Two Distinctive POMC Promoters Modify Gene Expression in Cushing’s Disease

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Abstract

**Context.** Mechanisms underlying pituitary corticotroph adenoma ACTH production are poorly understood, yet circulating ACTH levels closely correlate with adenoma phenotype and clinical outcomes.

**Objective.** We characterized the 5’ ends of proopiromelanocortin (POMC) gene transcripts, which encode the precursor polypeptide for ACTH, in order to investigate additional regulatory mechanisms of POMC gene transcription and ACTH production.

**Methods.** We examined 11 normal human pituitary tissues, 32 ACTH-secreting tumors, as well as 6 silent pituitary corticotroph adenomas (SCA) that immunostain for but do not secrete ACTH.

**Results.** We identified a novel regulatory region located near the intron2/exon3 junction in the human POMC gene, which functions as a second promoter and an enhancer. *In vitro* experiments demonstrated that CREB binds the second promoter and regulates its transcriptional activity. The second promoter is highly methylated in SCA, partially demethylated in normal pituitary tissue, and highly demethylated in pituitary and ectopic ACTH-secreting tumors. In contrast, the first promoter is demethylated in all POMC-expressing cells and is highly demethylated only in pituitary ACTH-secreting tumors harboring the *USP8* mutation. Demethylation patterns of the second promoter correlate with clinical phenotypes of Cushing’s disease.

**Conclusion.** We identified a second POMC promoter regulated by methylation status in ACTH-secreting pituitary tumors. Our findings open new avenues for elucidating subcellular regulation of the hypothalamic-pituitary-adrenal axis and suggest the second POMC promoter may be a target for therapeutic intervention to suppress excess ACTH production.

**Keywords:** ACTH, POMC, Cushing’s disease, pituitary adenoma
Introduction

Pituitary adenomas arise from differentiated anterior pituitary cell types, each exhibiting unique clinical features (1). POMC, the precursor protein cleaved to form ACTH, is abundantly expressed in corticotroph ACTH-secreting adenomas in patients with Cushing’s disease (2). The POMC protein is also expressed in several non-pituitary tissues, and autonomous ACTH secretion may be encountered with ectopic tumors, including those in the breast, lung, colon, pancreas, and thymus (3). In contrast to these neoplastic sources of excess ACTH, silent corticotroph pituitary adenomas (SCA) immunostain positively for but do not secrete ACTH (4). Although several mechanisms have been proposed to enhance autonomous adenoma ACTH production (5-7), pathogenesis of these tumors and mechanisms driving dysregulated POMC gene expression remain largely unclear. Corticotroph adenomas overexpress epidermal growth factor receptor (EGFR) (8) and EGFR signaling induces POMC transcription. In a subset of Cushing’s tumors, a mutant USP8 leads to attenuated EGFR ubiquitination and degradation (9,10). These gain-of-function somatic USP8 mutations are consistent with reports that EGFR signaling drives the cell cycle regulator E2F1, which also binds the human POMC promoter (11).

Patients not exhibiting mutations in the USP8 gene present with heterogeneous tumor phenotypes and clinical outcomes, suggesting that additional mechanisms distinct from the USP8-EGFR pathway are likely involved in overexpression of POMC and Cushing’s disease development (12,13).

Activity of the Pomc promoter, located within the −480/+34 bp region of the murine Pomp gene, was largely characterized using mouse corticotroph AtT20 cells (14). Several transcription factors, including TPIT (15,16), PITX1 (17), STAT3 (18), NEUROD1 (19), and TR4 (20), bind this region and regulate gene expression. In particular, TPIT and PITX1 binding sites are located in close proximity, and both transcription factors cooperate with
NEUROD1 to regulate corticotroph-specific Pomc gene expression (15,19,21,22). Thus, the proximal Pomc gene promoter regulates corticotroph-specific expression.

In analyzing 5’ ends of POMC mRNA derived from normal pituitary tissues, pituitary and ectopic ACTH-secreting tumors, and pituitary SCAs, we identified a second POMC promoter, located near the intron2/exon3 junction of the human POMC gene. This region contains a binding site for CREB, a methylation-sensitive transcription factor (23), and our results indicate that CREB binding plays a key role in POMC transcription from this region. We identified a CpG island in this locus, and show that DNA demethylation of the CpG island induced second promoter activity. The second promoter is partially methylated in normal pituitary tissue and highly demethylated in Cushing’s disease tumors, and methylation levels of the second promoter in the different ACTH-expressing tumor types correlate with their respective clinical phenotypes. Our findings suggest that the second POMC promoter may be a potential target for therapeutic intervention to suppress excess ACTH production.

**Materials and Methods**

**Study approval**

Post-surgical sample collection and tissue processing was approved by the Institutional Review Board (IRB) at Cedars-Sinai Medical Center (Protocol #2873) and Toranomon Hospital (Protocol #20171222). Informed consent was obtained prior to sample collection at each recruitment site. Human autopsy pituitary tissue collection was approved by the Cedars-Sinai IRB (Protocol #000375). Receiving and handling of samples was approved by the University of Minnesota IRB (Protocol #00005168). Experiments were performed at Cedars-Sinai Medical Center and University of Minnesota under the same IRB approvals.
Patient recruitment and sample collection

Male and female patients of any age were eligible for inclusion at either recruiting site after being diagnosed with Cushing’s disease according to standard guidelines. Specifically, patients had clinical signs of symptoms consistent with Cushing’s syndrome, biochemical evidence of hypercortisolism as determined by 24-hour urinary free cortisol, midnight salivary cortisol, and/or low-dose dexamethasone suppression test, as well as confirmed sellar lesion visualized on brain/pituitary MRI prior to surgery. Diagnosis of Cushing’s disease was confirmed by histopathology.

SCA was diagnosed at Cedars-Sinai Medical Center using histopathology of pituitary tumor specimens that stained positive for ACTH retrieved from patients exhibiting neither clinical nor biochemical evidence of hypercortisolism. Ectopic ACTH-secreting tumors were diagnosed with histopathology of non-pituitary tumor specimens that stained positive for ACTH retrieved from patients exhibiting clinical and/or biochemical evidence of hypercortisolism who do not meet diagnostic criteria for pituitary Cushing’s disease.

Fresh tumor tissue was collected during surgical tumor resection at Cedars-Sinai Medical Center or Toranomon Hospital. Formalin-fixed, paraffin-embedded (FFPE) specimens used for USP8 hotspot mutation analysis (described below) were also obtained from both sites.

