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Driver mutations in *USP8* wild-type Cushing's disease

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Abstract

Background. Medical treatment in Cushing's disease (CD) is limited due to poor understanding of its pathogenesis. Pathogenic variants of ubiquitin specific peptidase 8 (USP8) have been confirmed as causative in around half of corticotroph tumors. We aimed to further characterize the molecular landscape of those CD tumors lacking USP8 mutations in a large cohort of patients.

Methods. Exome sequencing was performed on 18 paired tumor–blood samples with wild-type USP8 status. Candidate gene variants were screened by Sanger sequencing in 175 additional samples. The most frequent variant was characterized by further functional in vitro assays.

Results. Recurrent somatic hotspot mutations in another deubiquitinase, USP48, were found in 10.3% of analyzed samples. Several possibly damaging variants were found in TP53 in 6 of 18 samples. USP48 variants were associated with smaller tumors and trended toward higher frequency in female patients. They also changed the structural conformation of USP48 and increased its catalytic activity toward its physiological substrates histone 2A and zinc finger protein Gli1, as well as enhanced the stimulatory effect of corticotropin releasing hormone (CRH) on pro-opiomelanocortin production and adrenocorticotropic hormone secretion.

Conclusions. USP48 pathogenic variants are relatively frequent in USP8 wild-type tumors and enhance CRH-induced hormone production in a manner coherent with sonic hedgehog activation. In addition, TP53 pathogenic variants may be more frequent in larger CD tumors than previously reported.

Key Points

1. The ubiquitin system plays a major role in Cushing's disease tumorigenesis with 2 deubiquitinases affected by mutations.
2. TP53 somatic variants are more frequent in corticotroph tumors than previously believed, while BRAF mutations are quite rare events.

Importance of the Study

Infrequent genetic defects in response to the hypothalamic stimulation pathway in pituitary tumors diminished its role in corticotroph tumorigenesis. We here show that somatic activating mutations in the deubiquitinase *USP48* gene may induce corticotroph tumorigenesis by enhancing corticotroph tumor response to hypothalamic stimulation. This may influence future therapies in this rare tumor entity. In a large number of cases we show that *BRAF* V600E variants are extremely rare in corticotroph pituitary tumors, while *TP53* pathogenic variants seem to be more frequent than previously assumed, especially in the larger tumors.

Cushing's disease (CD) is caused by pituitary corticotroph adenomas hypersecreting adrenocorticotropin hormone (ACTH). With an overall incidence of 1–2 per million per year and a prevalence of 20–45 patients per million per year, it is considered a rare disease.[1] CD is associated with increased morbidity and mortality, the latter mainly due to cardiovascular consequences of glucocorticoid excess.[2] To this day, pituitary surgery, already pioneered by Harvey Cushing in 1932,[3] is still the therapy of choice, which results in remission in about 70% of patients, but in a significant number of patients recurrences occur.[4] Despite an increase in medical treatment options in the last years, they often only ameliorate the clinical manifestations without long-lasting responses and could not achieve cure of the disease.[5] The pathogenetics of corticotroph adenomas has for a long time remained obscure. Until recently, only very small studies, using a targeted approach for gene mutations in the context of the main known endocrine familial genetic disorders, have been performed, with mainly disappointing results. In a negligible fraction of CD cases, alterations associated with the syndromes McCune–Albright, MEN4, Carney complex, and tuberous sclerosis have been reported.[6,7] In the last few years, access to high-throughput sequencing has led to a flurry of disease-specific genetic analyses for many tumor entities.[8] In rarer diseases, like CD, these analyses have been delayed due to material scarcity. For CD, the breakthrough came in 2014 when the first study using next-generation exome sequencing revealed recurrent somatic mutations in a hotspot region of the ubiquitin specific protease 8 (USP8) gene.[9,10] Subsequent studies using targeted USP8 sequencing[11–15] confirmed that hotspot mutations in *USP8* are responsible for 40–60% of CD tumors. While our study was ongoing, Chen et al[16] reported mutations in a gene encoding for another deubiquitinase, namely *USP48*, and frequent alterations in the *BRAF* proto-oncogene. In this study, we have screened a group of tumors without *USP8* mutations for possible new driver mutations using exome sequencing with confirmatory Sanger sequencing and we have analyzed the possible impact of these new mutations on CD. In addition, we screened for the *BRAF* V600E mutation reported by Chen et al.[16] Furthermore, we provide additional/new mechanistic explanations about the mode of action of these mutations.

Materials and Methods

Patient Cohort

Fresh frozen and/or formalin-fixed paraffin embedded (FFPE) corticotroph tumors were obtained from 237 patients (181 female) with CD, including 16 patients with corticotroph tumor progression causing Nelson syndrome (as defined by Perez-Rivas et al[15]). CD was diagnosed according to the current guidelines[17] based on lack of response to the 1 mg overnight dexamethasone suppression test (ie, serum cortisol >1.8 µg/dL or >50 nmol/L), elevated urinary free cortisol (UFC), and late-night salivary cortisol (taking into account locally established reference ranges for both parameters). Baseline ACTH values >20 pg/mL and characteristic responses to high-dose 8 mg overnight dexamethasone suppression testing and corticotrophin releasing hormone (CRH) stimulation testing (100 µg human CRH i.v.) or inferior petrosal sinus sampling were required for the final diagnosis of CD in these patients. Expert histological examination confirmed the presence of corticotroph tumor in all samples. All patients gave written informed consent and the study was approved by the ethics committee of each institution (LMU nos. 152-10 and 643-16, UKW no. 85/12).

