Supplementary Methods

Cell lines.—Thyroid cancer cells were grown in DMEM containing 10% fetal bovine serum (FBS). Nthy-ori 3-1 (NTHY) were grown in RPMI-1640 medium supplemented with 10% FBS. PC cells were cultured in Coon’s modified Ham F12 medium supplemented with 5% calf serum, and a mixture of 6 hormones (6H), including thyrotropin (TSH, 10 mU/ml), hydrocortisone (10 nM), insulin (10 μg/ml), apo-transferrin (5 μg/ml), somatostatin (10 ng/ml) and glycyl-histidyl-lysine (10 ng/ml) (Sigma Chemical Co., St. Louis, MO).

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mmol/L of L-glutamine and 100 U/ml penicillin-streptomycin (Invitrogen Groningen, The Netherlands).

Antibodies, compounds and proteins.—Anti-phospho p44/42 MAPK (#9102), recognizing MAPK (ERK1/2) when phosphorylated either individually or dually on Thr202 and Tyr204, anti-p44/42 MAPK (#9101) and anti-phospho(Ser/Thr) PKA substrate (#9621) were purchased from Cell Signaling (Beverly, MA, USA). Anti-tubulin (#T9036) and anti-HIS (#H1029) were from Sigma Chemical Company (St. Louis, MO, USA). Anti-phospho-CREB (S133) (#06-519), anti-CREB (#06-863), and anti-PP2A (#05-421) were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-p300 (sc-584), anti-GFP (sc-8334), anti-c-Myc (sc-40), and anti-cyclin D1 (sc-718) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech (Little Chalfort, UK). MEK1/2-
inhibitor U0126 was from Cell Signaling. The metallocproteinase-inhibitor BB94 was from British Biotech (Oxford, UK). The γ-secretase inhibitors, COMP X and DAPT, the 3’,5’ cyclic-AMP analog N6-benzoyl-cAMP, the adenylate cyclase (AC) activator forskolin (FSK), and the PKA-inhibitor H-89 were from Calbiochem (La Jolla, CA, USA). The RET tyrosine kinase inhibitor ZD6474 was kindly provided by Astra Zeneca Pharmaceuticals (Macclesfield, UK). Recombinant human CREB protein was from BIOSOURCE (Camarillo, CA, USA), recombinant human PP2A catalytic subunit was from Cayman (Ann Arbor, MI, USA) and recombinant PKA catalytic subunit was from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

**Cell growth and staining** — The rate of DNA synthesis was measured by the 5'-bromo-3'-deoxyuridine (BrdU) Labeling and Detection Kit from Boehringer Mannheim (Germany). Cells were seeded on glass coverslips, pulsed for 1 h with BrdU (final concentration 10 μM), fixed and permeabilized. Coverslips were incubated with anti-BrdU mouse monoclonal and rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Philadelphia, Pennsylvania) and mounted in Moviol on glass slides. Cell nuclei were identified by Hoechst 33258 (final concentration 1 mg/ml; Sigma Chemicals Co) staining. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss) (equipped with a 100X lens) interfaced with the image analyzer software KS300 (Zeiss).

**Protein studies** — Cells were lysed in a buffer containing 50 mM HEPES; pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM MgCl₂, 10 mM NaF, 10
mM sodium pyrophosphate, 1 mM Na$_3$VO$_4$, 2 mM PMSF, and 10 g/ml aprotinin. After clarification at 10,000 x g for 20 min, lysates containing comparable amounts of proteins (modified Bradford assay, Bio-Rad, Munich, Germany), were subjected to Western blot. Immune complexes were detected with the enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech). Signal intensity was analyzed with the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software.

For immunoprecipitations, after preclearing with protein-G or protein-A sepharose beads, the lysates were incubated overnight at 4°C with 2 μg of antibody. Antibody-antigen complexes were collected with 40 μl of protein-G or protein-A sepharose beads for 2 h at 4°C with gentle rotation. The samples were centrifuged, washed, eluted in sample buffer and run on SDS-polyacrylamide gel.

Nuclear extraction was performed as described elsewhere (1). Briefly, cells were harvested in lysis buffer [10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF)], supplemented with 60 mM NaF, 60mM β-glycerophosphate and protease inhibitors (aprotinin, leupeptin and pepstatin; 40 mg/ml) and lysed by shearing with 15 passages through a 26-gauge needle mounted in a 1 ml syringe. Nuclei were recovered by centrifugation at 3,000 x g for 10 m. Nuclear proteins were extracted in 50 mM Tris-HCl, pH 7.5, containing 0.3M sucrose, 0.42 M KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 20% glycerol, 2 mM DTT, 0.1 mM PMSF, 60 mM NaF, 60 mM β-glycerophosphate, leupeptin and aprotinin.

Pull down assay—Cells were serum-starved for 18 h and lysed in ice-cold buffer. Protein
lysates (2 mg) were incubated for 2 h with 30 μg of GST fusion proteins after 2 h of pre-clearing with glutathione Sepharose beads. Pellet beads were collected by centrifugation (1,000 x g) for 3 m and washed with lysis buffer. The beads were resuspended in 2X Laemmli buffer and subjected to Western blotting.

Reporter assay — The pGL3-CRE (cAMP response element), pGL3-NFKB (nuclear factor kappa B), pGL3-AP-1 (activator protein 1), pGL3-SRF (serum response factor), pGL3-Gli (Glioma-associated oncogene homolog, Sonic hedgehog pathway) reporter contain five repetition of the same site (respectively CRE, NFKB, AP-1, SRF and Gli elements) upstream the Firefly luciferase cDNA. The pGL3-CycD1 reporter contains a cyclin D1 (CCND1) natural promoter (2-4). To evaluate the TCF/LEF transcriptional activity, we used a pair of luciferase reporter constructs, TOP-FLASH and its negative control FOP-FLASH (Upstate Biotechnology). TOP-FLASH contains three copies of the TCF/LEF binding site; FOP-FLASH contains a mutated TCF/LEF binding site. TCF/LEF activity was determined from the TOP-FLASH:FOP-FLASH ratio (5).

Twenty-four hours after transfection cells were serum-starved overnight. Firefly and Renilla luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega Corporation). Light emission was quantitated using a Berthold Technologies luminometer (Centro LB 960, Bad Wildbad, Germany) and expressed as a percentage of residual activity compared to untreated cells. In all cases, the total amount of transfected plasmid DNA was normalized with empty vector DNA-transfected cells. The average results of three independent assays ± SD are reported.
Chromatin immunoprecipitation (ChIP)—ChIP was performed with the chromatin immunoprecipitation assay kit (Upstate Biotechnology Inc.), following manufacturer’s instructions. Briefly, chromatin was fixed by directly adding formaldehyde (1% v/v) to the cell culture medium. CREB-bound chromatin was immunoprecipitated with mock or CREB antibodies. Then, cross-linking was reversed and input DNAs as well as immunoprecipitated DNAs were analyzed by PCR using primers spanning the CRE site of the human CCND1 promoter. The primers used to amplify the promoter sequence were:

CRE-forward (5’-AACGTCACACGGACTACAGG-3’);
CRE-reverse (5’-TGTTCCATGGCTGGGCTTT-3’).

Supplementary references


