

Ubiquitin-Specific Protease 8 Mutant Corticotrope Adenomas Present Unique Secretory and Molecular Features and Shed Light on the Role of Ubiquitylation on ACTH Processing

Antonella Sesta^a Maria Francesca Cassarino^a Mariaros Terreni^b
Alberto G. Ambrogio^a Laura Libera^a Donatella Bardelli^a Giovanni Lasio^c
Marco Losa^d Francesca Pecori Giraldi^{a, e}

^aIstituto Auxologico Italiano IRCCS, Neuroendocrinology Research Laboratory, Cusano Milanino, Milan, Italy;

^bDepartment of Pathology, Ospedale San Raffaele, Milan, Italy; ^cDepartment of Neurosurgery, Humanitas

Clinical and Research Center, Rozzano, Italy; ^dDepartment of Neurosurgery, Ospedale San Raffaele, Milan, Italy;

^eDepartment of Clinical Sciences and Community Health, University of Milan, Milan, Italy

Keywords

Ubiquitylation · Cushing's disease · Gene expression profiling · POMC · ACTH-secreting adenomas · Ubiquitin-specific protease 8

Abstract

Background: Somatic mutations in the ubiquitin-specific protease 8 (USP8) gene have recently been shown to occur in ACTH-secreting pituitary adenomas, thus calling attention to the ubiquitin system in corticotrope adenomas. **Objectives:** Assess the consequences of USP8 mutations and establish the role of ubiquitin on ACTH turnover in human ACTH-secreting pituitary adenomas. **Methods:** USP8 mutation status was established in 126 ACTH-secreting adenomas. Differences in ACTH secretion and POMC expression from adenoma primary cultures and in microarray gene expression profiles from archival specimens were sought according to USP8 sequence. Ubiquitin/ACTH coimmunoprecipitation and incubation with MG132, a proteasome inhibitor, were performed in order to establish whether ubiquitin plays a role in POMC/ACTH degradation in corticotrope ad-

enomas. **Results:** USP8 mutations were identified in 29 adenomas (23%). Adenomas presenting USP8 mutations secreted greater amounts of ACTH and expressed POMC at higher levels compared to USP wild-type specimens. USP8 mutant adenomas were also more sensitive to modulation by CRH and dexamethasone in vitro. At microarray analysis, genes associated with endosomal protein degradation and membrane components were downregulated in USP8 mutant adenomas as were AVPR1B, IL11RA, and PITX2. Inhibition of the ubiquitin-proteasome pathway increased ACTH secretion and POMC itself proved a target of ubiquitylation, independently of USP8 sequence status. **Conclusions:** Our study has shown that USP8 mutant ACTH-secreting adenomas present a more "typical" corticotrope phenotype and reduced expression of several genes associated with protein degradation. Further, ubiquitylation is directly involved in intracellular ACTH turnover, suggesting that the ubiquitin-proteasome system may represent a target for treatment of human ACTH-secreting adenomas.

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A.S. and M.F.C. contributed equally to this work.

Introduction

The pathophysiology of Cushing's disease received considerable impetus from the recent discovery that corticotropin (ACTH)-secreting pituitary adenomas may harbor somatic mutations in the ubiquitin-specific protease 8 (*USP8*) gene [1, 2]. Indeed, in the early months of 2015, 2 independent investigators reported that gain-of-function *USP8* mutations can be observed in one-third of ACTH-secreting adenomas, leading to enhanced catalytic activity of the deubiquitinating enzyme. The ubiquitin-protease system is deputized to peptide-specific proteolytic degradation through binding of ubiquitin to target peptides, which are subsequently degraded by the 26S proteasome complex [3]. Deubiquitinases such as *USP8* are an integral part of the ubiquitin-protease complex and act to remove and recycle ubiquitin moieties.

The studies performed following the detection of *USP8* mutations in Cushing's disease [1, 2] revealed that mutations mostly occur in exon 14 and disrupt binding of 14-3-3 proteins, which leads to increased deubiquitinase activity. As *USP8* is involved in epidermal growth factor (EGF) receptor regulation [4] and, in turn, EGF is known to play a role in corticotrope tumoral development [5, 6], studies evaluated the role of *USP8* on the EGF receptor system and, indeed, demonstrated that *USP8* mutants inhibit EGF receptor degradation and increase EGF activity [1, 2]. These and other studies were performed on the murine corticotrope cell line AtT-20 or other easily transfected cell lines, for example, HeLa, Cos-7, PC12, HEK293T cells. No study has yet evaluated the consequences of *USP8* mutations on human corticotrope adenomas themselves. We attempted to answer this question with 3 different approaches: first, we evaluated ACTH secretory status and *POMC* expression in corticotrope adenoma primary cultures according to *USP8* mutation status; second, we assessed differences in the gene expression profile in *USP8*-mutated or wild-type corticotrope adenomas and; third, we evaluated whether the *POMC* peptide is itself a target for ubiquitylation and whether inhibition of the ubiquitin-proteasome system (UPS) affects ACTH corticotrope turnover in human corticotrope adenomas in vitro.

Methods

Specimens

One hundred twenty-six ACTH-secreting pituitary adenomas were collected during transsphenoidal surgery for Cushing's disease. The diagnosis had been established by standard criteria [7, 8]

and series comprised 29 men and 97 women, aged from 14 to 76 years median 40.1 years, 40% macroadenomas and 60% microadenomas, 23% surgical failures, and the remainder surgical remissions. As per our previous publications [9–11], the presence of corticotrope cells in fresh adenoma specimens was assured by abundant ACTH secretion in primary cultures [12]; as regards formalin-fixed specimens, abundant *POMC* and absent *GH*, *PRL*, *PIT1*, *LHB*, *FSHB* expression was documented by microarray analysis [13].