DNA Extraction and Sanger Sequencing

Results of USP8 sequencing of some of the analyzed tumors have been previously reported elsewhere ("JCEM 2015 cohort,"[14] "Clin Endo 2018 cohort,"[18] and "EJE 2018 cohort"[15]; Supplementary Figure 1). DNA from 80 FFPE cases (54 female) ("new FFPE cohort") was extracted using the QIAamp DNA FFPE Tissue Kit. In addition, DNA from 44 snap frozen tumors (37 female) ("new cryo

cohort") was extracted using the Maxwell RSC Tissue DNA Purification Kit and the Maxwell 16 instrument (Promega). Exon 14 of the *USP8* gene was amplified using GoTaq DNA polymerase (Promega) and sequenced as previously described.[14,15,18]

Exome Sequencing

We detected *USP8* pathogenic variants in 20 out of the cohort of 44 snap frozen tumors. We obtained high-quality paired leukocyte DNA from 18 out of 24 *USP8* wild-type (wt) patients with snap frozen tumors ("new cryo cohort"; 15 CD, 3 Nelson syndrome; 10 female; Supplementary Table 1). Exome sequencing was performed as previously described.[9]

Sanger Sequencing

Primers used to amplify the regions of interest of *USP8*, *USP48*, *BRAF*, and *FAT1* genes are listed in Supplementary Table 2. PCR was performed on 50 ng of genomic DNA in a final volume of 25 µL containing 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM dNTPs (deoxyribonucleotide triphosphates), and 1.25 U Platinum Taq DNA Polymerase (Invitrogen) for 30 cycles (denaturation 94°C for 20 sec, annealing 58°C for 30 sec, and elongation 72°C for 30 sec). PCR products were sequenced using the QuickStart Cycle Sequencing Kit (ABSciex) on a CEQ8000 DNA Analyzer (ABSciex). PCR primers for *USP8*, *USP48*, and *BRAF* were used for both amplification and sequencing. *FAT1* sequencing primers are shown in Supplementary Table 2.

Functional Regions of USP48 and Sequence Alignment

Sequence similarity analysis was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Thereby, human *USP48* (NCBI GenBank ID: 84196) was compared with sequences from other species. The 3D representation of the USP domain structure of USP48 has been developed using the SWISS-MODEL (based on Uniprot sequence: Q86UV5), and the effect of the pathogenic variant on the structure has been performed using University of California San Francisco Chimera 1.10 software for Mac. The 3D representations of human USP18 and USP7 are based on their structure deposited on the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB-PBD; accession numbers 5CHT and 1NB8, respectively).

Plasmids

The mammalian expression vector pcDNA3.1$^+$/C-(K)-DYK containing the open reading frame clone of human *USP48* (NM_032236.7) was purchased from Genescript (Clone ID OHu20169). The c.1245C>T/p.Met415Val point variant was introduced using the Q5 Site Directed Mutageneis Kit (New England Biolabs). The POMC-luc luciferase reporter vector that has the human pro-opiomelanocortin (POMC) proximal promoter upstream of the luciferase gene was purchased from Panomics.

Cell Culture and Transfection

Murine corticotroph tumor AtT-20 cells were obtained and authenticated by the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 10^5 IU/L penicillin-streptomycin at 37°C and 5% CO_2 and used until passage 14. Cell culture materials were purchased from Gibco, Sarstedt, and Sigma-Aldrich. Cells were plated in 48-well plates at 50 000 cells per well and transfected with *USP48* constructs or empty pcDNA3.1 vector (as control) using SuperFect (Qiagen). After being in serum-free medium overnight, cells were treated with CRH (Sigma) for 6 hours. We determined luciferase with the Beetle-Juice firefly luciferase assay (PJK). Each condition was in triplicates. Transfection with the RSV-β-Gal construct monitored the transfection efficacy. Data are presented as ratio of luciferase to β-galactosidase activity.

RNA Interference

Gli1 knockdown was achieved with a set of three 27mer small interfering (si)RNA duplexes (SR421553, OriGene). A set of scrambled siRNAs was used as negative control and siRNA against Gli2 as an independent control.[19]

ACTH Measurement in Cell Supernatant

ACTH was determined in cell supernatants using a radioimmunoassay as previously described.[20] In brief, transfected cells were serum washed overnight and, where indicated, treated with CRH for 24 hours. Each condition was in quadruplicates.

Cell Viability

Cell viability was determined 48 hours after transfection using the WST1 colorimetric assay and measured at 450 nm (Roche Applied Science) as previously described.[21] Each condition was in quadruplicates.

Deubiquitination Assays

HeLa cells were transfected with plasmids encoding indicated FLAG-tagged USP48 variants. Twenty-four hours after transfection, cells were lysed in 400 µL TNT150 (Tris-HCl 7.4, 1% Triton X-100, 150 mM NaCl) + 1:100 protease/phosphatase inhibitor cocktails (Sigma) for 30 min on ice; lysates were briefly sonicated and clarified by centrifugation. FLAG-tagged proteins were purified using protein G agarose and anti-FLAG M2 antibody (Sigma) at 4°C for 4 h, and eluted in deubiquitylase (DUB) buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 3 µL 3xFlag-peptide (5 mg/mL). Eluted proteins were incubated with purified K48 or K63-linked ubiquitin chains of 2–7 ubiquitin moieties for 30 min at 30°C.

Reactions were analyzed by immunoblotting with anti-ubiquitin antibody (Cell Signaling).