For ACTH assays, electrochemiluminescence immunoassay was used at Cedars-Sinai Medical Center (Quest Diagnostic) and Toranomon Hospital (Roche Diagnostics). For cortisol assays, chemiluminescent microparticle immunoassay (Abbott Architect i2000SR) was used at Cedars-Sinai Medical Center and electrochemiluminescence immunoassay (Roche Elecsys Cortisol II) was used in Toranomon Hospital.
Human autopsy pituitary tissue collection was approved by the Cedars-Sinai IRB (Protocol #000375). Specimens were collected and processed for formalin fixation within 24 hours of the patient’s death.

**Cell culture**

DMS79 cells (human small cell lung carcinoma), and COLO320 cells (human carcinoid-like colorectal carcinoma) were cultured in RPMI 1640 with L-glutamine (Cat# 11875-093, ThermoFisher) and 10% FBS. AtT20 cells were cultured in low glucose Dulbecco’s modified Eagle medium (Cat# 11885076, ThermoFisher) with 10% FBS.

**Analysis of human POMC transcription start sites**

We performed 5’-RACE (24) using RNA isolated from 4 pituitary ACTH-secreting tumors, 3 ectopic ACTH-secreting tumors, and POMC-producing DMS79 and COLO320 cell lines. Briefly, cDNA was prepared using a primer (CAGTCAGCTCCCTCTTGAACTCCA) that anneals to 3’ UTR of POMC transcripts. The G-tail was added at the 5’-end of the resulting cDNA by T4 DNA ligase, and 5’-ends were PCR amplified using a poly(C) primer (CCCCCCCCCCCCCCCC) and an antisense POMC primer (ACTCCAGGGGGAAGGCTCGCGCCGA). To concentrate the human POMC cDNA fragments, a second PCR was performed using an antisense POMC primer (TTGCCCTGCAGGGCCGGCGCCGCT) and the poly(C) primer. Resulting PCR products were cloned into pCR Blunt II TOPO and 5’-ends determined by Sanger sequencing. To avoid PCR artifacts, three independent PCR products were analyzed.
Luciferase reporter plasmids

Human POMC first promoter fragment (−428/+68) was cloned into the pGL3 Basic vector (Cat# E175-1, Progema) as previously described (11). POMC intron2/exon3 gene fragment (+6417/+7136) was PCR amplified from human genomic DNA (Jurkat cells) and cloned into pCR Blunt II TOPO vector. Deletion mutants of this fragments were generated, and Sal I and Hind III sites were introduced at 5’- and 3’-ends, respectively, by PCR. Resulting fragments were then cloned into the Xho I and Hind III sites in the pGL3 Basic vector. Mutations of STAT and CREB binding sequences in the second promoter fragment (+6657/+7136) were introduced by 2 steps PCR assembly (25), and mutated fragments cloned into the Xho I and Hind III sites in the pGL3 Basic vector.

To analyze enhancer activity, the second promoter region (+6657 to +7136) was cloned downstream of the luciferase gene in the pGL3 Basic with the first promoter (−480 to +68). The CpG-free luciferase reporter plasmid (pCpGL-basic vector) was kindly provided by Dr. Michael Rehli, University Hospital Regensburg, Germany (26), and used to analyze the methylation-dependent promoter activity as below. Human first promoter (−428 to +68), human second promoter (+6657 to +7136), mouse first promoter (−459/+80) and mouse second promoter (+4841/+5208) fragments were amplified, and Nhe I and Hind II sites were added by PCR. These fragments were cloned into the Spe I and Hind III sites in CpGL-basic vector. Final constructs were confirmed by Sanger sequencing.

In vitro methylation

Ten µg of CpG free plasmids were treated by methyltransferase (SssI) with or without 160 µM S-adenosylmethionine (SAM; Cat# B9003S, New England Biolabs, Frankfurt am Main, Germany) for four hours at 37°C. Methylation levels in the promoter region were digested with Hpa II and Msp I (2.5 U/µg DNA; New England Biolabs) for one hour at 37°C.
SssI-treated plasmid DNA was purified by phenol/chloroform, and digested DNA was analyzed by agarose gel electrophoresis. Purified plasmids were also used for luciferase assay.

**Luciferase assays**

Luciferase reporter assays were performed using 2.5 × 10^5 DMS79 cells or COLO320 cells with 0.7 μg luciferase reporter plasmids, or using 0.8 × 10^4 AtT20 cells with 0.5 μg luciferase reporter plasmids; 50 ng pRL-Renilla was used as an internal control plasmid. Cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and cultured in 0.5 mL medium in 24-well plates. Forty-eight hours post-transfection, cells were harvested and luciferase activity analyzed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase assays were repeated at least three times.

**DNA pull-down assay**

DNA pull-down assay was performed as described (23). A 120 bp DNA fragment (+6847/+6966) containing the CREB binding sequence or the mutated CREB sequence fragment was amplified by PCR using biotinylated primers. A 30 μL nuclear extract (100 μg protein) was added to 180 μL of 5 mM Tris (pH 8.0)-14% glycerol buffer and pre-incubated on ice for 30 min. Then, 15 μL of poly(dl-dC)(dl-dC) (7.5 μg) and 18 μL of 5-fold concentrated binding buffer (300 mM KCl, 60 mM HEPES [pH 7.9], 20 mM Tris-HCl [pH 8], 0.5 mM EDTA, 25% glycerol, 5mM DTT) and 6 μL of probe DNA (1 μg) were added, and the reaction incubated at room temperature for 15 min. DNA–protein complexes were collected with 10 μL Dynabeads M-280 Streptavidin magnetic beads (Cat# 11205D, Life Technologies, Carlsbad, CA). Bound protein was eluted from the DNA-Dynabeads complex, resolved by SDS-PAGE, and analyzed by immunoblotting using anti-CREB (Santa Cruz
Biotechnology, Dallas, TX, Cat# sc-186, dilution 1:1000). Antibody binding was detected using HRP-conjugated anti-rabbit with ECL (Cat# 934-1ML, Amersham, Little Chalfont, United Kingdom).