USP8 Sequencing

RNA was obtained from formalin-fixed or fresh specimens. About 100 ng RNA was reverse-transcribed (Superscript VILO cDNA synthesis kit; Life Technologies, Carlsbad, CA, USA) with the following oligonucleotide primers: 5' CTTGACCCAATCACTGGAAC3' (forward); 5'TTACTGTTGGCTTCCTCTTCTC3' (reverse) [14] for amplification of *USP8* exon 14, the most frequent site of mutations reported so far [14]. Touch-down polymerase chain reaction (PCR) was performed using gene ontology (GO) TAQ DNA polymerase (Promega, Madison, WI, USA) at 64–57 °C annealing. PCR products were purified by ExoProStar Illustra enzyme (Ge Healthcare, Chicago, IL, USA) and Sanger sequencing performed using the ABI PRISM Big DYE Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 3500 analyzer [13]. All chromatographs yielded informative sequencing data. Of note, *USP8* sequence was identical in the 10 samples obtained from both fresh and formalin-fixed specimens (1 adenoma carried the P720R mutation, all others wild-type *USP8* sequence)

Human Pituitary Adenoma Primary Culture

Specimens were established in culture according to our usual protocol [12, 15] and plated at 20,000–60,000 cells per well. Primary cultures were incubated in serum-free DMEM + 0.1% bovine serum albumin (BSA) containing 10 nM corticotropin-releasing hormone (CRH) or 10 nM dexamethasone (DEX). Wells treated with DMEM + BSA only represented control secretion. Medium was collected after 4 and 24 h for measurement of ACTH; after 24 h, RNA was extracted using Pure Link RNA mini kit (Invitrogen, Carlsbad, CA, USA).

Proteasome Inhibition

Adenomas were incubated with 0.01–100 nM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal MG132, a peptide aldehyde that selectively inhibits chymotrypsin-like proteolysis [16, 17]. Medium was collected and cell content extracted after 24 h for ACTH assay [18].

ACTH Assay

ACTH was measured by immunometric assay (Diasorin S.p.A. Saluggia, Italy) with all samples from the same specimen assayed in the same run. Intra-assay coefficient of variation is 7.9% and assay sensitivity is 1.2 pg/mL. Given the considerable variability in ACTH adenoma concentrations [12], responses were normalized to percent of secretion in wells incubated with DMEM + BSA only (control = 100%) for statistical analyses.

Ubiquitin-ACTH Co-Immunoprecipitation

Total protein content was extracted by RIPA Lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1%, NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific, Rockford, IL, USA) sup-

plemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Protein concentration was measured using Bradford assay (BioRad, Hercules, CA, USA). Cell lysates (150 µg) were incubated at 4 °C overnight with anti-ubiquitin rabbit polyclonal primary antibody (1:2,000 dilution, Abcam, Cambridge, UK) and nonimmune IgG (i.e., nonspecific control). Ubiquitin-precipitated cell extracts were captured on Protein A/G PLUS-agarose (Santa Cruz Biotechnology Inc., Dallas, TX, USA) then separated on SDS-PAGE using a 4–12% gradient (NuPage gel in Tris-glycine, Life Technologies, Carlsbad, CA, USA) under denaturing conditions. Proteins were transferred to Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) and the membrane blocked with 5% non-fat milk, incubated with anti-ACTH rabbit polyclonal antibody against the entire 1–39 sequence (1:1,000 dilution Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase-conjugated secondary goat polyclonal anti-rabbit antibody (1:10,000 dilution; Invitrogen, Camarillo, CA, USA). Blots were developed using enhanced chemiluminescence technique. Bioinformatic prediction of ubiquitylation sites on human POMC protein sequence (GenBank Accession # AAH65832) was performed at www.ubpred.org.

Microarray Analysis from Archival Specimens

RNA was extracted from formalin-fixed paraffin-embedded adenomatous samples using Recover All Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA), as previously described [13]. Samples were subjected to RNA quality control, that is, reverse-transcription and real-time PCR amplification of ribosomal protein L13A (*RPL13A*) [19], using Taqman probe Hs03043885_g, (Applied Biosystem, Foster City, CA, USA). All samples yielded the expected 81 bp transcript at <30 cycles, attesting to adequate quality RNA. RNA (300 ng) was analyzed on Human HT_12 v4 Bead Chip (Whole Genome DASL High Throughput assay, Illumina, San Diego, CA, USA). Fluorescence was imaged and data captured into HiScan, a high-resolution laser imager (Illumina, San Diego, CA, USA). Array data have been deposited at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=itsvwwkuzjvpsj&acc=GSE93825>.

Differentially Gene Expression Analysis

Genome studio software (Illumina, San Diego, CA, USA) was used to assess differential expression after rank invariant normalization (Genome Studio, Illumina, San Diego, CA, USA). Only genes with Benjamini and Hochberg *p* value ≤0.05 were considered and Diff Scores calculated based on *p* value transformation according to the difference between average signals in mutant and wild-type specimens. Volcano plot [20] was used to illustrate differential expression: magnitude of over- and underexpression of a given gene versus the significance of the comparison.

Functional Annotation and GO

Two approaches were used to extract biological features from gene lists: DAVID version 6.7 [21] was used to annotate and classify significant genes and perform functional annotation clustering. Minimum value of enrichment score for significant clusters was 1.3. Clusters were annotated to GO project, including molecular function, cellular component, biological process, Kyoto Encyclopedia of Genes and Genomes, and protein information resource. Further, Cytoscape with Cluego plug-in [22] was used to identify molecular interaction networks with K score = 0.4 and

Benjamini-Hochberg *p* value ≤0.001. Biological pathways included were GO project, Kyoto Encyclopedia of Genes and Genomes, and Inter Pro for protein domains [23].