HeLa cells were transfected with plasmids encoding hemagglutinin (HA)-tagged histone H2A or Gli1, His^6-tagged ubiquitin, and indicated FLAG-tagged USP48 variants. Cells were lysed in urea buffer (8 M urea, 10 mM imidazole in phosphate buffered saline) and ubiquitinated proteins were recovered on Ni-NTA agarose (Qiagen). Purified His^6-ubiquitin protein conjugates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with anti-Flag M2 (Sigma), anti-HA (Cell Signaling, 6E2), anti-Gli1 (Cell Signaling, V812), and anti-USP48 (Bethyl, A301-190A) antibodies.

Statistical Analysis

Results from the *POMC* luciferase reporter assay, ACTH radioimmunoassays, and cell viability assay are shown as mean ± standard deviation, and were analyzed using Student's *t*-test. Correlation between mutational status and sex was analyzed using the χ^2 test; for other clinical parameters, the Kruskal–Wallis test with Dunn's multiple comparison test was used as appropriate. $P < 0.05$ was considered statistically significant.

Results

Recurrent USP48 Pathogenic Variants in Corticotroph Adenomas

Exome sequencing of 18 *USP8* wt CD tumors (15 CD, 3 Nelson syndrome; 10 female) revealed the presence of the same recurrent pathogenic variant in 3 (17%) samples: g.22056252C>T; c.1245C>T in exon 10 of the gene encoding for the deubiquitinase *USP48* (Supplementary Figure 2A, Supplementary Tables 1 and 3). That pathogenic variant was present only in the tumor tissue. No pathogenic variant was detected in the corticotroph tumors derived from the 3 patients with Nelson syndrome. The g.22056252C>T; c.1245C>T variant leads to the non-synonymous amino acid exchange p.Met415Ile that resides in a region of the catalytic domain well conserved in the protein orthologues across a variety of vertebrate species (Supplementary Figure 2A). Targeted sequencing of the *USP48* region around the hotspot pathogenic variant in 175 additional CD tumors revealed the *USP48* p.Met415Ile genetic variant in 16 further samples, and a c.1243A>G (p.Met415Val) variant in one corticotroph tumor (Supplementary Figure 2B). No pathogenic variant was detected in the leucocytes of the same patients (Supplementary Figure 2C).

Altogether, we found pathogenic variants affecting the Met415 of *USP48* in 20/193 (10%) CD tumors. Both wt and mutant alleles were detected in the tumor tissue, consistent with a heterozygous state of *USP48* pathogenic variant. No tumor in our cohort carried both *USP8* and *USP48* pathogenic variants.

Tumors with *USP48* pathogenic variants were associated with smaller tumor size and were more frequently found in female patients (Table 1). We did not observe significant differences in age at diagnosis, body mass index, basal ACTH, basal cortisol, and cortisol response to low-dose dexamethasone suppression between patients with pathogenic *USP48* variants and tumors wt for both *USP8* and *USP48*.

USP48 Pathogenic Variants Affect the Structure of the Catalytic Domain

An analysis performed in an in silico 3D model of the catalytic subunit of USP48 protein revealed that Met415 is positioned on a beta sheet of the "palm" structure. The exchange of the large amino acid methionine to a smaller isoleucine (or valine) almost doubles the distance to the nearest opposite amino acids Ala182 and Phe103 that are situated on the "thumb" structure (from 3.16 Å to 7.24 Å for Ala182 and from 2.02 Å to 4.20 Å for Phe103; Fig. 1). It is also increased in the case of the valine exchange from 3.16 Å to 4.64 Å for Ala182 and from 2.02 Å to 4.57 Å for Phe103. This modification presumably leads to a conformational change of the typical "palm-finger-thumb" structure of the catalytic domain in a more open position. The

Table 1. Summary of the examined clinical data in the total patient cohort

	All ($n = 237$)	USP8 Mut+ ($n = 91$)	USP48 Mut+ ($n = 20$)	Wild Type ($n = 126$)
Sex F/M §	181/56	**84/7, $\chi^2 = 23.7$, $P < 0.001$**	17/3, $\chi^2 = 3.5$, $P = 0.05$	80/46
Age at diagnosis (years; mean ± SEM) #	45.1 ± 1.1	**38 ± 1.5, $P < 0.001$**	47.8 ± 3.3, $P = 0.5$	50.1 ± 1.5
Body mass index (kg/m²; mean ± SEM) #	29.7 ± 0.5	**31.1 ± 1, $P = 0.01$**	30.1 ± 2.3, $P = 0.4$	28.3 ± 0.6
Basal plasma ACTH (pg/mL; mean ± SEM) #	772.1 ± 376.4	928.7 ± 614.1, $P = 0.8$	148.6 ± 56.5, $P = 0.7$	739.7 ± 545.6
Basal serum cortisol (µg/L; mean ± SEM) #	34.4 ± 5.7	23.0 ± 1.2, $P = 0.09$	55.8 ± 33.1, $P = 0.7$	42.5 ± 10.8
Tumor size (mm; mean ± SEM) #	14.4 ± 0.7	**11 ± 0.8, $P < 0.001$**	**10.3 ± 1.8, $P = 0.01$**	17.2 ± 1.1

Differences in clinical parameters between the group carrying USP8 mutations (USP8 mut+) and USP48 mutations (USP48 mut+) and the group carrying neither USP8 nor USP48 mutations (wild type) were analyzed using either chi-square test (§) or Kruskal–Wallis test with Dunn's multiple comparison test (#). A *P* value <0.05 was considered statistically significant (bold).

