Research Article

O-GlcNAcylation Is Essential for Rapid Pomc Expression and Cell Proliferation in Corticotropic Tumor Cells

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Abbreviations: ACTH, adrenocorticotropin; ALS, amyotrophic lateral sclerosis; CAMKII, Ca2+/calmodulin-dependent protein kinase II; cDNA, complementary DNA; CRH, corticotrophin-releasing hormone; Ctrl, Control; DMSO, Dimethyl sulfoxide; DRB, 5,6-dichlorobenzimidazole 1-beta-D-ribofuranoside; ERK, extracellularly regulated kinase; ETHcD-MS, electron-transfer/higherenergy collision dissociation–mass spectrometry; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PCR, polymerase chain reaction; PKA, protein kinase A; POMC, proopiomelanocortin; PRL, prolactin; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; Std, standard; TBS-T, Tris-buffered saline-Tween 20; WGA, wheat germ agglutinin.

Received: 8 April 2021; Editorial Decision: 19 August 2021; First Published Online: 21 August 2021; Corrected and Typeset: 30 September 2021.

Abstract

Pituitary adenomas have a staggering 16.7% lifetime prevalence and can be devastating in many patients because of profound endocrine and neurologic dysfunction. To date, no clear genomic or epigenomic markers correlate with their onset or severity. Herein, we investigate the impact of the O-GlcNAc posttranslational modification in their etiology. Found in more than 7000 human proteins to date, O-GlcNAcylation dynamically regulates proteins in critical signaling pathways, and its deregulation is involved in cancer progression and endocrine diseases such as diabetes. In this study, we demonstrated that O-GlcNAc enzymes were upregulated, particularly in aggressive adrenocorticotropin (ACTH)-secreting tumors, suggesting a role for O-GlcNAcylation in pituitary adenoma etiology. In addition to the demonstration that O-GlcNAcylation was essential for their proliferation, we showed that the endocrine function of pituitary adenoma is also dependent on O-GlcNAcylation. In corticotropic tumors, hypersecretion of the proopiomelanocortin (POMC)-derived hormone ACTH leads to Cushing disease, materialized by severe endocrine disruption and increased mortality. We demonstrated that POMC mRNA splicing and stability, O-GlcNAcylation contributes to this new mechanism of fast hormonal response in corticotropes. Thus, this study stresses the essential role of O-GlcNAcylation.
Approximately 16.7% of people develop a pituitary adenoma in their lifetime (1). Consequently, around 10,000 pituitary adenomas are diagnosed yearly in the United States alone, accounting for 15% of central nervous system tumors (2–4). The pathophysiology of pituitary adenomas is diverse. They are defined clinicopathologically as one of the following subtypes: adrenocorticotropic hormone (ACTH)-, growth hormone (GH)-, thyrotropin-, and gonadotropin (luteinizing hormone [LH]/follicle-stimulating hormone [FSH])-secreting, prolactin (PRL)-secreting, and nonsecreting. Secreting adenomas present subtype-specific clinical symptoms. For example, GH-secreting tumors may induce acromegaly, leading to physical impairment, body pain, a significantly reduced quality of life, and increased mortality (5, 6). On the other hand, ACTH-secreting adenomas cause Cushing disease, with complex and heterogeneous clinical presentations including facial plethora, moon face symptoms, reproductive and sexual disturbances, visceral obesity, hypertension, diabetes, and dyslipidemia, which particularly increases mortality rate in this subgroup of pituitary adenomas (7, 8). Finally, PRL-secreting adenomas are associated with amenorrhea, galactorrhea, sexual dysfunction, and infertility (9). The other subtypes of pituitary adenomas, including nonsecreting subtypes, may lead to hypopituitarism by compressing surrounding tissue, decreasing patient quality of life, and increasing the risk of cardiovascular events and premature death (6, 10). In addition, pituitary adenomas may also compress the optic chiasm, leading to visual defects (11) or invade neighboring dural structures in more than 50% of patients (12, 13). Therefore, even in the absence of hormonal disruption, these neurologic dysfunctions greatly affect patients’ quality of life and require medical care.

Medical treatment for functional pituitary tumors is an ever-growing field. Current medical management of acromegaly includes somatostatin receptor ligands (octreotide, octreotide acetate, lanreotide, and pasireotide) and GH receptor antagonists (pegvisomant) (14, 15). In addition, the dopamine agonist cabergoline is also used alone or in combination with somatostatin receptor ligands (14, 15). For ACTH-secreting tumors, 3 drug categories exist targeting the pituitary (pasireotide, cabergoline), the adrenal (ketoconazole, metyrapone, mitotane), or the glucocorticoid receptors (mifepristone) (8, 16). However, these treatment protocols require lifelong treatment and confer a significant financial burden because they often treat the symptoms rather than eliminate the tumors. So far, the lack of mechanistic insight limits the discovery of new treatment protocols (17), with surgery remaining the only treatment for most pituitary adenomas. However, resection is particularly challenging for small microadenomas with dramatic clinical sequelae, such as Cushing-associated adenomas, necessitating arduous resection, often associated with a higher morbidity rate (18). Therefore, increasing our molecular understanding of pituitary adenoma pathophysiology is essential to find better targeted treatments and improve clinical outcomes.

Despite their prevalence and significant associated morbidities, the tumorigenesis of pituitary adenomas remains poorly understood. Although a small subset of pituitary adenoma cases is familial (most often associated with MEN1 mutations), the vast majority are sporadic with no clear common etiology (9, 19, 20). In addition, the heterogeneity of pituitary adenomas, emerging from diverse endocrine cell types, is highly challenging and requires targeted studies to understand the specific molecular signaling pathways dysregulated in each tumor subtype. In this article, we focus on ACTH-secreting pituitary adenomas because of their potential for profound endocrine disruption and lack of medical therapies targeted at their underlying pathophysiology.