Real-Time PCR

RNA (100 ng) was reverse transcribed (Superscript-Vilo cDNA synthesis kit; Life Technologies, Carlsbad, CA, USA) and quantitative real-time PCR performed on a 7900 HT sequence Detection System (Applied Biosystem, Foster City, CA, USA), using Platinum Quantitative PCR Supermix-UDG with premixed ROX. Taqman assay (Applied Biosystem, Foster City, CA, USA) was used for quantification of the following genes: *POMC* (probe Hs00174947_m1), *CRH-R1* (probe Hs01062290_m1), *NR3C1*, that is, glucocorticoid receptor (probe Hs00230813_m1) and *RPLP0*, that is, housekeeping gene (probe Hs99999902_m1). Basal expression data ($2^{-\Delta Ct}$) were calculated and normalized to *RPLP0*; expression after treatments was analyzed as $2^{-\Delta\Delta Ct}$ and expressed in fold change from baseline [9].

The following probes were used for validation experiments: *APIG2* (Hs00960175_g1) for downregulated genes and *CMTM8* (Hs00418243_m1) for upregulated genes. Expression data ($2^{-\Delta Ct}$) were normalized to *RPLP0*; validation was carried out in 15 *USP8* mutant and 16 wild-type specimens not used for microarray analysis.

Statistical Analysis

Differences between specimens harboring *USP8* mutant or wild-type sequence were established by ANOVA, Mann-Whitney test, or χ^2 test, as appropriate, using Statview 4.5 (Abacus Concepts, Berkeley, CA, USA). Significance was accepted for *p* < 0.05, and data are given as mean ± SEM.

Results

USP8 Mutation Carriers

Out of 126 adenomas, 29 carried heterozygous *USP8* mutations. Eighteen specimens carried missense mutations in p.S718 or p.P720; 10 adenomas presented the p. S719 deletion, and one adenoma carried a novel 15-bp deletion (p.P720_723 del). Specimens from female patients were more likely to present *USP8* mutations compared to specimens from male patients (28.9 vs. 3.5%, *p* < 0.005) as only one adenoma from a male patient presented a p.P720R mutations. Age (38.5 ± 2.45 vs. 40.6 ± 1.46 years, NS) as well as size of the adenoma (48.2 vs. 37.1% macroadenomas, NS) was comparable among *USP8* mutant and wild-type sequence carriers.

USP8 Mutant Adenomas Secrete More ACTH and Are More Sensitive to Corticotrope Modulators

Primary cultures were available in 84 ACTH-secreting adenomas: 59 wild type for *USP8* and 25 *USP8* mutation carriers (8 with p.S719 deletion, 16 with p.P720 and p.S718 missense mutations and 1 adenoma with

Fig. 1. Constitutive ACTH secretion and *POMC* expression in *USP8* mutant and wild-type adenomas. Medium and gene expression was assessed after 24 h incubation with plain medium. * Denotes $p < 0.05$.

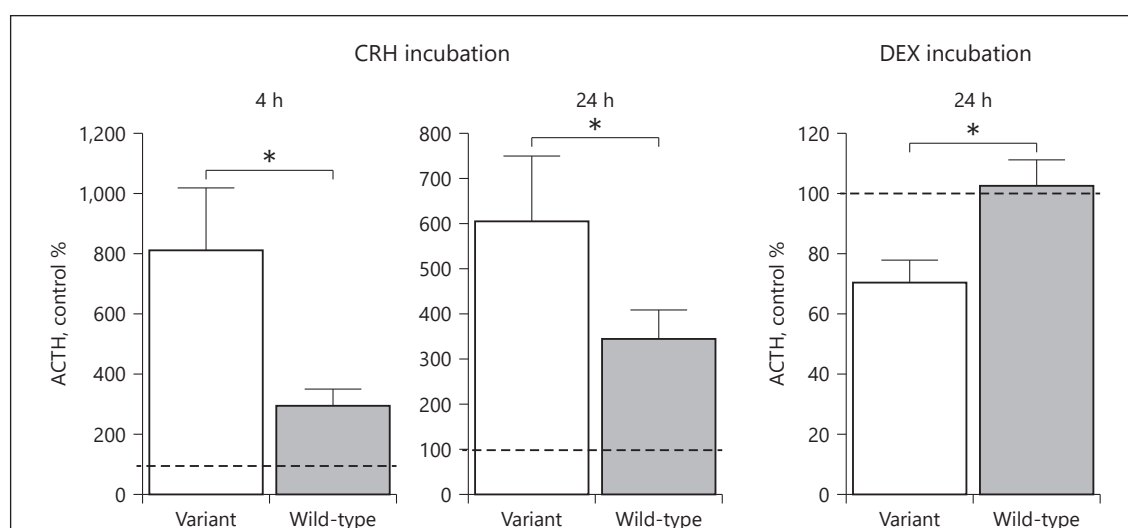
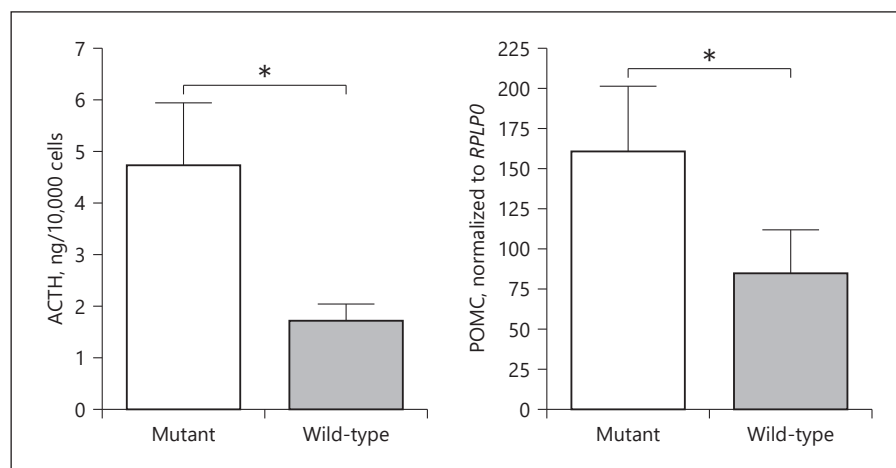


Fig. 2. ACTH secretion during CRH and dexamethasone incubation in *USP8* mutant and wild-type adenomas. Medium concentrations are expressed relative to incubation with plain medium set at 100% (dotted line). * Denotes $p < 0.05$.