**Construction of full and truncated POMC expression plasmids and detection of products by ELISA**

Full length (+3799/+3940 and +6828/+7499), Del-A (+6929/+7499) and Del-B (+6996/+7499) POMC cDNA fragments were PCR amplified and cloned into HindIII and XhoI sites in pMF neo expression vector. One µg of plasmids was transfected into 5 x 10^5 COLO320 cells by lipofectamine 2000 (Invitrogen), cultured for 48 hours, and the cultured media was collected for ELISA assays. 96-well plates were coated with anti-ACTH antibody (1 ng/well, Abcam, Cambridge, United Kingdom, Cat# ab20358) overnight at 4°C and then blocked with 1% BSA/PBS for 1 h at room temperature. Fifty µL of cultured supernatant were added into two wells and incubated for 1 hour at room temperature. After washing with PBS, biotin-conjugate monoclonal anti-HA antibody (1 µg, Sigma-Aldrich, St. Louis, MO, Cat#B9183) was added and incubated for 1 h at room temperature. Antibody binding was detected with Streptavidin-HRP conjugate (Life Technologies, Cat# SNN1004), TMB-ELISA substrate (Thermo-Fisher Scientific, Waltham, MA, Cat#34028) and Stop Solution TMB (Thermo-Fisher Scientific, Cat# N600). Absorbance at 450 nm was measured by an ELISA plate reader, and a calibration curve used to calculate ACTH concentrations.

**DNA extraction and methylation-specific PCR assay**

Methylation-specific PCR assay for the POMC promoter was performed as described (27). Genomic DNA was purified from paraffin embedded tumor tissues and DNA bisulfite modification using the EpiTect Fast Bisulfite Conversion Kit from formalin fixed paraffin
embedded tissues (Qiagen, Hilden, Germany). Methylation specific primers for PCR analysis were designed using MethPrimer (http://www.urogene.org/methprimer/) and used for methylation-specific PCR analysis (28). PCR was performed using EpitScope MSP kit (Clontech Laboratories, Mountain View, CA), and amplified using a PCR program with 45 cycles at 95°C for 5 sec, 55°C for 30 sec, and 72°C for 1 min. Primers used for bisulfate DNA sequencing are as follows: human first promoter: (methylated-specific) 5’-TAGTTTTTAAATAGGGGAAATCG, 5’-CGAAAAATAAAAATTACCTACGTACGTA, (demethylated-specific) 5’-TAGTTTTTAAATAGGGGAAATTTG, 5’-CAAAAAATAAAATTACCTACACATA; and human second promoter: (methylated-specific) 5’-GTTAGGGGTTCTTTTTATGTTTTT, 5’-ATAACGTACTTCCGAAAATTCTCG, (demethylated specific) 5’-GTTAGGGGTTCTTTTTATGTTTTT, 5’-ATAACGTACTTCCGAAAATTCTCG, (demethylated specific) 5’-GTTAGGGGTTCTTTTTATGTTTTT, 5’-

Bisulfite sequencing

Bisulfite sequencing of the POMC promoter region was performed as described (29). Briefly, each bisulfite-converted DNA was amplified by PCR, PCR products were cloned into pCR2.1-TOPO (Invitrogen), and 6 clones from each tumor sample were sequenced. Primers used for bisulfite sequencing were: 5’-CGAGTTAGGAAGGTAGGGAT, 5’-TTAAATTTGTACATCCCTATACAAA; mouse second promoter: (methylated-specific) 5’-ACGAGTTTTTCATGAGTAATTC, 5’-TGGAGTTTTTCATGAGTAATTC, 5’-ACGAGTTTTTCATGAGTAATTC, 5’-TGGAGTTTTTCATGAGTAATTC, 5’-TGGAGTTTTTCATGAGTAATTC, 5’-TGGAGTTTTTCATGAGTAATTC, 5’-TGGAGTTTTTCATGAGTAATTC.
**Sequencing of USP8 hotspot mutations**

Genomic DNA was extracted from fresh samples using DNeasy Blood and Tissue Kit (Qiagen) or from FFPE samples using AllPrep DNA/RNA FFPE kit (Qiagen). The presence of USP8 hotspot mutations was determined by Sanger sequencing. The following primers were used for PCR amplification and sequencing: 5’-TCCAACTCATAAAGCCAAGCCACAGAT and 5’-TGGCTTGTTCCTCCGATTAACTGTTGG.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed using AtT20 cells as described (30). Cells were fixed in 1% formaldehyde, 4.5 mM HEPES (pH8.0), 9 mM NaCl, 0.09 mM EDTA, and 0.045 mM EGTA for 10 min at room temperature, and sonicated using a Bioruptor (Diagenode, Denville, NJ) in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl pH 8.0) with proteinase inhibitor (Sigma-Aldrich, Cat#P8340). Pre-cleared lysates were incubated overnight at 4°C with 2 µg of anti-STAT3 (Santa Cruz Biotechnology, Cat# sc-482); anti-STAT5 (Santa Cruz Biotechnology, Cat# sc-836); anti-CREB (Santa Cruz Biotechnology, Cat# sc-186); or normal rabbit IgG (Santa Cruz Biotechnology; sc-2027). DNA fragments were isolated from immunoprecipitated chromatin and analyzed by qPCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCR primers used were: mPomc 5’-GCCGAGACTCCCATGTT, 5’-GTGGCCCATGACGTACT.

**Statistics**

Differences in methylation status between groups in each parameter were analyzed using t-test with Welch’s correction. Probability of p<0.05 was considered as significant. Correlation of methylation status and plasma ACTH/cortisol levels was assessed by
Spearman’s correlation. Results of luciferase assays and ELISA were analyzed by student t-test, and p<0.05 is considered significant.

Results

**Identification of a second human POMC promoter in both corticotroph adenomas and ectopic ACTH-secreting tumors**

We analyzed transcription start sites of the POMC promoter in seven human pituitary tumor samples. We performed 5’-rapid amplification of cDNA ends (RACE) using RNA obtained from four ACTH-secreting pituitary tumors and three ectopic ACTH-secreting tumors resected from thymus, lung, and liver, as well as from two human cell lines that express POMC and ACTH (DMS79 [human small cell lung carcinoma] and COLO320 [human carcinoid-like colorectal carcinoma]) (Fig.1A and Table 1). When mapping positions of the 5’-ends of POMC transcripts to the human POMC gene, we found start sites clustered in three regions (Fig.1A,B). The first region at the 5’-end mapped just downstream of the previously characterized TATA-box in the POMC proximal promoter (region A, Fig.1B), with the most abundant 5’ ends located approximately 30 bp downstream of the TATA box sequence (Fig.1A,B). The second region (region B) mapped to the coding sequence in the POMC exon2 (Fig.1B). All of the 5’-ends in region B were located within the POMC mRNA sequence and were mainly identified using RNA isolated from tumor tissue; as 5’ends were rarely found in freshly isolated cell lines, they were likely derived from degraded RNA.