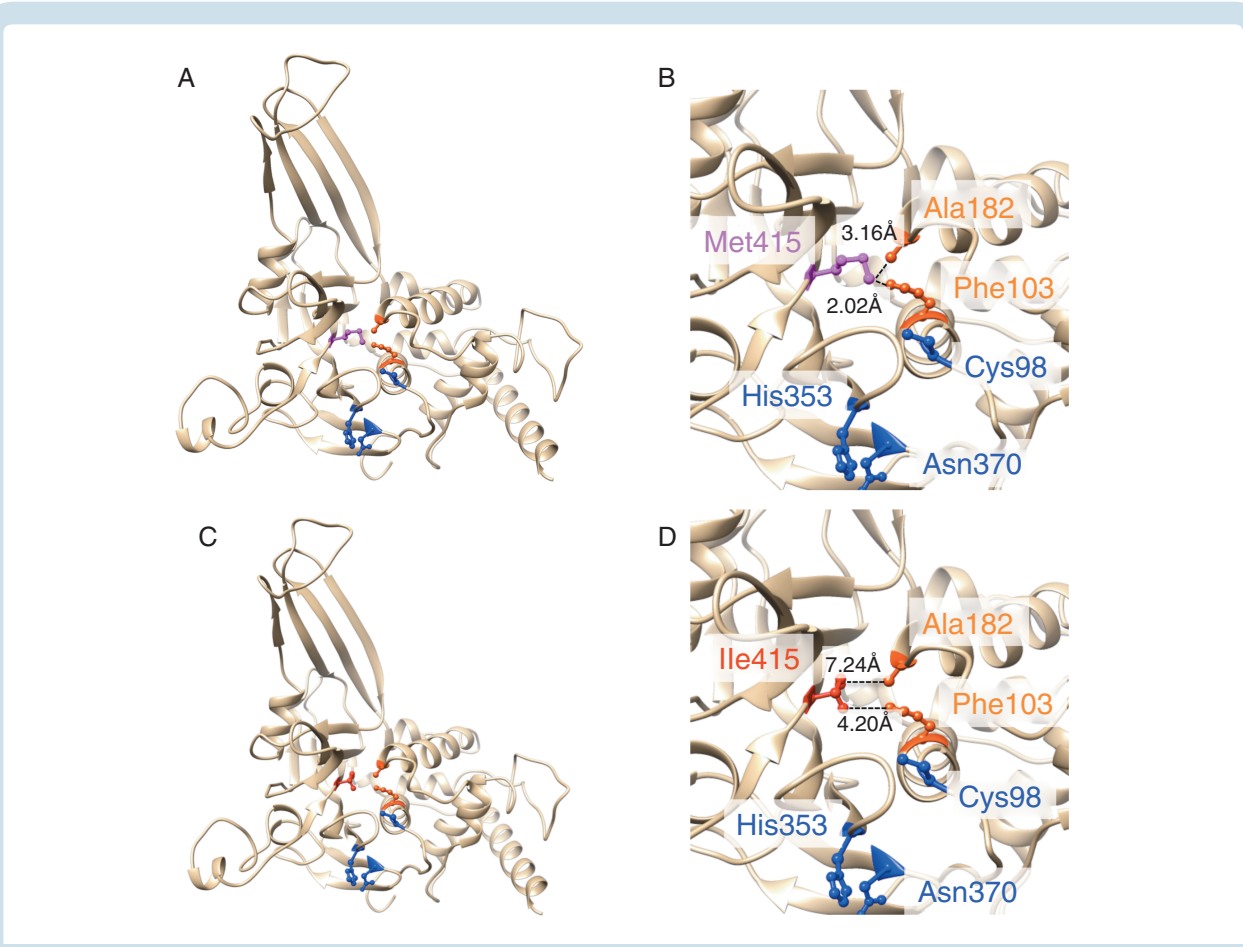

Fig. 1 Theoretical 3D model of the catalytic subunit of USP48 protein variants. Structure of the USP domain of USP48 for the wt (A and zoomed-in view in B) and the p.Met415Ile variant (C and zoomed-in view in D), developed using the SWISS-MODEL server based application (based on Uniprot sequence: Q86UV5) showing in violet Met415 and Ile415, respectively, and in orange the nearest neighboring amino acids, Ala182 and Phe103. The distance between the amino acids is represented by dotted lines and for Met415 amounts to 3.16 Å to Ala182 and 2.02 Å to Phe103, while it is increased for the mutated Ile415 to 7.24 Å to Ala182 and 4.20 Å to Phe103. The catalytic triad Cys98/His353/Asn370 is represented in blue in the foreground.

importance of this region for the USP structure is also underlined by the fact that the protein sequence in the USP48 region affected by the mutation is very well conserved between related deubiquitinases, like USP7 and USP18 (Supplementary Figure 3A). In those proteins, the amino acids homologous to the USP48 Met415 occupy the same position at the interface between a beta sheet of the "palm" and a double helix in the "thumb" of the catalytic subunit (Supplementary Figure 3B and C).

USP48 Pathogenic Variants Influence the Intensity and Specificity of Its Substrate Deubiquitination

Incubating purified FLAG-tagged proteins USP48 wt and p.Met415Ile mutant with K48-linked ubiquitin chains of 2–7 ubiquitin moieties in vitro showed decreased levels of long-chain ubiquitin oligomers in mutant compared with wt USP48 (Fig. 2A). Similarly, the mutant showed a higher deubiquitinase activity on K63 chains as well (Supplementary Figure 4). These data indicate that the mutant has a higher deubiquitinating activity and that the p.Met415Ile variant is an activating pathogenic variant.