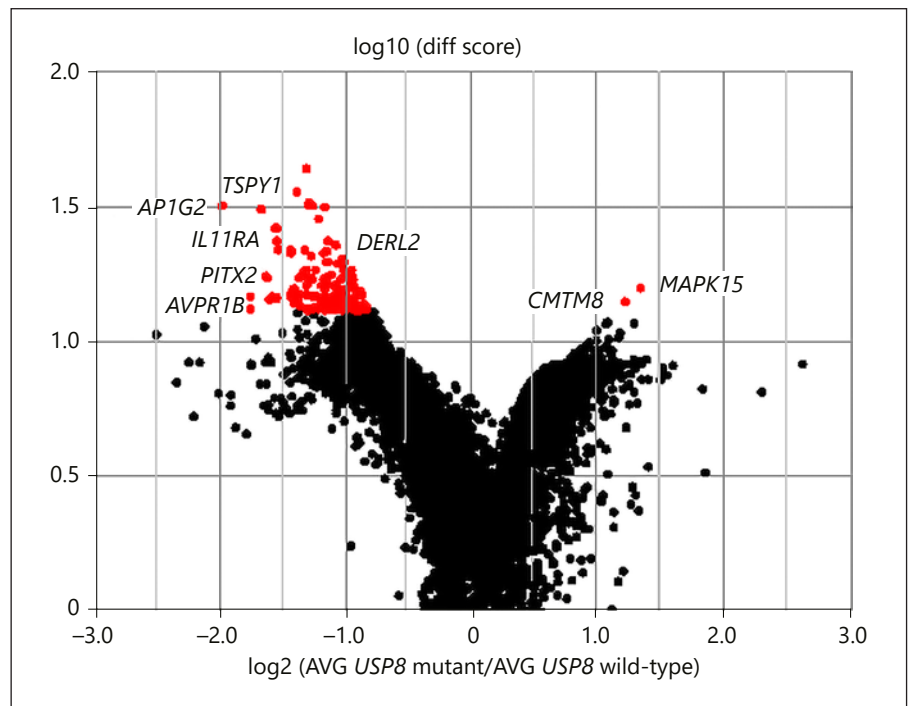
p.P720_723 deletion). Spontaneous 24 h ACTH secretion was significantly greater in specimens carrying *USP8* mutations compared to wild-type adenomas as was *POMC* expression (Fig. 1).

In regard to the response to CRH stimulation, *USP8* mutant adenomas presented a more marked response to CRH, as assessed in terms of percent increase (Fig. 2) and absolute increase (4 h: 5.79 ± 2.23 ng/10,000 cells vs. 1.30 ± 0.52 ng/10,000 cells, $p < 0.005$; 24 h: 10.8 ± 3.63 vs. 3.23 ± 1.51 pg/10,000 cells, in *USP8* mutant vs. wild-type, respectively, $p < 0.0005$). Further, expression *CRH-R1* was greater in *USP8* mutation carriers (0.153 ± 0.04 vs. 0.070 ± 0.023 normalized expression, respectively, $p < 0.005$).

Changes in *POMC* during incubation with CRH were not associated with *USP8* mutation status (24 h: 1.37 ± 0.158 vs. 1.198 ± 0.264 fold change from baseline, respectively, NS). CRH stimulation had been performed during the diagnostic work-up in 68 patients, 13 carrying *USP8* mutant and 55 *USP8* wild-type adenomas. No correlation between in vivo and in vitro responses was observed for either group ($r = 0.336$, NS and $r = 0.157$, NS for *USP8* mutant and wild-type adenomas, respectively).

In addition, *USP8* mutation carriers appeared more sensitive to the inhibitory effect of dexamethasone than *USP8* wild-type specimens, assessed as percent from control (Fig. 2) and absolute decrease (-2.42 ± 1.41 vs. -0.62

Fig. 3. Volcano plot shows results of differential expression analysis between *USP8* mutant and wild-type adenomas. Significant genes (diff score ≥ 13 : upregulated genes; diff score ≤ -13 : downregulated genes) are shown as red dots and selected genes identified. *USP8*, ubiquitin-specific protease 8.



± 0.51 ng/10,000 cells at 24 h, in *USP8* mutant versus wild-type, respectively, $p < 0.05$). No differences were observed after 4-h incubation with dexamethasone (174.9 ± 93.29 vs. $120.4 \pm 17.77\%$ control, NS, 0.13 ± 0.18 vs. -0.09 ± 0.09 ng/10,000 cells, respectively, NS), in keeping with the modest effect of dexamethasone after short-term incubation in adenomatous cells in vitro [12], nor in the effect of dexamethasone on *POMC* expression (24 h: 0.74 ± 0.068 vs. 0.94 ± 0.149 fold change from baseline, respectively, NS). Interestingly, *NR3C1* was expressed at greater levels in *USP8* mutation carriers compared to *USP8*-wild-type adenomas (0.94 ± 0.084 vs. 0.50 ± 0.085 normalized expression, respectively, $p < 0.005$). High dose dexamethasone testing had been performed in 63 patients, 14 carrying *USP8* mutant and 49 *USP8* wild-type adenomas. As for CRH, no correlation between in vivo and in vivo responses was observed ($r = -0.307$, NS and $r = 0.368$, NS for *USP8* mutant and wild-type adenomas, respectively).