In the third region detected, region C, located near the junction of intron2 and exon3 (Fig.1B), 5’-ends were identified in RNA from tumor tissue as well as isolated from COLO320 cells; 34% mapped to intron2 (Fig.1B). As 5’-ends of partially degraded RNA would not contain sequences corresponding to intron2 (31), these results suggest that POMC
transcripts start from this region, while an additional POMC promoter is located upstream of these 5’-ends. Notably, unlike in region A, where 5’-ends of POMC mRNA were identified in all cell types tested, few 5’-ends mapped to region C in DMS79 cells, suggesting that POMC mRNA transcribed from region C may be cell-type specific.

**CREB regulates activity of the second promoter**

The 5’-ends of POMC mRNA in region C are located between +6331 and +7120 bp downstream of the previously identified POMC transcription start site (11) (Fig.1C). This region does not contain a characteristic TATA box and is GC rich. To test whether this sequence possesses promoter activity, we cloned DNA fragment +6177 to +7136 and its deletion mutants into a luciferase reporter plasmid and promoter activities were analyzed by luciferase reporter assays in COLO320 and DMS79 cells (Fig.2A). Promoter activities were detected in all three cloned fragments: +6177 to +7136, +6417 to +7136, and +6657 to +7136 (Fig.2A, fragments a-c). Strongest activity was detected in fragment c at +6657 to +7136 in both COLO320 and DMS79 cells, suggesting that negative regulatory elements may be present in +6177 to +6657, upstream of fragment c. As 3’ deletion constructs (Fig.2A, fragments d and e) exhibited reduced promoter activity in both cell lines, we concluded that the deleted 264 bp sequence likely contains critical regulatory promoter sequences. We therefore termed fragment c (+6657 to +7136) as the POMC second promoter. Analysis of transcription factor databases for this POMC second promoter identified potential consensus binding motifs for STAT (TTCCCGGGAAA, +6877 to +6887, Fig.1C) and CREB (GTACGTCA, +6924 to +6931, Fig.1C) transcription factors.

To assess the contribution of these transcription factors to activity of the second POMC promoter, we introduced point mutations in potential STAT (TTCCCGGGAAA to TTGAATTCAAA) and CREB (GTACGTCA to GGGTACCA) binding sequences in the
luciferase construct for fragment c (Fig.2B). Point mutations in CREB, but not STAT, binding motifs reduced promoter activity in both cell lines (Fig.2B). To further validate this observation, we performed an in vitro DNA pulldown assay using nuclear extracts prepared from COLO320 (Fig.2C). Immunoblotting with anti-CREB antibody showed that DNA probes with wild-type CREB sites, but not CREB site mutations, were bound by CREB protein (Fig.2C). Taken together, these results demonstrate that sequences between +6657 and +7136 (fragment c) may serve as a POMC promoter and transcription start site, regulated by CREB.

**Regulation of the second promoter is distinct from the first promoter**

We next compared activity of the second promoter with the first promoter located upstream of exon1 (-428 to +68 including region A) that we previously cloned (11). Both promoters were active in DMS79, COLO320, and mouse corticotroph AtT20 cell lines (Fig.2D). Activity of the two promoters was similar in DMS79, however the second promoter was more robust in COLO320 and AtT20 cells (Fig.2D), consistent with cell-type specificities.

Next, we examined species specificity of the second promoter. GC-rich and CREB and STAT binding sequences are conserved in the intron2/exon3 junction region in the mouse Pomc gene as well as other species (Fig.2E). We cloned this GC-rich fragment from the mouse Pomc gene (Fig.2F) into luciferase reporter plasmids, and activity was analyzed in mouse-derived AtT20 cells. We detected the mouse second promoter in this fragment and its activity was stronger than that of the first promoter in non-stimulated AtT20 cells (Fig.2G). As the first Pomc promoter is stimulated with corticotroph releasing hormone (CRH) and leukemia inhibitory factor (LIF) (18,32), we analyzed promoter activity after these treatments (Fig.2G). Activity of the first promoter was increased 24-fold with CRH/LIF stimulation,
consistent with known increased POMC expression in response to CRH (33). By contrast, the second mouse promoter activity was downregulated in response to these stimuli (Fig.2G), indicating distinct responses to hypothalamic/pituitary signaling.

ACTH is produced from POMC mRNA transcribed from the second promoter

To determine whether second promoter-dependent transcripts are translated to ACTH, we sought ATG sequences in-frame with the ACTH sequence in human POMC mRNA and identified four ATG (ATG1 to ATG4) sequences upstream of and within the ACTH coding sequence (Fig.3A). ATG1 is located nearest the 5’ end and is considered the putative translation start site for the full length POMC polypeptide. ATG2, ATG3, and ATG4 are located in exon3, within the previously defined region C; specifically, ATG2 and ATG3 are located upstream of the ACTH coding sequence, and ATG4 is within the ACTH sequence. As POMC is a complex precursor peptide and is cleaved into several small peptide hormones with different roles, we considered that translation might start from ATG2-4.

To test this possibility, we generated three POMC constructs with a HA-tag at the 3’ end of the ACTH coding sequence, cloned the sequences into the EF1α promoter-driven expression vector pMF (Fig.3A), and transfected the resulting constructs into COLO320 cells. Recombinant HA-tagged ACTH was detected in the culture supernatant of all transfectants (Fig.3A), indicating that second promoter-dependent POMC transcripts lead to production of secreted ACTH.

The second POMC promoter exhibits enhancer activity

As the second promoter sequence is located within intron2 and exon3, we considered whether it may function as an enhancer for the first promoter. We therefore constructed luciferase reporter assays by cloning the 596 bp human POMC first promoter sequence (-428
to +68) upstream of luciferase and the 480 bp human POMC second promoter sequence (+6657 to +7136) downstream of the luciferase gene in both sense and antisense directions (Fig.3B). Luciferase assays using DMS79, COLO320, and AtT20 cells showed that the second promoter fragment enhanced the first promoter activity approximately 2-fold in both directions (Fig.3B), suggesting that the second POMC promoter sequence also functions as an enhancer for human POMC gene expression.