While pituitary adenomas have been studied for decades, much remains to be learned about their tumorigenesis, growth, and function. Whole-exome sequencing of pituitary adenomas has demonstrated a low number of somatic mutations not involved in their tumorigenic potential (20). Next-generation sequencing of the mitochondrial genome in pituitary adenomas has revealed many novel variants in these tumors, again not correlating with clinicopathological features (21). While DNA methylation and histone acetylation markers have been linked to invasiveness, the functional role of epigenetic changes in the endocrine and proliferative components of pituitary adenomas has not been studied further (22). Taken together, our understanding of tumorigenesis and hypersecretory function of pituitary adenomas is highly limited when viewed through a genetic lens, and nongenetic mechanisms must therefore be explored to pave the way for novel treatment protocols.
With this goal in mind, we questioned the importance of the O-GlcNAc posttranslational modification in pituitary tumor development. In short, this modification consists of the reversible addition of a single sugar residue (N-acetylglucosamine) onto serine or threonine of nuclear, cytoplasmic, and mitochondrial proteins (Fig. 1). Two essential enzymes regulate this cycling—O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA)—which reversibly add or remove O-GlcNAc residues, respectively. With more than 7000 O-GlcNAcylated human proteins described to date and counting (23), these posttranslationally modified proteins are involved in essential signaling such as transcription/translation, cell cycle, metabolism, cell survival, and protein degradation (23). Highly dependent on extracellular glucose concentrations, this nutrient-sensing modification adapts cell signaling in response to changes in nutrient availability (24). Finally, O-GlcNAcylation is dysregulated in many pathological conditions such as neurodegenerative and cardiovascular diseases and cancers (25-27).

Increased overall O-GlcNAcylation levels are consistently observed in cancer cells, including breast, lung, and colorectal (28, 29). Further, O-GlcNAcylation competes with phosphorylation sites on oncogenes and tumor suppressors to increase proliferation and promote invasion, making it an essential mediator in tumor formation and progression (28-31). Finally, ACTH-secreting pituitary adenomas increase glucose transporter cell surface expression in response to corticotrophin-releasing hormone (CRH) (32), increasing glucose uptake and presumably O-GlcNAcylation levels. Despite this, the involvement of O-GlcNAc cycling has never been investigated in pituitary tumors. We therefore hypothesize that O-GlcNAcylation contributes to pituitary tumor development. More precisely, we believe that O-GlcNAcylation affects multiple aspects of these tumors, including their hyperproliferative and hyperendocrine abilities.

In this study we used a combination of normal and tumorous human pituitary tissue and the AtT-20 murine pituitary adenoma cell line to study the role of O-GlcNAcylation in pituitary adenoma etiology. We demonstrate that the O-GlcNAc cycling enzymes OGT and OGA are significantly upregulated in ACTH-secreting tumors, correlating with their aggressiveness and ACTH secretion levels. Accordingly, O-GlcNAcylation is essential for pituitary adenoma cell proliferation, a common function for O-GlcNAcylation both in development and adult cells. This finding identifies O-GlcNAcylation as a potential therapeutic target in pituitary adenomas. As the predominant cause for clinical intervention, we next investigated pituitary adenoma hypersecretory ability. In AtT-20 ACTH-secreting pituitary adenoma-derived cells, the endocrine function is characterized by the expression and secretion of proopiomelanocortin (POMC)-derived hormones on CRH stimulation. For the first time, we demonstrated that CRH transiently stabilizes the pool of available Pomc messenger RNA (mRNA) to quickly raise Pomc levels, independently of Pomc promoter activation consequent to the CRH-downstream signaling pathway. Interestingly, this mechanism exists for many hormones, including thyrotropin, LH, and FSH mRNA (33-38), and has been suggested for POMC prehormone as well (39). Furthermore, O-GlcNAcylation is essential for this novel regulatory pathway because it enhances the proper splicing of Pomc mRNA and prevents its decay. Finally, we present a list of O-GlcNAcylated targets responsive to CRH stimulation and involved in mRNA processing. While these results are preliminary, we are hopeful that the expansion of this research in pituitary adenoma subtypes and more extensive clinical samples will highlight some of these O-GlcNAcylated proteins as druggable targets for corticotropic pituitary adenomas treatment.

**Materials and Methods**

**Normal Pituitary Gland and Pituitary Adenoma Collection**

A terminally ill 74-year-old male amyotrophic lateral sclerosis (ALS) patient was enrolled for postmortem brain/pituitary collection under an institutional review board–approved study. To date, ALS ontology is not correlated with the pituitary gland’s molecular alteration or O-GlcNAc defect (40), and no abnormality was noted on the pituitary at the autopsy. The pituitary gland was flash-frozen on collection and used as normal pituitary tissue. Patients with pituitary adenomas provided consent before surgery under an institutional review board–approved protocol. Pituitary adenomas were frozen and stored promptly following the

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**Figure 1. O-GlcNAcylation of proteins.** Two percent to 3% of glucose (Glc) entering cells is shuttled through the hexosamine biosynthesis pathway (HBP) to produce UDP-GlcNAc, the nucleotide donor for O-GlcNAcilation. O-GlcNAc transferase (OGT) adds the GlcNAc residue to serine or threonine (S/T) residues. O-GlcNAcase (OGA) removes the sugar residue.
resection. Patients were chosen based on the availability of a sufficient amount of banked tissues post pathology analysis to create a representative panel of the various pituitary adenoma subtypes. Mammosomatotropic tumors were characterized by GH+/PRL+ on histology, corticotrophic tumors by ACTH+ on histology, and nonsecreting tumors by negative hormone staining. Cushing disease was defined by signs and symptoms of cortisol excess and elevations of late-night salivary cortisol levels on 2 separate samples. Furthermore, if imaging was equivocal, inferior petrosal sinus sampling was performed to confirm pituitary etiology and provide guidance for laterality for surgical planning.

Acromegaly patients were defined by signs and symptoms of GH excess followed by laboratory values indicating elevations of insulin-like growth factor-1 levels and then confirmed with a GH suppression test in which a failure to suppress was indicative of acromegaly. Preoperative clinical diagnostic and pathological data for each participant is presented in Supplementary Table S1 (41). Two nonsecreting, 2 mammosomatotropic (1 with acromegaly at recurrence), and 4 corticotrophic tumors (3 Cushing disease and 1 hypopituitarism preoperative clinical diagnosis) were analyzed in this study.

Cell Culture

AtT-20 cells were maintained in Dulbecco’s modified Eagle’s medium containing either 4.5 g/L (Standard, Std) or 1 g/L (low) glucose, 10% (v/v) fetal bovine serum, and 1% penicillin/streptomycin at 37 °C in a 5% (v/v) CO2-enriched humidified atmosphere. Cells were cultivated in Std or low glucose media for at least 24 hours before the start of the experiments.