Analysis of ACTH secretion and *POMC* expression in basal and modulated conditions in adenomas carrying different *USP8* mutations, that is, p.S718, p.S719, and p.P720, did not reveal clear-cut differences according to the affected amino acid (spontaneous ACTH concentrations: $F = 0.619$, NS; *POMC* expression: $F = 0.673$, NS; ACTH change during CRH incubation: $F = 0.997$, NS; ACTH change during DEX incubation: $F = 0.419$, NS).

USP8 Mutant Adenomas Present a Different Gene Expression Profile

Comparisons of gene profiles in 5 *USP8* mutant vs. 34 wild-type specimens allowed identification of over- or underexpressed genes according to *USP8* status. Volcano plot (Fig. 3) shows genes upregulated with diff score ≥ 13 and genes downregulated with diff score ≤ -13 in *USP8* mutant vs. *USP8* wild-type adenomas. Comparison of gene profiles revealed that 2 genes, *CMTM8* (CKLF-like MARVEL transmembrane domain containing 8) and *MAPK15* (mitogen-activated protein kinase 15), both involved in EGF signaling, were upregulated in *USP8* mutation carriers. One hundred forty-four genes were downregulated in *USP8* mutant compared to wild-type adenomas (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000500688), which allowed functional analysis. Cytoscape revealed major clustering of downregulated genes in pathways related to endoplasmic reticulum-associated protein degradation, response to topologically incorrect proteins, Golgi-associated vesicle, ribosomal large subunit biogenesis, endosome membrane recycling, and cell-to-cell junction (Fig. 4). The latter 2 biological functions were also identified by DAVID with 58 genes included in the membrane component (enrichment score 1.56, $p = 0.0026$) and 7 genes in the cell-to-cell adhesion component (enrichment score 1.3, $p = 0.013$).

Fig. 4. Cytoscape analysis showing identified networks (K score = 0.4; Benjamini-Hochberg $p \leq 0.001$) among genes down-regulated in *USP8* mutant versus wild-type adenomas. ERAD, endoplasmic reticulum-associated protein degradation.

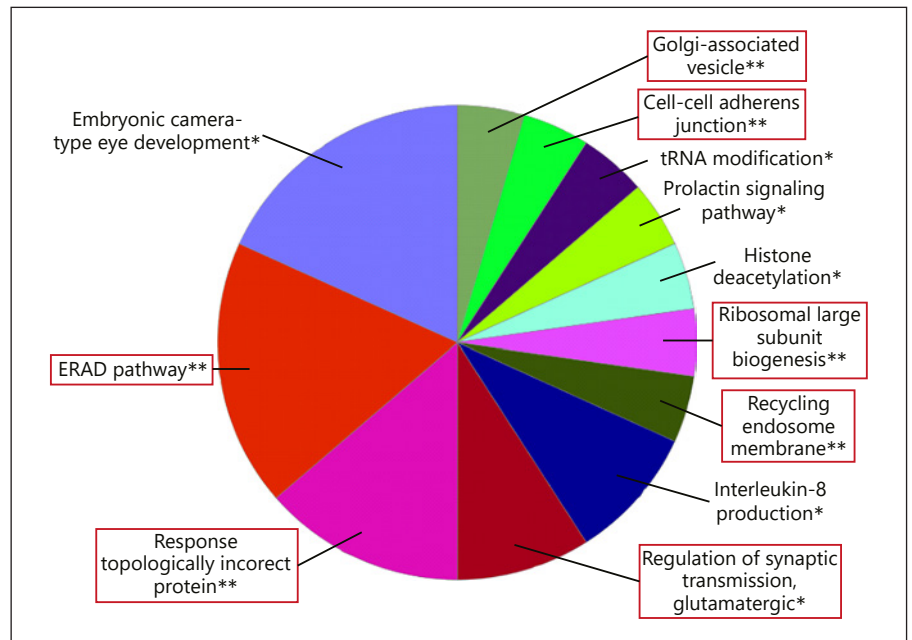
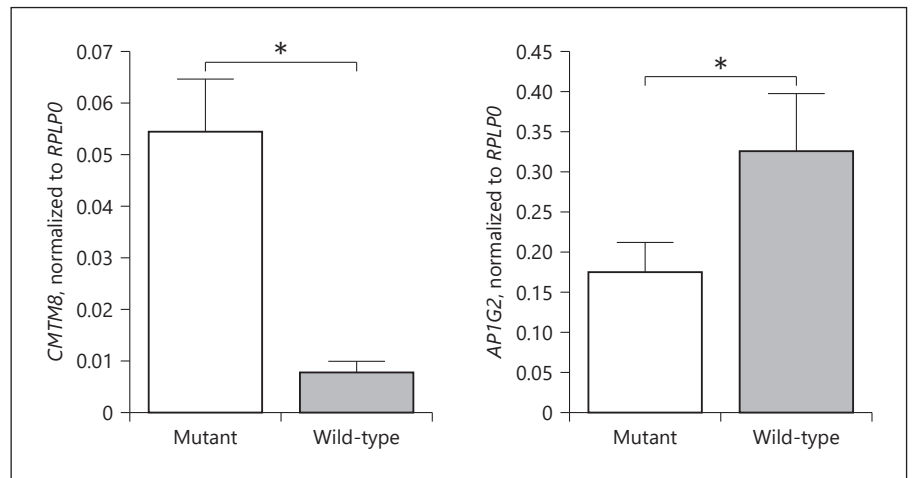


Fig. 5. RT-PCR for *CMTM8* and *APIG2* expression in *USP8* mutant and wild-type adenomas. * Denotes $p < 0.05$.



Validation of over- and underexpressed genes was carried out in a separate set of 15 *USP8* mutant and 16 *USP8* wild-type adenomas. Quantitative RT-PCR for *CMTM8* and *APIG2* (adaptor-related protein complex 1 gamma 2 subunit) confirmed overexpression and underexpression in *USP8* mutant adenomas compared to wild-type specimens, respectively (Fig. 5).