We then analyzed the effect of the Met415Ile variant on the activity of USP48 toward its reported physiological substrates histone H2A[22,23] and Gli1.[24] Consistent with the effects on purified ubiquitin chains, the USP48-p.Met415Ile mutant decreased ubiquitination of H2A and Gli1 levels more efficiently than USP48 wt (Fig. 2B and C). Furthermore, USP48 overexpression was accompanied by increased Gli1 protein levels. These results indicate that the p.Met415Ile variant stimulates catalytic activity of USP48 and thereby enhances the deubiquitination of its substrates.

USP48 Pathogenic Variant Potentiates CRH-Induced ACTH Synthesis

Given the relative frequency of *USP48* pathogenic variant in CD tumors, we next examined the impact of the USP48-p.Met415Ile variant on tumor cell growth and ACTH secretion using the immortalized murine AtT-20 corticotroph tumor

Fig. 2 K-48 deubiquitinating activity of USP48 wt and pMet415Ile mutant. (A) Deubiquitination assay with immunopurified FLAG-tagged USP48 variants and purified K48-linked ubiquitin chains, containing 2 to 7 ubiquitin moieties. Upper panel shows the immunoblot of the deubiquitination reactions using anti-ubiquitin antibodies. Lower panel shows purified USP48 proteins. (B) Deubiquitination assay and purification of His^6-ubiquitinated histone H2A extracted from HeLa cells, transiently overexpressing FLAG-tagged USP48 variants and HA-tagged histone H2A. (C) Deubiquitination reaction using total extracts of cells transiently overexpressed HA-tagged Gli1 and FLAG-tagged USP48 variants in HeLa cells showing enhanced deubiquitination of Gli1 by mutant USP48 resulting in an increase in Gli1 levels. - = naive cells, WT = cells transfected with USP48 WT, Mut = cells transfected with USP48 Met415Ile mutant, CA: cells transfected with the Cys98Ala catalytically inactive USP48 variant.

cells. Neither wt nor mutant USP48 affected cell growth as determined by a colorimetric cell viability assay (Fig. 3A). Overexpression of USP48-p.Met415Ile had a small but significant stimulatory effect on human *POMC* promoter activity (139% ± 23% vs mock transfected, $P < 0.05$; Fig. 3B) and ACTH secretion (140% ± 4%, $P < 0.05$; Fig. 3C). In contrast, USP48 wt overexpression did not affect ACTH synthesis.

As the stimulatory action of mutant USP48 on basal ACTH synthesis was only modest, we examined whether it influences the tumor response to CRH, which is the physiological corticotroph trophic factor. AtT-20 cells displayed a weak response to 100 nM CRH treatment (138% ± 7% for ACTH and 135% ± 5% for *POMC* promoter activity vs untreated, $P < 0.05$; Fig. 3D and E). Overexpression of USP48 wt potentiated the stimulatory CRH action (173% ± 13% for ACTH secretion and 171% ± 15% for *POMC* promoter activity vs CRH treated mock transfected controls, $P < 0.01$; representative example in Fig. 3D and E). This was further amplified by overexpressing the USP48-p.Met415Ile mutant (214% ± 23% for POMC promoter activity and 209% ± 21% for ACTH, $P < 0.01$; Fig. 3D and E).

Knocking down *Gli1* suppressed basal human *POMC* promoter activity (75% ± 17% suppression) and abolished the stimulatory action of CRH in cells overexpressing mock or the various USP48 forms. In addition, USP48-p.Met415Ile overexpression did not increase human *POMC* promoter activity in si*Gli1* cells contrary to what was observed in cells transfected with scrambled control or si*Gli2* (Fig. 3F).

Recurrent TP53 Pathogenic Variants in Corticotroph Adenomas

Exome sequencing revealed somatic pathogenic variants in the *TP53* gene in 6 out of 18 (33%) USP8 wt CD tumors (including all 3 tumors from patients with Nelson syndrome; S16–S18; Supplementary Tables 1 and 3). The Nelson tumors presented with: one deletion of a big fragment of chr17 consistent with a probable copy number loss (chr17:g.7576876-7577303del) and 2 stop codons in exons 7 and 8, respectively (chr17:g.7577567A>T; p.Cys238Stop and chr17:g.7577127C>A; p.Glu271Stop). The *TP53* variants in the other 3 CD tumors were point amino acid exchanges. Two were already described as pathogenic in a germline setting (chr17:g.7577548G>A; c.733G>A; p.Gly245Ser; rs28934575 and chr17:g.7577120G>A; c.818G>A; p.Arg273His; rs28934576). The third (chr17:g.7578227G>C; c.622G>C; p.Asp208His) affected the Asp208, which as part of the S6–S7 loop of p53 DNA binding domain is modulated by the interaction with DNA and is predicted by computational analyses to be a regulatory site for p53 transcription-independent functions.[25] We were not able to determine the *TP53* mutational status in the remainder of our cohort, as sequencing the entire open reading frame would require large amounts of DNA not possible to obtain from the majority of our CD tumor samples.

FAT1 and BRAF Pathogenic Variants Are Not Frequent in Corticotroph Adenomas

Previous whole exome sequencing efforts have reported genetic variants in 2 additional genes: *FAT1* and *BRAF*. *FAT1* variants were initially detected in exons 21 and 27 in 2 out of 10 corticotroph tumors.[9] However, targeted sequencing on exons 21, 25, 26, and 27 in 62 corticotroph tumors revealed no pathogenic variants on these regions of the *FAT1* gene. The *BRAF* pathogenic variant V600E was reported in 15/91 (16.5%) CD tumors.[16] In our series, sequencing 94 corticotroph tumors revealed only one tumor with *BRAF* V600E variant from a female CD patient with a pituitary macroadenoma.