Drugs and Small Interfering RNA

Lyophilized CRH (Sigma) was reconstituted in phosphate-buffered saline with 10-mM acetic acid for stability and used at a final concentration of 28 µM. Thiamet G (100 nM final concentration) and 5S-GlcNAc (100 µM final concentration) were dissolved in sterile distilled water. 5,6-dichlorobenzimidazole 1-beta-D-ribofuranoside (DRB) (50 µM final concentration) and OSMI4 (5 µM final concentration) were resuspended in dimethyl sulfoxide (DMSO), and corresponding control (Ctrl) samples were treated with a similar amount of DMSO. Cells were treated with inhibitors for 16 hours unless otherwise specified.

Mission esiRNA targeting mouse Oga (Magea5) was purchased from Sigma (No. EMU020941). Silencer Negative Control small interfering (siRNA) was also purchased from Sigma (No. AM4611). One microgram of siRNA was transfected in one well of a 6-well using Lipofectamine RNAiMAX transfection reagent according to the manufacturer protocol (Thermo Fisher Scientific). Cells were treated with siRNA for 24 hours.

RNA Extraction, Complementary DNA Synthesis, Polymerase Chain Reaction (PCR), and Quantitative PCR

mRNA was isolated with the PureLink RNA Mini Kit (Thermo Fisher Scientific) following the kit instructions. RNA concentrations were measured using an LVis microplate (BMG Labtech). Complementary DNA (cDNA) was then synthesized with SuperScript IV VILO Master Mix with ezDNase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The resulting cDNA was diluted sequentially to a 1:100 dilution and used as templates for polymerase chain reaction (PCR).

PCR was performed using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and loaded on 2% agarose gels in Tris-acetate-EDTA buffer. Quantitative PCR (qPCR) reaction was then performed using PowerSYBR qPCR Master Mix (Thermo Fisher Scientific). Specific primers for each reaction are as follows: Pmc Ex-Ex-F: GGAGAGAAAGCCGAGTCAC AA; Pmc Ex-Ex-R: GGGACCCCGTCTTGTCCTATAA; Pmc Intron-F: TTGTTTCTGGAGTGGCCCCC; Pmc Intron-R: CTGACCTGCGGGGTTTTCA; Actin-F: AGA TCAAGATCATGTCCTCTCTC; Actin-R: AGCGAGGTC AGTAACAGTCC; Ogt-F: TCCGGGAATCACCCTACTTCA; Ogt-R: TACCATATCCGGGCTCTTA; Oga-F: CG GTGTCTGGAAGGGTTTTA; Oga-R: GTTGTCAGC TTCTCCACTG, qPCR was performed on a QuantStudio 3 instrument (Applied Biosystems) with the recommended settings. The data were collected and processed using DataConnect (Thermo Fisher Scientific), and 2^-ΔΔCq were calculated and plotted using Prism 9 (GraphPad Software).

Protein Lysis, Protein Gel Electrophoresis, and Western Blotting

Samples were lysed for 5 minutes in radioimmuno- precipitation assay lysis buffer (10-mM Tris-HCl, 150-mM NaCl, 1% Triton X-100 [v/v], 0.5% NaDOC [w/v], 0.1% sodium dodecyl sulfate [w/v], and protease inhibitors; pH 7.5), vortexed and centrifuged 10 minutes at 18 000g at 4 °C. Sample lysates were resolved on 8% Tris-glycine gels and transferred onto nitrocellulose. Membranes were washed with ultra-purified water and labeled with No-Stain Protein Labeling Reagent (Thermo Fisher Scientific) according to kit instructions. Next, the membranes were blocked for 45 minutes with 5% [w/v] nonfat milk in Tris-buffered saline-Tween 20 (0.1% [v/v]) buffer.
Primary antibodies were added to the blocking solution, and the blots were incubated overnight at 4 °C with gentle agitation. Following primary incubation, blots were washed 3 times with 10 mL of TBS-T for 10 minutes and incubated with antimouse and antirabbit fluorescent-conjugated secondary antibodies in a 1:20 000 dilution for 1 hour at room temperature. Three additional TBS-T washes with 10 mL in 10 minutes were performed, and the blot signal was captured using Odyssey Fc (LI-COR).

Antibodies

Antibodies for Western blotting were used as follows: anti-O-GlcNAc (No. ab2739-Abcam): 1:1000; Anti-actin (No. A2066, Sigma Aldrich), 1:1000; Anti-Phospho-ERK1(T202/Y204)/ERK2(T185/Y187) (No. AF1018, R&D Systems), 1:500; Anti-ERK1/ERK2 (No. AF1576, R&D Systems), 1:500; Anti-Phospho-p90RSK (S380) (No. 9341S, Cell Signaling), 1:500; Anti-Rsk1/rsk2/rsk3 (No. 9355T, Cell Signaling), 1:500; Anti-Phospho-Nurr77 (S351) (No. 5095S, Cell Signaling), 1:500; Anti-Nurr77 (No. NB100-56745, Novus Biologicals), 1:500; Anti-Phospho-CREB (S133) (No. 9198S, Cell Signaling), 1:500; Anti-CREB (No. 9104S, Cell Signaling), 1:500.

Luciferase Reporter Assay

AtT-20 cells were concurrently transfected using Lipofectamine 3000 (Thermo Fisher Scientific) with 2 plasmids—one containing the pRL Renilla luciferase gene under the control of the cytomegalovirus promoter (No. E2261, Promega) and the other containing the firefly luciferase gene under the control of the Pome promoter (~646 bp to +65 bp) (No. 17553, Addgene). Forty-eight hours after transfection, luciferase activity was measured using the dual-luciferase assay protocol (Promega). Transfection was performed 48 hours before measurement of the promoter activity.

Wheat Germ Agglutinin Pull-down

O-GlcNAcylated Proteins were pulled down using the Triticum Vulgaris Lectin (WGA) - MagneZoom Kit (BioWorld) according to the manufacturer’s protocol. Magnetic beads with bound proteins were washed with 200 µL of 166 mM ammonium bicarbonate. The buffer was removed using a magnetic rack, and the beads were suspended in 100 µL of 40% Invitrosol (Invitrogen) and 100 mM ammonium bicarbonate. Proteins were reduced with 5-mM TCEP for 30 minutes at 37 °C and alkylated with 10 mM iodoacetamide for 30 minutes at 37 °C in darkness. A total of 2 µg of tryspin/LysC mixture (Thermo Scientific Pierce) were added, and the digest was allowed to proceed overnight at 37 °C. After digestion, the supernatant was removed using a magnetic rack, acidified with trifluoroacetic acid, and desalted according to the manufacturer’s directions with the PreOms Phoenix kit.