**DNA methylation regulates activities of the first and second promoters**

As both promoters contain CpG-rich sequences, we assessed whether promoter activities may be regulated by DNA methylation. We constructed two luciferase reporter plasmids, with the first and second promoters cloned into the CpG sequence-free pCpGL luciferase reporter plasmid (26) (Fig.4A). To methylate CpG sequences in the inserted promoters, reporter plasmids were treated with methyltransferase (SssI) with or without substrate S-adenosylmethionine (SAM). We then examined promoter methylation levels by digestion of methylation-sensitive Hpa II and methylation-insensitive Msp I restriction enzymes, both of which recognize the 5’-CCGG sequence (Fig.4B). Plasmid DNA treated by SssI with SAM were digested by Msp I but not Hpa II, indicating high methylation levels. Luciferase reporter assays using these plasmids showed that activity of both promoters was strongly inhibited by DNA methylation in both DMS79 and AtT20 cells (Fig.4C), suggesting that activity of both promoters is regulated by demethylation and may be conserved in mouse and human.

Considering that varying POMC expression and ACTH levels in Cushing’s tumors may be reflective of promoter activities regulated by DNA methylation, we next analyzed DNA methylation levels of these promoter regions by a PCR-based method (28). We assessed three ACTH-secreting pituitary corticotroph tumors and two ectopic ACTH-
secreting tumors, and compared their respective methylation levels with the average methylation level of 11 normal pituitary specimens obtained from autopsy processed within 24 hours of death and used as controls (Table 2). As expected, given the variable 5’RACE results (Fig.1A), methylation levels of ACTH-secreted tumors varied widely (Fig.4D). The first and second promoters in two pituitary ACTH-secreting tumors (pituitary #1 and #2) as well as in one of the ectopic tumors (Ectopic #1) were highly demethylated. Interestingly, in these samples, 5’RACE detected substantial numbers of transcription starts in the second promoter (Fig.1A). Methylation levels of the second promoter in pituitary #3 was high (Fig.4D), and no transcripts were detected from the second promoter site (Fig.1A), suggesting that methylation levels correspond to transcriptional activities. By contrast, in Ectopic #2, the second promoter was more demethylated than the first promoter, and 3-fold more mRNA in this sample were transcribed from the second promoter. In summary, POMC gene expression levels in ACTH-secreting tumors seems to be regulated by DNA methylation in both first and second promoters.

DNA methylation regulates POMC production in ACTH-secreting tumors

We then analyzed methylation/demethylation levels in both first and secondary promoter regions in 11 normal pituitary autopsy specimens, 32 pituitary ACTH-secreting tumors, two ectopic ACTH-secreting tumors, and six SCAs (Table 2 and Table 3). In pituitary ACTH-secreting tumors, both the first and second promoters were significantly demethylated compared with normal pituitary tissue, and the degree of demethylation was greater in the second promoter (Fig.5A). By contrast, in SCAs, which do not produce ACTH, although the first promoter was significantly demethylated, the second promoter was highly methylated compared with normal pituitary (Fig.5B). Differences in methylation patterns between the first and second promoters were even more striking when comparing pituitary
ACTH-secreting tumors and non-secreting SCAs (Fig. 5C). As only two ectopic ACTH-secreting tumor samples were available for study, statistical analysis was not possible. However, visual inspection showed that the first promoter was highly demethylated in one specimen, while the second promoter was highly demethylated in both (Fig. 5D).

To assess methylation/demethylation positions in the second promoter, we performed bisulfite-conversion-based DNA sequencing of the CpG islands (+6689 to +6965) in four normal pituitaries, four ACTH-secreting pituitary tumors, one ectopic ACTH-secreting tumor, and two SCAs, all randomly selected from the full set (Table 2 and Table 3). The 5’-half of the CpG islands in normal pituitary specimens were highly methylated, and nearly the entire region was highly methylated in SCAs that express POMC but do not secrete ACTH (Fig. 5E). By contrast, pituitary ACTH-secreting tumors and ectopic ACTH-secreting tumor specimens that express POMC and actively secrete ACTH were highly demethylated (Fig. 5E). These results suggest that methylation status underlies regulation of POMC production from the second promoter in different POMC-expressing tissue types.

Demethylation of the second POMC promoter correlates with aggressive features of Cushing’s disease

We next investigated whether demethylation of the second POMC promoter is associated with tumor-specific and clinical features of ACTH-secreting pituitary corticotroph tumors (Table 3). The first promoter was markedly demethylated in USP8-mutated tumors (Fig. 6A), while the second promoter was demethylated in specimens derived from patients with more adverse phenotypic characteristics (34), including macroadenoma (Fig. 6B) and tumor recurrence (Fig. 6C), defined as biochemical evidence of hypercortisolism in patients who previously achieved resolution of hypercortisolism, as well as Crooke cell changes (Fig. 6D), all indicating an aggressive phenotype. Neither age nor sex was associated with
methylation status of either promoter (Fig. 6E, F). These results suggest that second promoter demethylation is associated with aggressive features of Cushing’s disease unrelated to USP8 mutation and EGFR signaling. We also observed that methylation levels of both first and second promoters correlated with serum ACTH (Fig. 7A, B), but not serum cortisol levels (Fig. 7C, D).

**LIF signaling may regulate CREB binding to the second promoter by activating STAT3/5**

Although we identified potential binding sequences in the second promoter for both STAT and CREB (Fig. 1C), luciferase reporter assays (Fig. 2B) and DNA binding assays (Fig. 2C) showed a regulatory role for CREB in the second promoter but did not reveal a contribution for STAT. We therefore performed in vivo chromatin-immunoprecipitation (ChIP) assay using AtT20 cells, which are highly methylated (Fig. 8A), and treated cells with LIF to induce potential STAT activation. Methylation-sensitive CREB binding to this region was not detected until 24 hours later, while binding of STAT3 and STAT5 was detected after 12 hours (Fig. 8B-D).

**Discussion**

Analyzing 5’ ends of POMC transcripts in pituitary and ectopic ACTH-secreting tumors, we identified an additional regulatory region located near the intron2/exon3 junction of the POMC gene. This region appears to function as a second POMC promoter with activity regulated by DNA methylation and as an enhancer of the first POMC promoter situated upstream of exon1.