Proteasome Inhibition Increases ACTH Levels

Incubation with MG132, an inhibitor of the ubiquitin-protease pathway, was performed in 11 adenomatous specimens (7 *USP8* wild-type and 4 *USP8* mutation carriers). ACTH concentrations in both medium and cell content increased significantly during MG132 incubation

(Fig. 6), indicating that proteasomal degradation plays a role in maintaining ACTH corticotrope turnover. No significant differences were observed between *USP8* mutant (2 with p.S719del, 1 with p.P720_723del and 1 with p.P720R) and wild-type adenomas (1 nM MG132: ACTH in medium: $F = 1.96$, NS, ACTH content: $F = 0.056$, NS; 10 nM MG132: ACTH in medium: $F = 0.08$, NS, ACTH content: $F = 0.014$, NS; remaining comparisons all NS).

POMC Is a Target of Ubiquitylation

In order to identify whether the POMC/ACTH peptide is a direct target of the UPS, as we have recently shown in rat corticotropes [18], we performed ubiquitin/

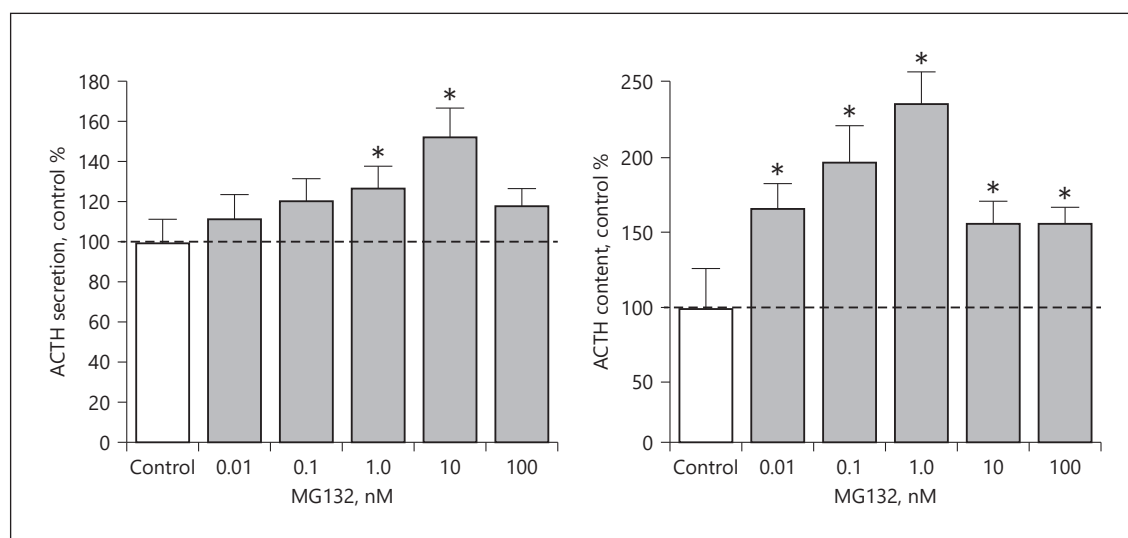
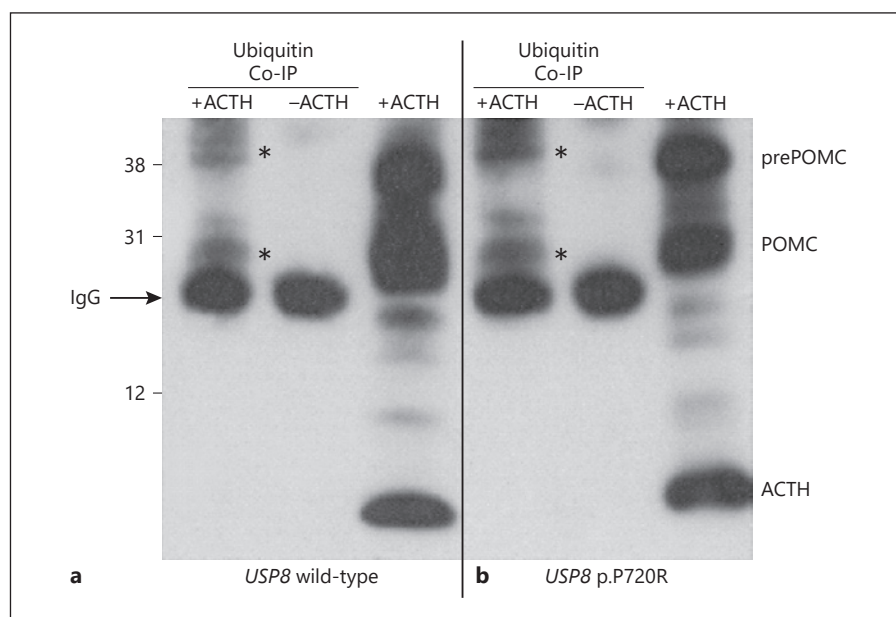


Fig. 6. ACTH concentrations in medium and cell content in corticotrope adenoma primary cultures treated with 0.01–100 nM MG132 for 24 h (white bars). Dashed line represent unchallenged wells set at 100% (control; grey bar). * Denotes $p < 0.05$ versus control.

Fig. 7. Ubiquitin/ACTH coimmunoprecipitation in corticotrope adenoma primary culture cell extracts. Two representative samples are shown, *USP8* wild-type (**a**) and *USP8* mutant (**b**). For each sample, right most lane shows input blotted for ACTH: prePOMC (~39 kDa), POMC (~29 kDa), and ACTH (~6 kDa) are visible. Middle lane shows ubiquitin immunoprecipitation without ACTH blotting: arrow identifies IgG light chains (~23 kDa) visible in both middle and left lanes. Left lane shows ubiquitin-tagged ACTH-blotting fragments: * identifies ubiquitylated POMC and pre-POMC. No band was observed at the expected size for ubiquitylated ACTH. Co-IP, co-immunoprecipitation.