Mass Spectrometry

Each sample was analyzed on a Thermo Scientific Orbitrap Fusion Lumos MS via 2 technical replicate injections in 2 methods (90 or 180 minutes). Further details regarding these 2 methods can be found in the supplementary methods (41).

Pathway Enrichment

Pathway enrichment was performed using the Reactome analysis tool (https://reactome.org/PathwayBrowser/#TOOL=AT) (42).

Proliferation Assay

Proliferation assays were performed using the Cell Proliferation Kit I (MTT) (Sigma Aldrich) according to the manufacturer’s protocol. Briefly, cells were seeded at 5 x 10³ cells well in a 96-well plate. Each condition was performed in 6 replicates, and measurements were averaged. A total of 10 µL of the MTT reagent were added to each well for 4 hours. Then, 100 µL of the solubilization solution was added overnight before measuring absorbance at 600 nm.

Tunel Assay

Apoptotic flux was measured using the EZClick TUNEL—in situ DNA Fragmentation/Apoptosis Assay Kit (BioVision) according to the manufacturer’s protocol. Positive Ctrl cells were treated with DNase according to the kit’s instructions.

Results

Clinical Corticotrophic Tumor Aggressiveness Correlates With Overexpression of O-GlcNAc Enzymes

In all literature published on the topic, O-GlcNAc cycling is increased in tumors and contributes to cell-cycle regulation (28, 43). Therefore, we investigated whether pituitary adenomas also overexpressed the O-GlcNAc cycling enzymes OGT and OGA. A pituitary gland from a recently deceased ALS patient was used as a control (Ctrl) nontumorous pituitary tissue. For
each pituitary adenoma subtypes, OGT and OGA expression levels were measured by qPCR and normalized by ACTIN (Fig. 2A and 2B).

Nonsecreting pituitary tumors showed a slight but significant upregulation of OGT (n = 2), and OGA (n = 1) compared to the normal pituitary gland (Fig. 2A and 2B). On the other hand, none of the mammosomatotrophic tumors showed deregulations of OGT or OGA (Fig. 2A and 2B). All the corticotropic pituitary adenomas (n = 4) exhibited a significant increase in OGT and OGA expression vs Ctrl pituitary (Fig. 2A and 2B). Interestingly, one tumor (ID No. 599) showed a 5-fold increase in OGT and OGA expression relative to Ctrl pituitary tissue and was clinically particularly aggressive, characterized by very high plasma ACTH and cortisol, large tumor diameter at presentation, and invasion into the clivus (Supplementary Table S1) (41). Among all tumor subtypes, plasma ACTH positively correlated with OGT and OGA expression (respectively, $R^2 = 0.81, P = .008$ and $R^2 = 0.88, P = .002$, Pearson correlation), suggesting that O-GlcNAcylation may be involved in the hormonal function of pituitary adenomas, especially as it pertains to ACTH secretion. In contrast, no correlation was found between OGT and OGA expression and tumor size (respectively, $R^2 = 0.38, P = .17$ and $R^2 = 0.27, P = .26$, Pearson correlation) (Supplementary Fig. S1) (41). Thus, we decided to focus our study on understanding the impact of O-GlcNAcylation on ACTH-secreting adenomas.

**O-GlcNAcylation Is Essential for AtT-20 Cell Proliferation**

We first wondered if O-GlcNAcylation participates in the proliferation signaling in corticotropic pituitary adenomas as shown previously in other tumor environments...
The murine AtT-20 cells were selected for this study because they possess both proliferative and endocrine abilities reminiscent of human ACTH-secreting adenomas.

Cell proliferation was measured daily by the MTT proliferation assay. Measurement of cell proliferation was measured daily by spectrophotometry in 6 replicates per condition (Fig. 3A). Because O-GlcNAcylation is a nutrient-sensing modification highly dependent on glucose level (24), AtT-20 were cultivated both in standard (Std Glc, 4.5 g/L) or low (low Glc, 1 g/L) glucose media leading to high or low basal O-GlcNAcylation levels (Fig. 3B). As expected, switching to low Glc media lowered the overall O-GlcNAcylation in AtT-20 cells compared to Std media (Fig. 3B). Additionally, cells were treated daily with O-GlcNAc transferase inhibitor 5S-GlcNAc or O-GlcNAcase inhibitor Thiamet-G to decrease or increase O-GlcNAcylation levels (Fig. 3A). The efficiency of both inhibitors was confirmed by Western blot (Supplementary Fig. S2A and S2B) (41). In Std and low Glc media, 5S-GlcNAc treatment decreased cell proliferation in AtT-20 cells over the course of 3 days (Fig. 3A). It was not due to increased apoptosis as measured by Tunel assay (Supplementary Fig. S3) (41). While the effect was moderate compared to serum-free media, cell proliferation was halved compared to the Ctrl condition (Fig. 3A). Likely because their proliferative abilities are already elevated, AtT-20’s treatment with Thiamet-G did not increase proliferation and was comparable to Ctrl levels in both media conditions (Fig. 3A). Overall, this emphasized the essential role of O-GlcNAcylation in pituitary adenoma AtT-20 cells and their potential as a druggable target to slow the expansion of ACTH-pituitary adenomas.

While extensive proliferation and expansion of pituitary adenomas is deleterious when invading neighboring structures, the first clinical signs of these tumors are often hormonal. Therefore, we next investigated the role of O-GlcNAcylation on the endocrine function of corticotrope pituitary adenoma cells.

**Corticotrophin-Releasing Hormone Stimulation Increases O-GlcNAcylation Levels**

In AtT-20 cells—and normal corticotropes—CRH stimulation induces prohormone Pomp mRNA and protein, which then gets converted to ACTH.

Thus, we first incubated AtT-20 cells with CRH for 4 hours to measure to levels of total O-GlcNAcylated protein and the expression of both O-GlcNAc enzymes, Ogt and Oga (Fig. 3B). Interestingly, CRH treatment increased overall O-GlcNAcylation of proteins as observed by western blot both in Std and low Glc media (Fig. 3B).
Conjointly, a slight increase in Ogt expression was observed by qPCR in low Glc media accompanying the increase in total O-GlcNAcylated proteins (Supplementary Fig. S4A) (41). No changes in Oga were noted (Supplementary Fig. S4B) (41).

This suggested that CRH promoted a quick rise in O-GlcNAcylation, likely on signaling molecules, but did not dramatically alter O-GlcNAc enzyme expression. Thus, we next examined the impact of O-GlcNAcylation on CRH-downstream signaling.