DNA methylation regulates promoter activities, with hypermethylation leading to repression of gene expression and hypomethylation allowing for gene activation (35). Promoter methylation has been shown to be an epigenetic determinant of ectopic POMC
expression in normal and tumorous tissues (36), and POMC promoter demethylation was reported with ectopic ACTH secretion by a pheochromocytoma (37). However, we report here that methylation patterns in the first and second POMC promoters were strikingly different, suggesting different roles for methylation in POMC-expressing ACTH-secreting tumors depending on which promotor is activated.

We found the first promoter demethylated in 6 pituitary ACTH-secreting tumors that harbor gain-of-function USP8 mutations. USP8-mutated pituitary ACTH-secreting tumors show enhanced EGFR signaling (9,10), which we showed increases activity of the POMC first promoter (8). These findings suggest that the USP8-EGFR pathway regulates the first promoter and that methylation status of this promoter may contribute to driving the corticotroph adenoma phenotype. By contrast, the second promoter was demethylated in tumors harboring other markers of an aggressive phenotype (34), including size ≥10 mm, recurrent disease, and Crooke cell changes, suggesting that methylation status of the second POMC promoter is involved in driving inherent corticotroph tumor behavior.

Of note, differences in methylation status were also apparent when comparing ACTH-secreting pituitary ACTH-secreting tumors and non-secreting SCAs, suggesting that methylation of the second promoter may play a role in POMC gene silencing in SCAs. Several mechanisms have been proposed to explain the distinctive pathogenesis of SCAs versus pituitary ACTH-secreting tumors and how they might affect POMC activity (4), although promoter methylation has not been proposed. Phenotypic transition of SCA to active Cushing’s disease is uncommon (38,39), and the potential contribution of sequential hypomethylation of the second promoter to activate POMC expression and ACTH secretion requires study.

The second POMC promoter contains closely located binding sites for CREB and STAT, which are highly methylated in normal pituitary tissue. We show here that CREB
activates POMC transcription by the second promoter, but CREB binding occurs later, after STAT3/5 binding, suggesting step-wise binding in second promoter transcriptional regulation (Fig.9). We have shown step-wise regulation for the highly methylated Foxp3 enhancer (23), which also contains STAT and CREB binding sites in close proximity. In that setting, ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET2 bind with STAT5, inducing demethylation of the Foxp3 enhancer, which allows methylation-sensitive CREB to bind and activate transcription in T cells (40). Our results suggest the possibility of a similar mechanism for the second POMC promoter, with STAT3/5 cooperating with TET or other proteins to demethylate the second promoter for CREB-dependent promoter activation.

Of note, robust constitutive baseline activity of the second promoter was downregulated by CRH and LIF stimulation, while the opposite reaction was seen with the first promoter. POMC gene expression is associated with a range of hypothalamic-pituitary-adrenal (HPA) axis functions, including acute stress response, negative feedback response by glucocorticoid administration, and constitutive active expression maintaining circadian rhythms (2). Further study of second promoter activation could elucidate both physiologic and pathologic phenomena associated with Cushing’s disease.

We identified a second POMC promoter regulated by methylation status in ACTH-secreting pituitary tumors. Demethylation of this promoter is associated with characteristics of aggressive pituitary ACTH-secreting tumor phenotypes independent of USP8 mutations, and may be regulated by CREB and stimulated by STAT3/5 activation. Our findings open new avenues for elucidating subcellular regulation of the HPA axis and suggest the second POMC promoter as a locus for targeted therapies in patients with Cushing’s disease phenotypes.
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Data Availability

All data generated or analyzed during this study are included in this published article.
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Table 1. Characteristics of ACTH-secreting tumors used for 5’-RACE.

| Tumor       | Age (years), sex | ACTH (pg/mL) | 24 UFC (µg/d) | Maximum diameter (mm) |
|-------------|-----------------|--------------|---------------|-----------------------|
| Pituitary #1| 29 F            | 54           | 281           | 8                     |
| Pituitary #2| 73 M            | 296          | 59            | 28                    |
| Pituitary #3| 33 F            | 44           | 239           | 4                     |
| Pituitary #4| 16 F            | 139          | 661           | 37                    |
| Thymus #1   | 20 F            | 42           | 1178          | 13                    |
| Lung #2     | 23 F            | 145          | 1030          | 100                   |
| Liver #3    | 40 F            | 1100         | N/A           | 20                    |

ACTH, adrenocorticotrophic hormone; RACE, rapid amplification of cDNA ends; UFC, urinary free cortisol.

ACTH and 24 UFC are preoperative values. Tumor with ≥10 mm maximum diameter is defined as a macroadenoma, and <10 mm as a microadenoma.
Table 2. Characteristics of tissue samples used for methylation analyses.

| Sample number | Tissue type | Sex | Age (years) |
|---------------|-------------|-----|-------------|
| 1             | N           | M   | 45          |
| 2             | N           | M   | 52          |
| 3             | N           | F   | 74          |
| 4             | N           | F   | 68          |
| 5             | N           | F   | 78          |
| 6             | N           | M   | 54          |
| 7             | N           | F   | 82          |
| 8             | N           | F   | 77          |
| 9             | N           | M   | 67          |
| 10            | N           | M   | 73          |
| 11            | N           | M   | 65          |
| 12            | SCA         | F   | 58          |
| 13            | SCA         | F   | 42          |
| 14            | SCA         | M   | 58          |
| 15            | SCA         | M   | 70          |
| 16            | SCA         | M   | 63          |
| 17            | SCA         | F   | 35          |
| 18            | EC (lung)   | F   | 23          |
| 19            | EC (thymus) | F   | 20          |

EC, ectopic Cushing’s tumor; N, normal pituitary; SCA; silent corticotroph adenoma. Samples #1, 2, 3, 4, 12, 13 and 18 were randomly selected for use in bisulfite methylation analysis sequencing.
Table 3. Characteristics of ACTH-secreting pituitary corticotroph tumors.