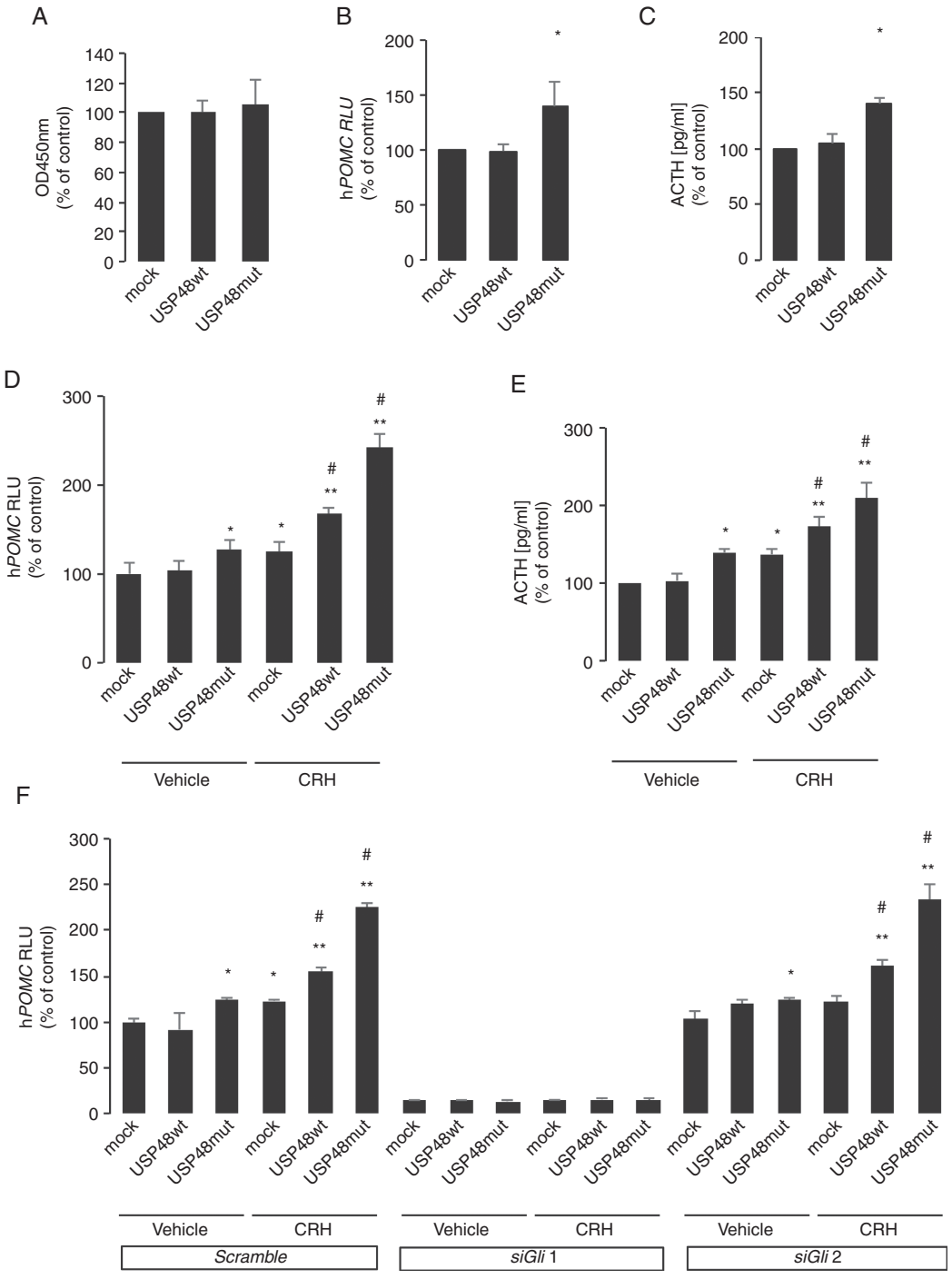

Fig. 3 USP48 potentiates CRH-induced *POMC* promoter activity and ACTH secretion. (A) Cell viability in AtT-20 cells transfected with USP48 wt and Met415Ile mutant. (B) Basal *POMC* promoter activity presented as percentage of empty vector (mock) control. Data are luciferase/β-galactosidase ratio, means of 3 experiments with each transfection condition done in triplicates. (C) Basal ACTH secretion in cells overexpressing USP48 wt and mutant determined by radioimmunoassay and expressed as percentage of empty vector control. Means of 7 experiments, with each condition in each experiment done in quadruplicates. $^{*}P < 0.05$. Representative experiment showing the impact of USP48 wt and mutant on (D) *POMC* promoter activity and (E) ACTH secretion induced by 100 nM CRH treatment for 6 and 24 hours, respectively. Representatives of 3 and 4 independent experiments, respectively. Data are percentage of mock vehicle treated control. $^{*}P < 0.05$ and $^{**}P < 0.01$ to mock vehicle treated control, $^{\#}P < 0.01$ to mock CRH treated. (F) Impact of *Gli1* knockdown with RNA interference on basal and CRH-induced *POMC* promoter activity in cells overexpressing USP48 wt or Met415Ile mutant. Cells were transfected for 48 hours and treated with 100 nM CRH for 6 hours. Data are percentage of mock and scramble transfected, vehicle treated control. Representative of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ to mock, scramble, vehicle treated control, $^{\#}P < 0.01$ to mock CRH treated.