O-GlcNAcylation Stimulates Corticotrophin-Releasing Hormone Downstream Signaling

CRH stimulates downstream signaling in corticotropes, including the mitogen-activated protein kinase (MAPK) signaling cascade, ultimately resulting in Pomc transcription (Fig. 4A).

In both Std and low Glc media, AtT-20 cells were treated with CRH for 4 hours. Analysis of the MAPK effectors and their activated/phosphorylated forms was performed by Western blot. In addition, AtT-20 cells in Std glucose media were also pretreated with 5S-GlcNAc to prevent O-GlcNAcylation (Supplementary Fig. S2A) (41). In low Glc/O-GlcNAcylation conditions, AtT-20 cells were also pretreated with Thiamet-G to increase total O-GlcNAcylation, independently of glucose availability (Supplementary Fig. S2B) (41). In Std Glc media, activation of the MAPK signaling pathway following CRH treatment has been shown to happen within 30 minutes (46, 47) (Supplementary Fig. S5) (41). Therefore, MAPK effector phosphorylation was minimal at the 4-hour time point in CRH-stimulated vs Ctrl cells (Fig. 4B). However, CRH stimulation of the MAPK signaling pathway was still visible in the low Glc condition at 4 hours post-CRH, including increased phospho-RSK and phospho-NURR77, representing slower signaling (Fig. 4B). However, both in unstimulated and CRH-treated AtT-20 cells, phospho-RSK and phospho-NURR77 were dramatically reduced when pretreated with the O-GlcNAc inhibitor 5S-GlcNAc (Fig. 4B). It emphasized a role for O-GlcNAcylation in regulating basal and stimulated MAPK signaling in corticotropic pituitary adenomas. Finally, increasing O-GlcNAcylation levels with Thiamet-G in low Glc media increased basal and CRH-stimulated phospho-RSK and phospho-NURR77 (Fig. 4B), confirming the critical role of O-GlcNAc in MAPK basal activation.

We next investigated whether alterations in MAPK signaling dependent on O-GlcNAcylation levels also affect Pomc mRNA, the ultimate product of the CRH signaling pathway.

Total Proopiomelanocortin Messenger RNA Availability Is Dependent on O-GlcNAcylation Level

At 4 hours post-CRH stimulation, Pomc expression was measured by reverse transcriptase–qPCR and normalized by Actin (Fig. 5A and 5B). In Std media, CRH induced Pomc expression (Fig. 5A). This was prevented by
5S-GlcNAc–mediated inhibition of O-GlcNAcylation, as well as by another OGT inhibitor, OSMI4 (Fig. 5A and Supplementary Fig. S2A and S2C) (41). Furthermore, low Glc/O-GlcNAc conditions suppressed the ability of CRH to induce Pomc expression (Fig. 5B). However, this aptitude was recovered by supplementing media with Thiamet-G or by transfection of siRNA against Oga and boosting proteins’ O-GlcNAcylation (Fig. 5B, Supplementary Figs. S2B, S2D, and S6) (41).

This experiment demonstrated that sufficient Glc level, and subsequent O-GlcNAcylation, are critical to promoting Pomc expression on CRH stimulation, which we presumed was due to enhanced Pomc Promoter activity.

O-GlcNAcylation Has a Limited Effect on Proopiomelanocortin Promoter Activity

A Pomc luciferase reporter assay was used to measure the Pomc promoter’s activation. Cells were cotransfected with 2 constructs: PGL3-Pomc-Luc, expressing the firefly luciferase under the control of the Pomc promoter, and pRL, expressing the Renilla luciferase under a general mammalian promoter. Std and low Glc conditions and O-GlcNAc inhibitors were used as previously described, in addition to CRH stimulation. Firefly luciferase activity was measured by luminescence and reported on Renilla used as the internal control for transfection efficiency (Supplementary Fig. S7).

Agreeing with total Pomc mRNA levels, CRH induced Pomc promoter activity in Std but not low Glc condition, demonstrated respectively by an increase or no increase in firefly luciferase activity (Fig. 5C and 5D). However, 5S-GlcNAc only mildly suppressed this stimulation (Fig. 5C). Simultaneously, Thiamet-G only slightly increased the basal promoter activation but did not rescue low Glc levels (Fig. 5D). This is in divergence with the effect of Thiamet-G on total Pomc mRNA levels. However, O-GlcNAcylation seemed to slightly increase the unstimulated basal level of Pomc promoter activation (Fig. 5D).
Overall, this suggested that glucose but not O-GlcNAcylation was critical for CRH-dependent Pomc promoter stimulation. However, because this experiment did not recapitulate the finding that O-GlcNAcylation is essential to increase Pomc expression upon CRH stimulation, we next searched for alternative pathways stimulated by CRH that would affect Pomc mRNA in an O-GlcNAc-dependent fashion.

O-GlcNAc-dependent Messenger RNA Processing Pathways Are Affected by Corticotrophin-Releasing Hormone Treatment

O-GlcNAcylated proteins from AtT-20 cells treated or untreated with CRH for 1 hour were pulled down using the wheat germ agglutinin (WGA) lectin. WGA-bound proteins were then analyzed by electron-transfer/higher-energy collision dissociation–mass spectrometry (EThcD-MS) to detect O-GlcNAcylated residues and quantify the relative changes in O-GlcNAcylated proteins on CRH treatment (Fig. 6A and 6B; Supplementary Tables S2 and S3) (41). A total of 1051 and 986 proteins were identified and mapped to the mouse proteome in control and CRH-treated AtT-20 cells, respectively. Among these, 399 O-GlcNAc sites were identified on 321 proteins (Supplementary Tables S2 and S3) (41). More than half of the proteins identified have already been identified in previous O-GlcNAcomics experiments (48), confirming the validity of our experiments (Supplementary Fig. S8 and Supplementary Table S4) (41). Measurement of the peak area for relative quantification highlighted 52 proteins differentially pulled down by WGA in control and CRH-treated AtT-20 cells (Fig. 6A and 6B; Supplementary Table S5) (41). Significantly deregulated proteins were inputted into the analysis tool of the Reactome platform (42) for enrichment analysis (Fig. 6C and Supplementary Tables S6 and S7) (41). mRNA processing pathways, including “processing of intron-containing pre-mRNA” and “mRNA splicing,” “transcription,” and “DNA repair,” were enriched in CRH-treated cells (Fig. 6C and Supplementary Table S6) (41). Depleted in CRH-treated cells (vs control) were protein-processing pathways like “translation” and “non-sense mediated decay (NMD),” a central pathway for mRNA degradation (Fig. 6C and Supplementary Table S7) (41).

