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Alterations in Protein Kinase A Substrate Specificity as a Potential Cause of Cushing's Syndrome

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1 **Abstract**

2 Cushing's syndrome is a severe endocrine disorder of cortisol excess, associated with major
3 metabolic and cardiovascular sequelae. We recently identified somatic mutations in the gene
4 (*PRKACA*) encoding the catalytic α ($C\alpha$) subunit of protein kinase A (PKA) to be responsible
5 for cortisol-producing adrenocortical adenomas (CPAs), which are a major cause of
6 Cushing's syndrome. In spite of previous studies on the two initially identified mutations
7 (L206R, 199_200insW), the mechanisms of action of the clinically highly relevant *PRKACA*
8 mutations remain poorly understood. Here, by investigating a large panel of *PRKACA*
9 mutations including all those identified so far in Cushing's syndrome, we unexpectedly find
10 that not all mutations interfere with the binding of regulatory (R) subunits as previously
11 hypothesized. Since several mutations lie in a region of PKA $C\alpha$ involved in substrate
12 recognition, we have investigated their consequences on substrate specificity by quantitative
13 phosphoproteomics. We find that all three mutations analyzed (L206R, 200_201insV and
14 d244-248+E249Q) cause major changes in the preference of PKA for its targets, leading to
15 hyperphosphorylation of several PKA substrates, including most notably histone H1.4 at
16 Ser36, which is required for and promotes mitosis. This is reflected by a 9-fold
17 hyperphosphorylation of H1.4 in CPAs carrying the L206R mutation. Thus, our findings
18 suggest that besides hampering binding to R subunits, *PRKACA* mutations act via altering
19 PKA substrate specificity. These findings shed new light on the molecular events leading to
20 Cushing's syndrome and provide an illustrative example of how mutations altering substrate
21 specificity of a protein kinase might cause human disease.

22 Introduction

23 Cushing's syndrome is an endocrine disease characterized by cortisol excess. If not
24 promptly recognized and treated, it is associated with severe morbidity and increased
25 mortality, mostly due to metabolic and cardiovascular sequelae, such as diabetes mellitus,
26 osteoporosis and hypertension (1,2). Beside ACTH-producing pituitary adenomas, cortisol-
27 producing adenomas of the adrenal are the major cause of endogenous Cushing's
28 syndrome (3).

29 Protein kinase A (PKA) is the main intracellular mediator of the ubiquitous second
30 messenger cyclic AMP (cAMP), which is produced in response to several hormones and
31 neurotransmitters (4,5). In its inactive state, PKA is a tetrameric complex composed of two
32 regulatory (R) and two catalytic (C) subunits (5), which exist in different isoforms – three for
33 C (α , β , γ) and four for R (I α , I β , II α , II β) subunits, each encoded by a separate gene (6).
34 Additionally, protein kinase X (PrKX) can also form holoenzymes with R subunits (7). In the
35 inactive complex, two short linear segments emanating from each R subunit, known as
36 inhibitory sequences, act as substrates (RII) or pseudo-substrates (RI) that occupy the active
37 site clefts of the C subunits, thus preventing the access of other substrates (5,8,9); this
38 keeps PKA in the inactive state (5,8,9). Binding of cAMP to two distinct sites on each R
39 subunit induces their dissociation from the C subunits, allowing the C subunits to
40 phosphorylate a large number of PKA substrates located in both the cytosol and the nucleus
41 (5,8,9).

42 Our group has recently discovered that somatic mutations in the gene coding for the C α
43 subunit of PKA (*PRKACA*) are responsible for Cushing's syndrome due to cortisol-producing
44 adrenocortical adenomas (CPAs) (10-13). These findings have been independently
45 confirmed by other groups (14-18). Very recently, rare mutations in the gene coding for the
46 C β subunit of PKA (*PRKACB*) have also been found in CPAs (19). Between 23 and 67% of
47 all CPAs carry a recurrent mutation (L206R) of a residue in the C α subunit that is involved in
48 the interaction with the R subunits (10,11,14-16). By screening a large group of CPAs, we

49 have in the meantime identified six additional *PRKACA* mutations (199_200insW,
50 S213R+insIILR, 200_201insV, W197R, d244-248+E249Q and E32V) in unilateral adenomas
51 of patients affected by overt Cushing's syndrome (10-12). With one exception (E32V), all
52 identified mutations lie in a hot spot region on the surface of the C α subunit that is adjacent
53 to the active site cleft and faces the R subunit (12).

54 Whereas all previous studies provide strong evidence for a causal role of *PRKACA*
55 mutations, the mechanisms linking these mutations to increased cell proliferation and cortisol
56 secretion in adrenocortical cells are still debated (10,14-16). One of the initial studies
57 suggested that the L206R mutation increases PKA activity by enhancing substrate
58 interaction and/or the catalytic activity of the enzyme (14). In contrast, two other studies
59 suggested that the L206R mutation might prevent binding of the R subunit (15,16). In strong
60 support of this possibility, we subsequently demonstrated that the two initially identified
61 mutations (L206R and 199_200insW) interfere with the formation of a stable complex with R
62 subunits, thus rendering mutated C α subunits constitutively active (13). However, we did not
63 observe increased catalytic activity (13). Whereas these data indicate that a reduced
64 interaction with R subunits is likely involved in the pathogenesis of Cushing's syndrome – at
65 least in the case of the two investigated *PRKACA* mutations (L206R and 199_200insW) –
66 additional mechanisms might play an equally important role.