Discussion

USP8 mutations are present on average in ~50% of corticotroph tumors,[9,10,14,15] leaving the rest with unknown genetic defect. Exome sequencing efforts have suggested the presence of additional genetic hotspots in the *USP48*,[16] *BRAF*,[16] and *FAT1* genes. In the present study, we performed whole exome sequencing on *USP8* wt corticotroph tumors and identified potential driver mutations in the *USP48* as well as the *TP53* genes. In contrast, analysis of 94 corticotroph tumors revealed the *BRAF* V600E pathogenic variant in only one case.

Two independent sequencing studies, Chen et al's and ours, discovered a single mutational hotspot (Met415) in the *USP48* gene in 10–20% of corticotroph tumors. *USP48*, like *USP8*, encodes for a deubiquitinase. USP48 was first identified in the rat brain as synUSP[26] and one year later in humans as USP31[27] (not to be confused with the newly designated USP31; Uniprot ID Q70CQ4). USP48 contains the USP domain at the N terminus followed by 3 domains present in ubiquitin-specific proteases (DUSP) and a ubiquitin-like domain (ULD) at the C end (Fig. 1A).[27] ULD motifs are widely spread in a structurally and functionally heterogeneous group of proteins from proteasomal shuttle factors to E3 ligases like Parkin as well as DUB enzymes.[28] The ULDs are neither processed nor conjugated to other cellular proteins and are involved in a diverse series of cellular functions, but most of them share the ability to interact with the 19S regulatory particle of the 26S proteasome.[28]

The pathogenic variants found in CD concentrate on a single amino acid (Met415) and lead to Met415Ile or Met415Val amino acid exchange. The Met415 amino acid is located in a region of the catalytic domain that is most conserved between related deubiquitinases. This region consists of one beta sheet and is part of the "palm" structure of the catalytic domain that is adjacent to the double helices of the "thumb" structure of the deubiquitinase. The "palm-finger-thumb" conformation of the catalytic subunit is characteristic for most deubiquitinases and is essential for "picking" ubiquitin chains from the ubiquitinated substrates.[29] Structural 3D predictive analyses revealed that pathogenic variants found in corticotroph tumors could change the distance between the affected Met415 and the neighboring amino acids, probably leading to a more open conformational change of the catalytic domain and resulting in higher deubiquitinating activity. Indeed, our in vitro assays revealed enhanced deubiquitinase activity of the USP48-p.Met415Ile variant on the previously reported USP48 substrate H2A.[22]

In an in vitro corticotroph tumor cell model, the USP48-p.Met415Ile variant displayed a moderate stimulatory effect on basal ACTH synthesis, but strongly potentiated the stimulatory action of CRH. During the last decades the establishment of monoclonal origin of corticotroph tumors and the finding of infrequent genetic defects in genes expressing its receptor (*CRHR1*) and downstream cAMP pathway (*GNAS1*, *PRKAR1A*) diminished the role of hypothalamic stimulation in corticotroph tumorigenesis.[7,30] It is therefore of interest to show that somatic activating pathogenic variants in the *USP48* gene may induce corticotroph tumorigenesis by enhancing corticotroph tumor response to hypothalamic CRH stimulation.

Among the potential USP48 substrates, Gli1 is of particular interest for corticotroph pathophysiology.[24] Gli1 (glioma-associated oncogene) is the downstream mediator of sonic hedgehog (SHH) signaling that is deregulated in corticotroph tumors.[31] We have previously shown that in adult corticotroph cells, CRH signaling is potentiated by the SHH/Gli1 pathway to stimulate ACTH synthesis.[19] Herein we show that Gli1 is pivotal for USP48 action on CRH-induced ACTH synthesis and suggest that the USP48 mutants decrease Gli1 ubiquitination that in turn stabilizes and amplifies the stimulatory action of CRH on ACTH synthesis.

It is notable that both deubiquitinases mutated in corticotroph tumors, USP8 and USP48, have among their targets members of the SHH pathway: Smoothened[32] and Gli1, respectively. SHH is a key regulator of pituitary stem/progenitor cells[33] and deregulated in corticotroph tumors; therefore, it is possible that the 2 mutational hotspots hijack the same pathway to trigger corticotroph tumorigenesis.

An unexpected finding in our study was the presence of *TP53* somatic pathogenic variants in 6 out of the 18 samples that have been exome sequenced (33%). All variants led to either deletion of a complete allele or to a truncated protein or were point variants that have been already described in a germline setting to be malignant due to loss of function.[34] The effect of homozygous TP53 defects on tumorigenesis in general is clear. The effect of the exclusively heterozygous somatic pathogenic variants alone cannot be easily predicted, especially as they do not occur simultaneously with the more ubiquitous *USP8* pathogenic variants. Nevertheless, these variants lead to the inactivation of one of the *TP53* alleles and thus decrease the cellular response to other oncogenes.[34] Also clinicopathologically, the tumors carrying the TP53 mutations showed signs that they are more aggressive with mostly higher Ki67 indices (>5) and trans-sphenoidal surgery number (>2). Interestingly, 4 of the patients carrying somatic *TP53* pathogenic variants also had recurrent pathogenic variants in 2 genes encoding for the chromatin remodeling complex protein DAXX (death domain-associated protein) and its interaction partner ATRX (alpha thalassemia/mental retardation syndrome X-linked).[35] *TP53* pathogenic variants have been up to now only rarely described in corticotroph tumors that were aggressive in nature and evolved to carcinomas.[36] Two other exome sequencing studies[9,16] have also described inactivating somatic *TP53* pathogenic variants in corticotroph tumors, but with lower prevalence (1/10 and 1/22, respectively). The higher incidence of *TP53* variants in our exome sequencing study may be due to a bias that we inevitably introduced when selecting exclusively *USP8* wild type tumors (which tend to be larger[14]) and favoring bigger macroadenomas (>10 mm). Fig. 4 provides a summary of the hypothesized mechanisms leading to CD tumor formation and ACTH hypersecretion.