|   | hPro1 (%) | hPro2 (%) | Micro/ Macro | Recur | Crooke | USP8 Mut | Sex | Age group | ACTH (pg/mL) | Serum cortisol (µg/dL) |
|---|-----------|-----------|--------------|-------|--------|----------|-----|-----------|--------------|------------------------|
| 1 | 17.9      | 2.7       | Macro        | N     | Y      | WT       | M   | ≥40       | N/A          | N/A                    |
| 2 | 19.1      | 17.8      | Macro        | Y     | Y      | WT       | F   | <40       | 19.1         | N/A                    |
| 3 | 44.0      | 67.3      | Micro        | Y     | N      | WT       | F   | <40       | 44.1         | N/A                    |
| 4 | 15.6      | 21.3      | Macro        | N     | Y      | WT       | M   | ≥40       | N/A          | N/A                    |
| 5 | 35.1      | 24.0      | Micro        | N     | N      | WT       | M   | <40       | N/A          | N/A                    |
| 6 | 9.4       | 16.7      | Micro        | Y     | N      | WT       | M   | <40       | 35.1         | N/A                    |
| 7 | 8.3       | 46.1      | Micro        | N     | N      | Mut      | F   | ≥40       | N/A          | N/A                    |
| 8 | 15.3      | 11.8      | Macro        | N     | Y      | WT       | F   | ≥40       | N/A          | N/A                    |
| 9 | 45.2      | 16.0      | Macro        | N     | N      | WT       | F   | <40       | N/A          | N/A                    |
|10 | 39.1      | 70.8      | Micro        | N     | N      | Mut      | F   | <40       | 17.4         | 107.7                  |
|11 | 43.7      | 17.2      | Macro        | Y     | N      | WT       | M   | <40       | 179.2        | 37.9                   |
|12 | 36.1      | 14.4      | Macro        | N     | Y      | WT       | F   | ≥40       | 96.0         | 16.5                   |
|13 | 34.2      | 69.9      | Macro        | N     | N      | WT       | M   | ≥40       | 388.0        | 10.3                   |
|14 | 12.9      | 4.8       | Macro        | N     | N      | Mut      | F   | <40       | 88.6         | 23.7                   |
|15 | 22.4      | 17.1      | Macro        | N     | Y      | Mut      | F   | ≥40       | 109.0        | 22.3                   |
|16 | 20.2      | 26.2      | Micro        | N     | N      | WT       | F   | ≥40       | 115.9        | 18.3                   |
|17 | 13.5      | 3.2       | Macro        | N     | N      | Mut      | F   | ≥40       | 136.9        | 45.9                   |
|18 | 11.4      | 66.5      | Macro        | N     | N      | Mut      | F   | <40       | 127.8        | 15.7                   |
|19 | 18.9      | 21.2      | Macro        | Y     | N      | WT       | F   | ≥40       | 91.7         | 17.4                   |
|20 | 62.0      | 2.6       | Micro        | N     | N      | WT       | M   | ≥40       | 140.9        | 17.3                   |
|21 | 44.4      | 22.0      | Micro        | N     | N      | WT       | M   | <40       | 86.2         | 16.3                   |
|22 | 38.7      | 4.6       | Micro        | Y     | Y      | WT       | F   | ≥40       | 69.8         | 19.9                   |
|23 | 41.4      | 37.2      | Macro        | Y     | N      | WT       | F   | <40       | 68.2         | 25.4                   |
|24 | 51.7      | 40.7      | Micro        | N     | N      | WT       | F   | ≥40       | 81.1         | 12.3                   |
|25 | 52.6      | 18.3      | Macro        | N     | N      | WT       | F   | ≥40       | 59.8         | 16.7                   |
|26 | 58.9      | 46.0      | Micro        | N     | N      | WT       | F   | ≥40       | 105.6        | 53.1                   |
|27 | 78.3      | 28.0      | Macro        | N     | N      | WT       | F   | ≥40       | 242.7        | 25.9                   |
|28 | 49.7      | 2.9       | Macro        | N     | Y      | WT       | F   | ≥40       | 72.1         | 10.0                   |
Crooke, Crooke’s cell changes; Macro, macroadenoma (defined as ≥10 mm); Micro, microadenoma (defined as <10 mm); Mut, mutated; Recur, recurrent disease.

ACTH and serum cortisol are preoperative levels. Samples #1, 6, 8 and 9 were randomly selected for use in bisulfite methylation analysis sequencing.
Figure Legends

Figure 1. hPOMC transcription start sites in pituitary and ectopic ACTH-secreting tumors and cell lines. (A) 5’-RACE analysis of human POMC mRNA isolated from human pituitary corticotroph adenomas (Cushing) and ectopic Cushing’s tumors (Ectopic Cushing) resected from thymus, lung, and liver (see Table 1), as well as ACTH-secreting DMS79 (three independent experiments, ex.1, ex.2, ex.3) and COLO320 cells (two independent experiments, ex.1, ex.2). The number of identified 5’-ends of POMC mRNA located in regions A, B, and C (depicted in B) are noted, with the number of major 5’-ends in region a located approximately 30 bp downstream of the TATA box sequence shown in parentheses. (B) Number of 5’-ends of POMC mRNA in pituitary ACTH-secreting (Cushing), ectopic ACTH-secreting tumors (Ectopic Cushing), and DMS79 and COLO320 cells in each region. Top, relative position of each region, with the major mRNA start site defined as position +1; relative positions of exon1, exon2, and exon3 are depicted in boxes. Left scale, number of 5’-ends located in region A; right scale, number of 5’-ends located in region B and region C. (C) DNA sequence in region C. Identified 5’-ends of POMC mRNA are shown in bold and flagged with an asterisk. The intron2 sequence is shown in lowercase letters and exon3 sequence in capital letters. Arrow indicates 5’-end of exon3. Potential STAT and CREB binding sequences are shown in italics and underlined. CpG sequences identified as potential methylation sites are underlined. The ACTH coding sequence is also underlined.
Figure 2. Activity of the second POMC promoter. (A) Left, structure of five luciferase (Luc) reporter plasmids (a-e). The inserted POMC gene fragments are depicted as boxes, and positions of the 5’- and 3’-ends are noted. Right, luciferase assays were performed using indicated cells (DMS79 and COLO320) and generated luciferase activities with indicated plasmids compared with negative control plasmid (no promoter; Basic). (B) Left, structure of luciferase reporter plasmids shown in A with mutated (mut) potential STAT, CREB, and STAT/CREB binding sequences (indicated by X) in fragment c in the second promoter (hPro2). Right, generated luciferase activities with indicated plasmids compared with negative control plasmid (no promoter; Basic) in DMS79 and COLO320 cells. (C) Biotinylated probes from DNA binding assay with wild-type (WT) and mutated (Mutant) CREB binding sites co-precipitated with proteins and analyzed by immunoblotting using anti-CREB. (D) Luciferase assays using reporter plasmids containing the first promoter (hPro1) (-428 to +68) and second promoter (hPro2) (+6657 to +7136) compared with negative control plasmid (no promoter; Basic) using DMS79, COLO320, and AtT20 cell lines. (E) Alignment of POMC second promoter STAT and CREB response elements by species. (F) DNA sequence in mPomc second promoter with the intron2 sequence shown in lowercase letters and exon3 sequence in capital letters. Potential STAT and CREB binding sequences are shown in italics and underlined, and CpG sequences identified as potential methylation sites are underlined. (G) Luciferase assays using reporter plasmids mouse first promoter (mPro1) (-459 to +80) and mouse second promoter (mPro2) (+44841 to +5209) compared with negative control plasmid (no promoter; Basic) with CRH and LIF stimulation or without stimulation in AtT20 cells.
Figure 3. ACTH production and enhancer activity of second promoter-dependent POMC mRNA transcripts. (A) Left, wild type (Full) and truncated (Del-A and Del-B) human POMC mRNA expression plasmids were constructed in the indicated cDNA encoding regions. Relative positions of four ATG sequences (ATG1-4) upstream of the ACTH coding sequence are shown. A HA-tag sequence (gray box) was inserted in the ACTH sequence (patterned box) in frame. Right, secretion of ACTH-HA tag fusion proteins analyzed by ELISA using anti-ACTH as a capture antibody and anti-HA as a detection antibody in COLO320 cells transfected with these plasmids and cultured for 48 hours. Three independent experiments (#1, #2 and #3) using each plasmid were performed. (B) Top left, structure of luciferase reporter plasmids with the 480 bp fragment (+6657 to +7136) inserted downstream of the hPOMC first promoter (hPro1; -428 to +68)/luciferase gene unit in both sense (S) and antisense (AS) directions in the second (hPro2) promoter. Top right and bottom, luciferase assays performed using the resulting plasmids (S and AS) and control plasmids with no enhancer fragment (-) and compared with no promoter/no enhancer fragment (Basic) in AtT20, DMS79, and COLO320 cells lines.