This analysis demonstrated that O-GlcNAcylations of pre-mRNA splicing, and mRNA stability factors, are modulated by CRH, identifying these pathways as potential mechanisms for the regulation of Pomc expression.

Proopiomelanocortin Messenger RNA Stability Is Regulated by O-GlcNAcylation

To investigate whether O-GlcNAcylation participates in Pomc mRNA stabilization, Pomc expression was measured in AtT-20 cells treated with the mRNA synthesis DRB. An 8-hour time course measuring Pomc expression normalized by Actin by qPCR confirmed the rapid decay of Pomc mRNA in less than 2 hours (Supplementary Fig. S9A) (41). While the basal level of Pomc expression was reduced by 2-fold in low Glc levels, DRB was still effective in preventing the neosynthesis of Pomc mRNA (Supplementary Fig. S9B) (41), confirming the compatibility with our previous experimental design. Concurrent treatment of DRB with CRH prevented Pomc mRNA decay in Std and low Glc levels, showing, for the first time, that CRH rapidly stabilizes Pomc mRNA (Fig. 7A).

Figure 6. Corticotrophin-releasing hormone (CRH) induces O-GlcNAc-dependent changes in messenger RNA (mRNA) processing. A, Fold change of O-GlcNAcylated protein pulled down with wheat germ agglutinin (WGA) following CRH stimulation (1 hour) in AtT-20 cells. B, Magnified view of A between –log10(p-value) of 1 to 2. C, Pathway enrichment analysis for O-GlcNAcylated proteins enriched or depleted in CRH-treated AtT-20 cells.
However, the effect was more permanent in Std Glc conditions and persisted 2 hours after DRB treatment (Fig. 7A). Thus, per the O-GlcNAcomic analysis, we demonstrated that, while Pomc mRNA is subject to rapid decay, CRH stimulation prevented Pomc mRNA degradation. We next investigated whether O-GlcNAcylation affects this process by concurrently treating AtT-20 cells with OSMI4 or Thiamet-G to decrease and increase O-GlcNAcylation levels (Supplementary Figs. S2B and S2E) (41). Following overnight OSMI4 treatment, Pomc mRNA decay was increased regardless of CRH treatment (Supplementary Fig. S2B and S2C) (41). A similar response was observed following 5S-GlcNAc treatment, another OGT inhibitor (Supplementary Fig. S9C) (41). The fact that OSMI4 also affected Pomc mRNA decay in Ctrl conditions suggested that overnight treatment with OSMI4 or 5S-GlcnAC likely downregulated the expression of critical mRNA regulatory protein, as previously published (49-52). Therefore, we repeated the experiment by adding OSMI4 only 1 hour before DRB treatment, thus affecting only the ability to cycle O-GlcNAcylation onto existing protein (Supplementary Fig. S9D and S9E) (41). While OSMI4 did not affect Pomc decay in Ctrl conditions, OSMI4 prevented the stabilization of Pomc mRNA in CRH-treated cells (Supplementary Fig. S9E) (41), suggesting that rapid O-GlcNAc cycling is regulating Pomc mRNA turnover on CRH stimulation. Inversely, in low Glc, cotreating cells with Thiamet-G and CRH stabilized Pomc mRNA compared to CRH alone (Fig. 7D and 7E), suggesting that O-GlcNAcylation is required for CRH-induced Pomc mRNA stabilization.

Overall, this experiment stressed the essential contribution of O-GlcNAcylation in CRH-mediated Pomc expression, specifically by promoting the stabilization of Pomc mRNA. Interestingly, improper splicing of mRNA is one of the mechanisms leading to mRNA decay and thus affecting mRNA’s half-life. mRNA splicing was also highlighted as enriched in CRH-stimulated cells as presented earlier (Fig. 6C and Supplementary Table S6) (41). Thus, we next investigated the possibility that O-GlcNAcylation and CRH stabilize Pomc mRNA by promoting proper splicing.

### Intron-containing Proopiomelanocortin Messenger RNA Levels Are Dependent on O-GlcNAcylation

Because the O-GlcNAcomic analysis showed that “processing of intron-containing pre-mRNA” was the most enriched O-GlcNAc-dependent pathway on CRH stimulation (Fig. 6C and Supplementary Table S6) (41), we investigated the impact of O-GlcNAcylation on intron-containing Pomc RNA. A specific set of primers spanning the last intron into the last exon was used to measure the level of intron-containing Pomc mRNA by PCR (Fig. 8A). Inhibition of OGT by OSMI4 led to increased intron-containing Pomc mRNA both in Ctrl and CRH-treated cells. OSMI4 also led to the appearance of shorter PCR products, likely...
corresponding to Pomc mRNA species with partially retained introns (Fig. 8B). In low-Glc conditions, increasing quantities of partially retained intron species of Pomc mRNA were noted compared to Std-Glc conditions. This correlated with the limited ability of CRH to induce Pomc mRNA stabilization in low Glc levels (Fig. 8B). On the other hand, when treated with Thiamet-G, intron-containing Pomc mRNA species were dramatically reduced, potentially because of enhanced proficiency in splicing (Fig. 8B).

Taken together, we showed that O-GlcNAcylation regulates Pomc mRNA intron splicing, hypothetically by facilitating proper mRNA splicing, contributed to Pomc stabilization by CRH.

Bioinformatic Analysis Reveals O-GlcNAcylated Targets Involved in Regulation of Messenger RNA Processing

To identify O-GlcNAcylated proteins responsive to CRH and potentially responsible for regulating Pomc mRNA, we explored O-GlcNAcylated proteins identified previously by WGA pull-down and mass spectrometry (Fig. 6A and 6B and Supplementary Tables S2 and S3) (41). In AtT-20 cells, 321 O-GlcNAcylated proteins and 399 O-GlcNAcylation sites were identified (Supplementary Table S4) (41). For those, we extracted information on functional domains and functions from UniprotKB and linked databases. Thus, 24 O-GlcNAcylated proteins were determined to be involved in mRNA binding and/or processing and are excellent targets for CRH-mediated regulation of Pomc mRNA. Additionally, 180 and 81 proteins carried phosphoserine and phosphothreonine sites, respectively (Fig. 9A and Supplementary Table S8) (41), and might be the subject of O-GlcNAcylation/ phosphorylation interplay. Therefore, we provide a condensed list of 24 O-GlcNAcylated proteins in pituitary adenomas cells that are likely involved in Pomc mRNA regulation on CRH stimulation (Fig. 9A).