ACTH co-immunoprecipitation. Western blotting on ubiquitin-precipitated cell extracts showed that POMC and its precursor, 39 kDa pre-POMC, are ubiquitylated, whereas no band corresponding to 1–39 ACTH at 6 kDa was detected in ubiquitin precipitates (Fig. 7). Further, the same bands were evident in both *USP8* mutant and wild-type adenomas, suggesting that deranged *USP8*

function did not lead to qualitative changes in POMC/ACTH peptides. The search for canonical ubiquitylation sites on the human POMC sequence revealed 5 potential residues with medium-high confidence, at lysine 76, 103, 129, 177, and 215.

Discussion

The UPS is a major player in intracellular protein degradation, regulating transcription factors, membrane trafficking, receptor signaling, cell-cycle control among others [3]. Proteolytic degradation by the UPS is a multi-step process requiring ubiquitylation of the target protein and detragadation by a 26S proteasome macromolecular complex. As with other posttranslational modification, ubiquitylation is reversible and specific proteases, that is, deubiquitinases, remove ubiquitin from tagged proteins thereby rescuing and recycling proteins marked for degradation. The balance between ubiquitylation and deubiquitination determines which proteins are degraded and which are not, thus subserving “proteostasis” [24].

Most recently, mutations in a deubiquitinase, *USP8*, have been shown to occur in a subset of human corticotrope adenomas [1, 2, 14, 25–30], thus proving the first somatic mutation to be described in Cushing’s disease. Mutations in another deubiquitase, *USP48*, have also been recently detected in corticotrope adenomas, mostly wild-type for *USP8* itself [31]. *USP8* is one of over 80 human deubiquitinases and is mainly involved in stabilization of endosomal sorting complexes [32] and receptor-activated tyrosine kinase downregulation [33]. Mutations reported in corticotrope adenomas usually occur in exon 14 [1, 2] and disrupt association with 14-3-3 protein and catalytic inactivation; it follows that most *USP8* mutations represent gain-of-function mutations with unchecked, constitutive deubiquitinase activity [2].

One well-known target of *USP8* is the EGF receptor [4], a tyrosine kinase and potent mitogen involved in corticotrope tumorigenesis [5, 6, 34–36]. Studies on cells transfected with mutant *USP8* demonstrated higher levels of EGF-R protein [1], reduced EGFR degradation, and enhanced EGF-R recycling [1, 2]. Further, treatment with the EGF-R inhibitor gefitinib attenuated ACTH secretion in 2 *USP8* mutant but not a wild-type corticotrope adenoma [1]. Experiments in the murine corticotrope cell line AtT-20 [2] also revealed that *USP8* mutant-transfected cells are more responsive to the stimulatory effect of EGF on both *POMC* transcription and ACTH secretion [5, 14].

Studies aimed at investigating the effect of mutant *USP8* have been performed in cell lines and little is known as yet on the consequences on human corticotrope tumors themselves in vitro. Our first aim was to establish whether *USP8* status affects ACTH synthesis and secretion in human pituitary ACTH-secreting adenomas in vitro and we could demonstrate that, indeed, adenomas

harboring *USP8* mutations secrete markedly greater amounts of ACTH. Of note, plasma ACTH levels have been reported to be both lower [2, 25], equal [14, 26, 28], and higher [1] in patients harboring *USP8* mutants compared to wild-type *USP8* carriers, attesting to the fact that baseline plasma ACTH is a poor indicator of corticotrope secretory activity [37]. ACTH synthesis in vitro was also increased as *USP8* mutant adenomas expressed nearly twice as much *POMC* as *USP8* wild-type specimens. This latter result tallies with findings in some 30 frozen adenomatous specimens, which revealed higher *POMC* expression in *USP8* mutant vs. wild-type specimens [1, 25].

In regard to the response to CRH, *USP8* mutation carriers exhibited a two-fold greater response than *USP8* wild-type adenomas and expressed the CRH receptor gene at higher amounts. In keeping with this more typical “corticotrope” secretory behavior, *USP8* mutant adenomas also appeared more sensitive to dexamethasone inhibition and expressed greater levels of the *NR3C1* receptor. Analysis of responses to CRH and high-dose dexamethasone in patients with *USP8* mutant adenomas yielded superimposable responses according to *USP8* status in our hands [28], whereas greater sensitivity to high-dose dexamethasone testing has been reported by others [14]. In the present series, no correlation between in vitro and in vivo responses in a given patient was observed for either *USP8* mutant and wild-type carriers; this result is not surprising as the correlation between in vitro and in vivo responses to CRH and dexamethasone is good but by no means absolute [12].

Given these differences in the secretory profile of *USP8* mutation carriers, we decided to compare the gene expression profiles in *USP8* mutant and wild-type corticotrope adenomas in our microarray data set [13]. Differential gene expression analysis according to *USP8* status revealed reduced expression of genes related to pathways associated with protein degradation, that is, response to topographically incorrect protein, endoplasmic reticulum-associated protein degradation, in *USP8* mutant adenomas which is in line with reduced activity of the ubiquitin-proteasome system given constitutive activation of the *USP8* deubiquitinase. The recycling endosome component also featured among *USP8* mutant downregulated functions in keeping with involvement of the endosome pathway in EGF-R recycling [2]. Further, both Cytoscape and DAVID identified functions related to membrane component and cell-to-cell adhesion among genes downregulated in *USP8* mutant adenomas, which suggests deregulation in additional membrane-related pathways. Genes of interest in this group (online suppl. Table 1) are,

SCAMP2 and *CDC42EP5*, involved in calcium-dependent granule exocytosis [38, 39], *ERGIC1* and *TMED9*, associated with endoplasmic reticulum-Golgi trafficking [40], *DERL2*, required for ER-associated degradation [41] and *APIG2*, an adaptin involved in immature secretory granule sorting [42].

