS Institute Inc., Cary, NC). Statistical significance was defined as a two tailed *p*-value less than or equal to 0.05. Qualitative and quantitative non-parametric comparisons were performed using Fisher's exact test and the Mann-Whitney U-test respectively. Multivariable comparisons were made using the Kruskal-Wallis analysis of variance. Correlation analyses were performed using Spearman's Rho method. Survival curves were generated using the Kaplan Meier method and compared using the log-rank test. Cox regression analysis was used to control for the effects of confounding variables on outcome.

GenBank accession numbers: *SCCRO*, AF456425, AF456426; Homo sapiens similar to RP42 homolog (LOC151625), XM_037433; Homo sapiens *RP42* mRNA, AF292100; RP42, GI Acc. # 6166507; Tes3, GI Acc. # 15826850; AY050926, 15292985; CG7427, 7294266; H38K22, 17553918; *Gli1*, NM_010296; 18S, U13369.

References for supplementary methods

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