In our relatively large cohort of 94 corticotroph tumors, *BRAF* V600E variant was an extremely rare event, found in only one case of a female patient with

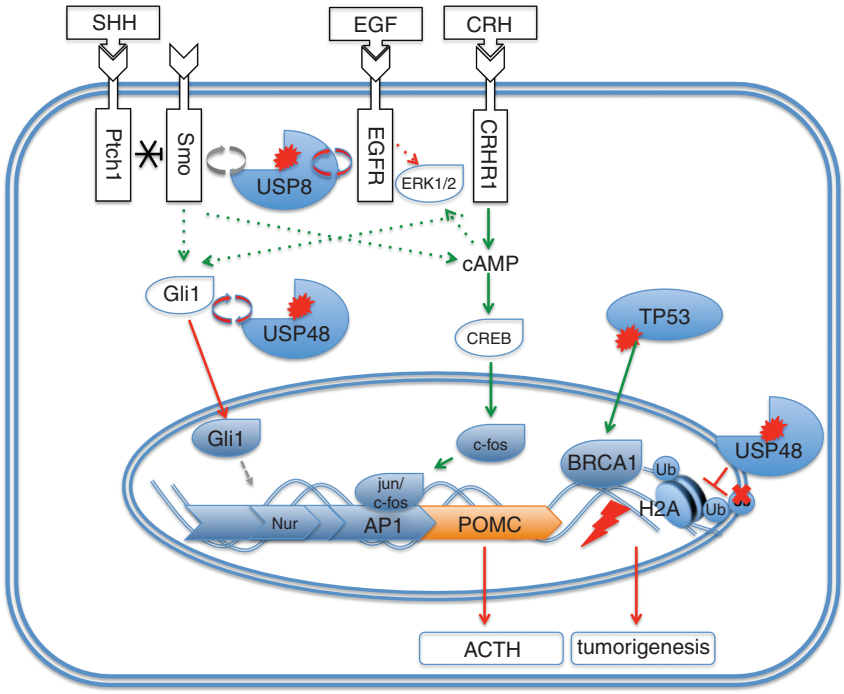

Fig. 4 Hypothesized mechanisms leading to corticotroph tumor formation involving the different recurrently mutated genes discovered in CD. The 3 genes most frequently mutated in CD (*USP8*, *USP48*, and *TP53*) are shown in the context of (patho)physiological corticotroph cell regulation. CRH acts on Nur and AP1 binding elements on the *POMC* promoter to activate transcription downstream to the cAMP/extracellular signal-regulated kinase (ERK) signaling pathways. Activated epidermal growth factor receptor acts via ERK1/2 to stimulate *POMC* transcription; activating USP8 mutations potentiate this effect by deubiquitinating and rescuing the receptor from lysosomal degradation.[13] SHH binding to Patched (Ptch1) relieves suppression of Smoothened (Smo) allowing for Gli1 activation; SHH and CRH crosstalk at Gli1 level to stimulate *POMC* transcription and ACTH secretion.[19] We hypothesize that activating mutations in the *USP48* deubiquitinase lead to increased levels of Gli1, which enhances basal and CRH-induced *POMC* transcription via an unknown at present mechanism. In *Drosophila* S2 cells, USP8 was shown to prevent Smo ubiquitination.[32] Activating *USP48* mutations may also lead to increased deubiquitination of histone H2A and thus to a decreased recruitment of DNA repair factors in case of DNA damage and increased tumorigenesis potential. This effect can also be triggered by inactivating mutations in *TP53*.[40] Green lines represent physiological, red lines pathological activity, and gray lines mechanism of action shown in other cell systems but not yet proven in corticotroph tumor cells. Dotted lines present indirect effects.

a macroadenoma. This is in clear contrast to a recent study that reported this pathogenic variant in 16.5% of corticotroph tumor cases.[16] It has to be noted that this is the only report of *BRAF* pathogenic variants in CD, whereas other sequencing efforts using both Caucasian and Asian populations did not find *BRAF* pathogenic variants in CD tumors and particularly with such relatively high incidence.[9,10,37] Similarly, although the *BRAF* V600E variant is frequent in papillary craniopharyngiomas,[38] it has been only rarely described in pituitary tumors and never within the context of CD.[39]

In conclusion, our data corroborate the presence of a second mutational hotspot in another deubiquitinase coding gene, the *USP48*, in 10% of USP8 wt corticotroph tumors. We showed that USP48 pathogenic variants have increased catalytic activity that leads to enhanced deubiquitination of its physiological substrates Gli1 and H2A. We provide evidence that Gli1 is at least in part responsible for the increased ACTH synthesis in USP48 mutant corticotroph cells, while we suspect that USP48-induced H2A deubiquitination dissociates it from DNA damage repair proteins and deregulates DNA repair. In addition, we demonstrate in a large number of 94 cases that *BRAF* V600E variants are an extremely rare event in corticotroph tumors. In contrast *TP53* pathogenic variants may be relatively more frequent than previously assumed, especially in larger tumors.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

Cushing's disease | genome sequencing | driver mutations | USP48 | TP53

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