Figure 4. First and second POMC promoter activity with DNA methylation. (A) Structure of luciferase reporter plasmids using the CpG sequence free luciferase vector pCpGL and first (hPro1; -428 to +68) and second promoter (hPro2; +6657 to +7136) fragments. Relative position of the CpG island and potential STAT and CREB binding sites on these fragments are indicated. (B) First (hPro1) and second (hPro2) promoter reporter plasmids treated with methyltransferase with (+) or without (-) SAM, digested with no enzyme (-), Hpa II, or Msp I, and analyzed by agarose gel electrophoresis. (C) Luciferase assays using first (hPro1) and second (hPro2) promoter reporter plasmids treated with (+) and without (-) SAM, with generated luciferase
activities compared to negative control plasmid pCpGLbasic (Basic). (D) Methylation levels in first (hPro1) and second (hPro2) promoters analyzed by bisulfite-conversion based methylation-specific PCR using DNA isolated from three pituitary ACTH-secreting tumors (Pituitary # 1, # 2, and # 3; see Fig.1A) and two ectopic ACTH-secreting tumors (Thymus #1 and Lung #2; see Fig.1A), with normal pituitary obtained at autopsy (normal) as control. Tumor characteristics are shown in Table 1 and Table 2.

Figure 5. DNA methylation of first and second promoters. (A-D) DNA methylation in the POMC first (hPro1) and second (hPro2) promoters analyzed by bisulfite-conversion based methylation specific PCR using DNA isolated from 11 normal autopsy-derived pituitaries (Normal), 32 pituitary ACTH-secreting tumors (Cush), two ectopic ACTH-secreting tumors (EC), and six silent corticotroph adenomas (SCA). Results were compared between (A) normal and Cush, (B) normal and SCA, (C) Cush and SCA, and (D) normal and EC. *p=0.0007; **p<0.0001; ***p=0.008; (E) CpG methylation sites in the POMC second promoter were analyzed by bisulfate sequencing using DNA isolated from four normal pituitary (N1-4), four pituitary ACTH-secreting tumors (C1-4), one ectopic ACTH-secreting tumor (EC1), and two SCAs (SC1,2). Circles indicate potential methylation sites in the CpG island at +6689 to +6965. DNA sequence of the CpG island is shown in Fig.1C. Positions of STAT and CREB binding sequences are indicated. Open and closed circles indicate demethylated and methylated sites, respectively. Tumor characteristics are shown in Table 2 and Table 3.

Figure 6. DNA methylation in first and second promoters correlates with pituitary ACTH-secreting tumor characteristics. DNA methylation levels in first (hPro1) and second (hPro2) promoters were analyzed by bisulfite-conversion based methylation specific PCR based on (A)
USP8 mutation status (USP8 wild type [WT] (n=26) vs USP8 mutated [mut] (n=6), *p=0.0029); (B) tumor size (microadenoma [Micro <10 mm] (n=12) vs macroadenoma [Macro ≥10 mm] (n=20), *p=0.0383); (C) recurrent disease (non-recurrent [Not recur] (n=24) vs recurrent [Recur] (n=8), *p=0.007); (D) Crooke cell changes (absent [non-Crooke] (n=22) vs present [Crooke] (n=10), *p=0.0006); (E) sex (Male [n=10] vs Female [n=22]); and (F) age (<40 [n=12] vs ≥40 years [n=20]). Characteristics of all 32 ACTH-secreting pituitary tumors are given in Table 3.

Figure 7. DNA methylation of first and second promoters correlate with ACTH and cortisol levels. Correlations between preoperative serum ACTH levels and methylation status of (A) first (hPro1) and (B) second (hPro2) promoters, and between morning serum cortisol levels and methylation status of (C) first (hPro1) and (D) second (hPro2) promoters. ACTH and cortisol values are given in Table 3.

Figure 8. In vivo binding of STAT3, STAT5, and CREB to the second promoter in LIF-stimulated AtT20 cells. (A) DNA methylation levels in first (mPro1) and second (mPro2) promoters analyzed by bisulfite-conversion based methylation specific PCR procedure in AtT20 cells. (B-D) ChIP assay of AtT20 cells stimulated with LIF for the indicated times using (B) anti-CREB (CREB), (C) anti-STAT3 (STAT3), and (D) anti-STAT5 (STAT5), with control antibody (IgG). *p<0.05 vs control.

Figure 9. Cartoon of possible step-wise binding in transcriptional regulation of the second promoter. STAT3/5 binds and demethylates the second POMC promoter, which allows CREB to bind the promoter, activating transcription.
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9