Materials and Methods

Determination of cell proliferation and flow cytometry

To determine cell proliferation, equal numbers of the inducible CoAA, CoAM, or vector-expressing cells were seeded in 12-well plates. Cell proliferation was determined every other day by counting cell numbers. For crystal violet staining, 24,000 of each cell were seeded in 12-well plates and were grown in the absence or presence of 0.5 mg/l of tetracycline. After 9 days of growth, cells were stained with crystal violet (Sigma, St. Louis, MO) for 10 min at room temperature.

To determine cell cycle progression, cells were treated with or without tetracycline for 72 hours followed by serum starvation for another 24 hours. After serum release, cells were harvested and fixed in 90% ethanol at 4°C, washed with PBS, and incubated at 37°C for 30 min in PBS containing 100 μg/ml RNAase A (Sigma Chemical Co. St. Louis, MO) and 100 μg/ml propidium iodide (Sigma Chemical Co. St. Louis, MO) followed by FACS analysis. Cellular DNA content was measured by the fluorescence intensity of propidium iodide stained cells according to the Becton-Dickinson protocol (Becton-Dickinson Immunocytometry Systems, San Jose, CA) on a FACScan flow cytometer (Becton-Dickinson Instruments, San Jose, CA). BrdU incorporation rate was measured by FACS analysis using FITC-conjugated BrdU antibody according to the manufacturer's instructions (BD Pharmingen, San Jose, CA). Histograms were analyzed with Flow Jo software.

Foci formation, soft agar assay and xenograft experiment

To assess contact inhibition, foci formation assays were carried out as previously described (13). The Student's *t* test was used to evaluate the data. To assess anchorage-independent growth, colony formation in soft agar was assessed as previously described. Cells (3×10^3/well in 24 well plate) from 293-CoAA, or 293-CoAM were seeded in 1 ml of 0.35% low-melting agarose (Cambrex, Walkersville, MD) over a 0.7% agar bottom layer in growth medium with (+) or without (-) tetracycline. Each assay was performed in triplicate on three or more independent occasions. Colony formation was counted after 20 days. The Student's *t* test was used to evaluate the data. Xenograft experiments were performed in 10 or 15 female athymic nude mice 4 to 5 weeks of age that were purchased from Harlan Sprague Dawley (Madison). Mice received two subcutaneous injections of 5×10^6 HEK293 stable cells/one each into their right and left flanks. Animals were maintained in pathogen-free conditions in autoclaved microisolator cages in the Transgenic Mouse Facility at Baylor College of Medicine. Serial tumor measurements were obtained at the indicated time points by caliper. Tumor volumes were calculated by the following formula: volume = $W^2 \times L \times 0.5$, where W is the distance across and L is the measurement lengthwise of the tumor. Animals were followed until any mouse developed a tumor measuring $1000mm^3$; they were then euthanized according to institutional policy. Statistical analyses for xenograft assays used one-way ANOVA followed by Tukey's *post hoc* multiple range test with the Instat package from GraphPad (San Diego, CA, USA). p values of < 0.05 were considered statistically significant, and p values of < 0.01 highly significant. In the figures, standard error of the mean (S.E.M) is indicated in all bar graphs.

Reverse transcription and real-time quantitative PCR

qRT-PCR analyses of endogenous *c-myc* and *ccnd1*, *skp2*, *cdkn1b*, *CoAA* and *CoAM* mRNAs were performed. Total RNA was isolated from six-well culture dishes using the RNeasy kit (QIAGEN, Hilden, Germany) followed by first strand cDNA synthesis using Superscript II (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Real-time PCR reactions were performed using the AIB Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) with the TaqMan reaction mixture according to manufacturer's instruction. Means and S.E.M from the three or five independent samples were then calculated. The Student's *t* test was used to evaluate the data. To avoid variations from different samples, the relative mRNA level of each gene was normalized against the beta-2-microgloblulin mRNA content of the same sample. Primer sequences are available upon request.

Western blot analyses and quantitation

Western blot analyses were performed following standard procedures using Immune Star (Bio Rad). β-actin was used for normalization controls. Antibodies were from various sources: Anti-FLAG (Sigma, St. Louis, MO) and anti-β-actin (Sigma, St. Louis, MO), Anti-Skp2 antibody (Zymed, South San Francisco, CA), anti-p27 antibody (Santa Cruz, Santa Cruz, CA), anti-Myc antibody (9E10, Covance, Berkeley, CA), anti-Cyclin D1 antibody (Santa Cruz, Santa Cruz, CA). Polyclonal anti-CoAA was generated in rabbits by immunization with GST-CoAA (580-669) fusion protein (Genemed Synthesis, San Antonio, TX). Each band was subjected to quantitation as described in Experimental procedures. Relative fold-changes were presented by setting each normalized protein to "1" relative to the amount shown in lane 1 unless stated otherwise. Quantification of the relative intensity from the image was done using Image J free software (NIH, USA).

Supplementary Figure legends

Figure S1. A (i) Western blot analysis with anti-FLAG antibody showing the expression levels of 2xFlag-CoAA-8xHis and 2xFlag-CoAM-8xHis in the indicated stable cells in the absence (-: lanes 1 to 3) or the presence (+; lanes 4 to 6) of tetracycline. (ii) Western blot analyses showing the endogenous CoAA, FLAG and β-actin in the indicated stable cells with the indicated tetracycline treatment.

Supplementary Table and Table legends

Table S1. Tumor growth of HEK293 xenografts

Cohort	Cells	No. of injected sites developing tumor	Tumor volume[a] (mean ± SEM)[a]	Days to palpable tumors[b] (mean ± SD)
1	CoAA-Dox	15/20	297.08± 54.448	13±2.5
2	CoAA+Dox	6/20	9.975 ± 4.27	25±1.3
3	CoAM-Dox	25/25	638.45± 47.28	10±1.2
4	CoAM+Dox	30/30	722.268± 65.45	10±2.2

Table S1. Tumor growth of HEK293 xenografts. Four cohorts of 10-15 mice each were inoculated with either 5 x 10^6 293-CoAA cells/flank or 5 x 10^6 293-CoAM cells/flank. 293-CoAA cells demonstrate less propensity for tumor development, a delayed latency period and a decreased growth rate compared with untreated xenografts.

[a] Mean tumor volume of all mice measured on the 19th day postinjection.

[b] Mean days from inoculation until tumor is first palpable in mice developing tumors.