Discussion

Clinical management of Cushing disease, defined as a hypercortisolism due to a corticotropic pituitary adenoma, is mainly dedicated to restoring normal cortisol secretion rather than eliminating the tumor. It is achieved efficiently through glucocorticoid receptor blockade (mifepristone) and steroidogenesis inhibition (ketoconazole, metyrapone, mitotane) (8). However, these therapeutic options often do not achieve complete restorations of normal cortisol levels, leading scientists to search for better, more targeted treatment options (53). To reach this goal, it seems imperative to better understand the unique cell biology of corticotropic pituitary adenomas and further our understanding of these tumors to guide drug discovery.

Pituitary adenomas have been studied through many different lenses, including broad studies such as epigenomics, mitochondrial genomics, and gene expression microarrays. These screening studies have highlighted many dysregulated pathways in various pituitary adenoma subtypes, including those involved in transforming growth factor-β signaling, amino and nucleotide sugar metabolism, and O-glycan biosynthesis (54). In addition, aberrations in the nutrient-sensitive O-GlcNAc posttranslational protein modification have been implicated in a vast array of tumor types (29). While this posttranslational protein modification competes for phosphorylation sites, O-GlcNAcylation is regulated by only 2 enzymes rather than various kinases and phosphatases. OGT and OGA are responsible for cycling O-GlcNAc moieties at serine/threonine residues, thereby modulating countless biochemical processes from transcription to RNA processing to cell-cycle regulation (23). The extensive involvement of O-GlcNAc cycling in tumor biochemistry and the existence of numerous enzyme inhibitors targeting O-GlcNAc cycling make it a compelling target for therapeutics. In pituitary corticotropes, OGT has been shown to be involved in glucocorticoid receptor-mediated transcripational repression (55). Further, glucose uptake appears to be upregulated at the cell membrane in response to CRH, which would provide an additional precursor for O-GlcNAc (32). It is therefore essential to understand the regulation of the O-GlcNAc cycling enzymes OGT and OGA in the context of pituitary adenomas.
Our study demonstrates that OGT and OGA are preferentially upregulated in corticotrophic pituitary adenomas (and, to a lesser extent, nonsecreting pituitary adenomas) (Fig. 2). Furthermore, we demonstrate that the degree of upregulation of OGT and OGA correlates well with the degree of hypersecretion of ACTH observed clinically. This indicates a potential role for O-GlcNAcylation in the aberrant regulation of ACTH secretion in pituitary adenomas.
Furthermore, we show a specific case of a particularly young patient with an aggressive, invasive corticotrope pituitary adenoma and marked associated hypercortisolism. We therefore speculate that O-GlcNAc enzymes may be a useful pathological biomarker for identifying invasive potential in pituitary adenomas and certainly seem to be a reliable marker of profound endocrine disruption.

Corticotrophic Pituitary Adenoma Proliferation Is O-GlcNAcylation Dependent

In numerous cancers, O-GlcNAcylation and/or O-GlcNAc enzymes are dysregulated (28, 43). Unsurprisingly then, many oncogenes and tumor suppressors are O-GlcNAcylated, including Ki-67 (56), β-catenin (57), E-cadherin (58), and p53 (59), all of which are markers of pituitary adenoma invasiveness (60). For some of these proteins, O-GlcNAcylation’s role has been well defined. For example, the O-GlcNAcylation of β-catenin stabilizes the protein and drives its transcriptional activity, resulting in increased proliferation (57, 61). Similarly, p53 O-GlcNAcylation affects its degradation in cancer cells (59, 62). Therefore, it is not surprising that preventing O-GlcNAcylation affects a network of O-GlcNAcylated oncogenes, ultimately resulting in suppressed pituitary adenoma proliferation in vitro (Fig. 3A). Consequently, we are suggesting that O-GlcNAcylation might be both a diagnostic tool and a druggable target to reduce pituitary adenoma progression (63). After establishing the importance of O-GlcNAcylation in regulating corticotrophic adenoma proliferation, we addressed the similarly important issue of hormone expression regulation in these cells.

O-GlcNAc Cycling Participates in Corticotrophin-Releasing Hormone–dependent Signaling Pathway

In stressful situations, circadian stimulation, and under other conditions, the hypothalamus releases CRH into the hypophysial portal circulation, which carries it to the target pituitary corticotropes, inducing them to synthesize, and secrete ACTH. To do so, the binding of CRH to its receptor triggers a signaling cascade, including both protein kinase A (PKA) and Ca\textsuperscript{2+}/Calmodulin-dependent protein kinase II (CAMKII)-dependent phosphorylation cascade leading to neosynthesis of Pomc mRNA and protein (64, 65) (Fig. 4A). Interestingly, stimulating pituitary adenoma cells with CRH leads to an overall increase in O-GlcNAcylated protein, demonstrating that O-GlcNAcylation is responsive to CRH stimulation (see Fig. 3B).

A relationship between O-GlcNAcylation and intracellular signaling was described shortly after its discovery. Owing to its interplay with serine and threonine phosphorylation, O-GlcNAc often counteracts phosphorylation-driven signaling, including interactions with insulin and various MAPK signaling pathways (66-70). For example, OGT attenuates insulin signaling by O-GlcNAcylation of proteins involved in numerous steps in the PI3 kinase signaling pathway (66). In addition, O-GlcNAcylation also affects p38 MAPK pathways by modifying JNK (c-Jun N-terminal kinase) and ASK1 (apoptosis signal-regulating kinase 1) (67). Finally, in gastric cancer, elevated O-GlcNAcylation promotes extracellularly regulated kinase (ERK) 1/2 signaling and cell proliferation (68), linking O-GlcNAcylation to extracellular signal-dependent MAPK/ERK signaling, which is central to the CRH responsiveness of corticotropes.