More closely pertaining to corticotrope pathophysiology, *AVPR1B*, the gene encoding for the vasopressin subtype 3 receptor, and *IL11RA*, encoding for the interleukin 11 receptor, involved in the ACTH/POMC response to desmopressin [11] and interleukin 11 [43], respectively, were among genes underexpressed in *USP8* mutant adenomas. *PITX2*, an expression factor involved in pituitary development, was also underexpressed in *USP8* mutant adenomas which is of particular interest given that *PITX2* knockout mice differentiate only the corticotrope lineage [44]. Other genes of interest to neuroendocrine and pituitary tumors which were underexpressed in *USP8* mutant corticotrope adenomas were neuropeptide S receptor 1 [45], fibroblast growth factor 2 antisense gene product or nudix hydroxylase 6 (a.k.a. *GFG1*) [46], and human testis specific protein Y-encoded 1, the latter giving rise to prolactin and ACTH-secreting pituitary adenomas in transgenic mice [47].

Interestingly, only 2 genes, *CMTM8* and *MAPK15*, proved significantly overexpressed at differential expression analysis in *USP8* mutant adenomas and both are linked to the EGF-R pathway. Upregulation of *CMTM8* (a.k.a. *CKLFSF8*), a tumor suppressor, chemokine-like factor, might represent a compensatory adaptation to EGF-R activation as it has been shown to attenuate EGFR-mediated signaling [48]. Likewise, *MAPK15* (or *ERK8*), a member of the MAPK family, acts as corepressor for EGF-activated genes such as estrogen-related receptor alpha [49]. Conversely, other MAP kinases, that is, *MAPK12*, and MAPK-associated proteins, that is, *MAPK8P2* and *MAPK8P3*, were downregulated in *USP8* mutant adenomas, suggesting tight regulation of factors involved in the EGF-MAPK pathway. *EGFR* gene expression did not differ among *USP8* mutant and wild-type adenomas (diff score 3.027, NS), in keeping with previous results [1, 25]. Lastly, differential expression of *USP8* itself did not reach significance (diff score -7.002, NS); previous results on *USP8* expression were contrasting, as mRNA levels appeared increased in *USP8* mutant adenomas according to one study [25] and comparable to wild-type in another [1].

As mentioned above, upon the discovery of *USP8* mutations in corticotrope adenomas, studies have focused on its effect on the EGF receptor system but we postu-

lated that ubiquitylation could target the ACTH biosynthetic pathway directly. Indeed, co-immunoprecipitation experiments showed that POMC and its precursor pre-POMC are direct targets of ubiquitylation. Of note, the POMC peptide contains several lysines, that is, canonical ubiquitylation sites, as well as other, potential ubiquitylation sites, for example, threonines, cysteines, and serines [50]. ACTH itself does not appear a target for ubiquitylation but this is not surprising as it is synthesized downstream to the endoplasmic reticulum [51] where ubiquitinases are known to act [52]. Interestingly, no qualitative differences were observed between *USP8* mutant and wild-type adenomas, suggesting that the UPS modulates ACTH turnover in normal as well as tumoral corticotropes. Indeed, we have recently shown that inhibition of the ubiquitin-proteasome pathway with MG132 led to an increase in ACTH secretion and intracellular content in normal rat pituitary primary cultures [18]. The present study shows that inhibition of the ubiquitin-proteasome pathway in human corticotrope adenomas leads to increased ACTH secretion and cell content. This could be observed in both *USP8* mutant and wild-type adenomas, indicating that gain-of-function *USP8* mutations are not a prerequisite for sensitivity to ubiquitin-mediated proteolysis. Indeed, a recent study on AtT-20 cells reported reduced ACTH secretion and increased apoptosis during incubation with an *USP8* inhibitor [53], supporting the concept that the interplay between ubiquitylation and deubiquitylation subserves corticotrope ACTH proteostasis.

In conclusion, our study has shown that *USP8* mutant corticotrope adenomas present a more “typical” corticotrope phenotype with pronounced ACTH synthesis and secretion and greater sensitivity to the 2 main corticotrope modulators, that is, CRH and glucocorticoids. Further, we have shown that genes associated with the proteosomal degradation machinery are dampened in *USP8* mutant adenomas and, at the same time, counter regulatory mechanisms may come into play to fine-tune the EGFR-MAPK pathway in these cells. In addition, the POMC peptide itself is a target of ubiquitylation and inhibition of the ubiquitin-protease pathway increased ACTH content and secretion supporting the concept that the ubiquitin system plays a direct role on ACTH synthesis and processing within corticotropes, in addition to its effect via EGF. In fact, the hypothesis of corticotrope-specific *USP8* substrates other than EGF-R had been put forward by one of the first publications on *USP8* mutations in Cushing’s disease [2].

Altogether, this evidence indicates that the UPS is a key component in human corticotrope pathophysiology and represents a potential candidate for targeted therapy, acting either upon the EGF-receptor or POMC processing, in ACTH-secreting adenomas. The detection of *USP8* mutant in these adenomas [1, 2] has indeed opened new scenarios for Cushing's disease.

Statement of Ethics

Informed consent for the secondary use of surgical specimens was collected at the neurosurgical units and the study was approved by the Ethical Committee of the Istituto Auxologico Italiano.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

A.S., M.F.C. and F.P.G.: contributed to conception and research design. A.S., M.F.C., L.L. and D.B. performed experiments. M.T., A.G.A., M.L. and G.L. carried out to pituitary sample and clinical data collection. A.S., M.F.C. and F.P.G. analyzed data and drafted the manuscript. A.S., M.F.C., M.L., G.L. and F.P.G. edited and revised manuscript; all authors approved final version of manuscript.

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