Of the proteins involved in CRH signal transduction, many are O-GlcNAcylated (23), including PKA (71), CAMKII (72), B-RAF (73), MEK1 (73), ERK 1/2 (74), RSK (75, 76), CREB (77), and NURR77 (78) (Fig. 4A). Therefore, we anticipated that O-GlcNAc modulation would affect the ability of CRH to induce downstream signaling. Not surprisingly, preventing O-GlcNAcylation reduces the basal activation of several MAPK effector proteins in AtT-20 cells (Fig. 4B). While little O-GlcNAcylation suppressed basal MAPK activation, we found no evidence of an interaction with CRH. Therefore, we concluded that O-GlcNAcylation increases the basal activation of the MAPK pathway in corticotropic cells, independently of CRH stimulation. In pituitary tumors, this is particularly relevant because CRH stimulus is usually not required for increased ACTH production (79). It also agrees with our observation that human ACTH-positive pituitary adenomas demonstrate increased expression of O-GlcNAc cycling enzymes that correlates with plasma cortisol levels (see Fig. 2).

While these observations indicate an intricate relationship between O-GlcNAcylation and corticotrope function, the lack of interaction between CRH and O-GlcNAcylation in MAPK activation suggests O-GlcNAcylation affects signaling downstream of the MAPK cascade.

O-GlcNAcylation Increases Available Proopiomelanocortin Messenger RNA (mRNA) Level by Stabilizing an Existing Pool of mRNA

One of the downstream products of the CRH/MAPK pathway is the neosynthesis of Pomc mRNA and, ultimately, secretion of ACTH. We first demonstrated that O-GlcNAcylation is essential for that outcome. Indeed, preventing O-GlcNAcylation precludes CRH induction
of Pomc expression (Fig. 5A). Since O-GlcNAcylation is highly glucose dependent, lowering the glucose level in AtT-20 cells prevents CRH-induced Pomc expression, which can be restored by artificially increasing O-GlcNAcylation (Fig. 5B). These data indicate that O-GlcNAcylation is necessary for CRH induction of Pomc expression, so it was surprising that O-GlcNAcylation only mildly affected Pomc promoter activation (Fig. 5C and 5D). We therefore concluded that the critical regulatory step whereby CRH and O-GlcNAcylation interact must also be posttranscriptional.

Using an O-GlcNAcomic approach and pathway enrichment analysis, we discovered that stimulation by CRH induces changes in O-GlcNAcylated proteins participating in mRNA processing. Specifically, we observed increased levels of O-GlcNAcylated splicing regulators and decreased levels of O-GlcNAcylated mRNA decay proteins (Fig. 6). Over recent years, a relationship between O-GlcNAc and mRNA metabolism has been demonstrated. In a meta-analysis of the O-GlcNAcome, we previously highlighted that the most enriched ontology of the human O-GlcNAcome is RNA processing, including various mRNA decay mechanisms and binding proteins (17). Many mRNA surveillance proteins are O-GlcNAcylated, including AUF1 (56, 80), ZFP36-like-1 (81), KSRR (56, 82), RHAU (80), CUG-BP1 (56, 83), and CUG-BP2 (83). Nevertheless, the function of their O-GlcNAcylation remains to be characterized. Based on the existing literature and the present O-GlcNAcomic analysis, we imagine that CRH, through downstream signaling, affects the O-GlcNAcylation of mRNA processing elements to regulate Pomc mRNA levels.

In Xenopus melanotropes, it was previously suggested that Pomc mRNA is posttranscriptionally regulated via an mRNA-degradation system (39). While in the context of light adaptation, this study concluded that Pomc mRNA induction did not require neosynthesis and led us to look deeper into Pomc mRNA decay. To this end, we demonstrated that an existing pool of Pomc mRNA is subjected to a rapid decay in unstimulated cells. Furthermore, we showed, for the first time, that CRH mitigates this decay, quickly resulting in a stabilization of Pomc mRNA in an O-GlcNAc-dependent manner (Fig. 7). While mRNA stabilization mechanisms have been studied for other pituitary hormones, the regulatory proteins involved in Pomc mRNA degradation remain unknown (33-35, 37, 38, 84-86). In light of our O-GlcNAcomic analysis, intron retention and associated degradation by nonsense-mediated decay appear to be the most plausible mechanism for Pomc mRNA stability. Indeed, we demonstrated intron retention in Pomc mRNA, which is modulated by changes in O-GlcNAcylation (Figs. 8 and 9B). Not surprisingly, intron retention has been previously demonstrated to be regulated by O-GlcNAcylation (52).

The specific factors that are O-GlcNAcylated to enhance splicing and thus stabilize the transcribed pool of Pomc mRNA are likely numerous given the complexity of splicing and the relative lack of specificity of the O-GlcNAc cycling enzymes. By integrating our O-GlcNAcomic analysis with data obtained from publicly available protein databases, we highlighted more than 20 O-GlcNAcylated proteins that are excellent candidates to regulate Pomc mRNA in an O-GlcNAc- and CRH-dependent manner. First, hnRNPU, a critical splicing factor, contains 2 known sites, both O-GlcNAcylated and phosphorylated, indicating a significant regulatory potential and a possible competition between those 2 modifications in response to CRH (Fig. 9A). In a previous study, hnRNPU was shown to be one of the myriad proteins phosphorylated by PKA, central to intracellular signal transduction in response to CRH stimulation (87). Another protein identified in our analysis, Papola, is phosphorylated and regulated by Erk and was found to be O-GlcNAcylated in our experiments, indicating another protein with significant potential for crosstalk between signaling pathways (88) (Fig. 9A). A deeper investigation of the direct competition between phosphorylation and O-GlcNAcylation on these targets, as well as their binding to mRNA, will be necessary to define the mechanism by which CRH stabilizes Pomc mRNA.

In conclusion, this study uncovered the importance of O-GlcNAcylation in regulating proliferation and endocrine function in corticotropin adenomas. While this study emphasized the role of O-GlcNAcylation in regulating Pomc mRNA turnover and the responsiveness to CRH stimulation, future work will be needed to demonstrate the clinical implications of this novel regulatory mechanism and identify potential sites for the pharmacologic manipulation of this pathway.

Acknowledgments

We first thank Dr Peter LaViolette, Allison Lowman, and Dr Mona Al-Gizawiy (Medical College of Wisconsin; MCW) for supporting pituitary tissue collection and banking. Furthermore, we thank Dr Hershel Raff (MCW) for his guidance with this project. We also thank Drs Anat Ben-Shlomo and Melmed Shlomo (Cedars-Sinai) for the AtT-20 cell line. Finally, we thank Dr David Vocadlo (Simon Fraser University) for sharing the OGT inhibitor SS-GlcNAc.

Financial Support: This work was supported by the National Institute of Child Health and Development (R00HD087430).

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Disclosures: The authors have nothing to disclose.
Data Availability: All data generated during this study are included in the article or in the data repositories listed in “References (41).”

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