67 Here, we report a comprehensive functional characterization of all *PRKACA* mutations
68 identified so far. Unexpectedly, our results indicate that besides interfering with the binding
69 of R subunits, *PRKACA* mutations cause major changes in the preference of PKA for its
70 targets, leading to hyperphosphorylation of several PKA substrates, including most notably
71 histone H1.4 at Ser36, which is required for and promotes mitosis (20).

72 **Methods**

73 **Plasmids**

74 Plasmids encoding human $R1\alpha$, $R11\beta$ and $R11\beta$ -FLAG were described earlier (13). A plasmid
75 encoding human wild-type $C\alpha$ was purchased from Origene. Mutations in the coding
76 sequence of $C\alpha$ were introduced by PCR as previously described (10). To generate a
77 plasmid encoding a FLAG-tagged $R1\alpha$ subunit, a fragment containing the entire coding
78 sequence of the human $R1\alpha$ subunit was amplified by PCR with a forward primer containing
79 the FLAG tag (amino-acid sequence DYKDDDDA) and a HindIII site at the 5' and a reverse
80 primer containing a NotI site at the 3'. This fragment was subsequently cloned between the
81 HindIII and NotI sites of pcDNA3.

82

83 **Tumor tissue**

84 A total of 8 snap-frozen tumor specimens from CPAs were investigated. All samples were
85 collected at the University Hospital of Würzburg. Six of these samples had been included in
86 previous reports from our group: three in Ronchi et al. (12) and three in Beuschlein et al.
87 (10). According to our previous sequencing data, four CPA samples harbored somatic
88 *PRKACA* mutations. In particular, three samples from patients with overt Cushing's
89 syndrome presented a L206R substitution and one sample from a patient with mild
90 autonomous cortisol secretion (subclinical Cushing's syndrome) presented a d244-
91 248+E249Q mutation. The four remaining CPAs were *PRKACA* wild-type. The collection of
92 the clinical data and the biomaterial for this retrospective study was approved by the ethics
93 committee of the University of Würzburg (approvals no. 93/02 and 88/11). Written informed
94 consent was obtained from all patients.

95

96 **Cell culture and transfection**

97 HEK293A cells were obtained from ATCC. Cells were cultured in Dulbecco's modified
98 Eagle's medium (DMEM) supplemented with 10% FCS, 0.1 mg/ml streptomycin and 100

99 U/ml penicillin at 37 °C, 5% (vol/vol) CO₂. HEK293A cells were seeded at a density of
100 3.5x10⁶ cells/15-cm Petri dish, 1.2 x 10⁶ cells/10-cm Petri dish or 0.25 x 10⁶ cells/well onto 6-
101 well plates and allowed to grow for 24 h, after which they were transfected with the Effectene
102 transfection kit (Qiagen) according to the manufacturer's protocol. A 1:8 ratio of transfected
103 C and R subunit DNAs was used to favor association between C and R subunits (10). All
104 experiments were performed 48 h after transfection. For SILAC labeling, the cells were
105 grown in light (Lys0, Arg0) or heavy (Lys8, Arg10) labeled DMEM supplemented with 10%
106 dialyzed FCS and 0.1 mg/ml streptomycin and 100 U/ml penicillin for at least 6 passages
107 before the experiment.

108

109 **Preparation of cell lysates**

110 Cells were washed with ice-cold phosphate-buffered saline (PBS), scraped off plates, and
111 resuspended in 300 µl 5/2 buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4). Thereafter, samples
112 were homogenized using an Ultraturrax device for 20 s on ice and centrifuged at 50,000 x g
113 for 30 min at 4 °C to remove membranes.

114

115 **Preparation of total cell lysates**

116 Cells were washed once with PBS. Then, 2 ml of lysis buffer (8M urea, 1 mM sodium
117 vanadate, 2.5 mM pyrophosphate, 1 mM β-glycerophosphate, 20mM HEPES, pH 8.0)
118 supplemented with the cOmplete Mini EDTA-free protease inhibitor cocktail (Roche) were
119 added to each 15-cm Petri dish and the cells were scraped into the buffer. Cell suspensions
120 were sonicated three times for 15 sec each and lysates were cleared by centrifugation at
121 4,000 rpm for 1 h at 15 °C.

122

123 **PKA activity assay**

124 PKA catalytic activity was measured on cell lysates using the PepTag non-radioactive
125 cAMP-dependent protein kinase assay (Promega), which uses fluorescent kemptide as a

126 substrate, following the manufacturer's instructions. Images of the gels were acquired with a
127 gel documentation system (Herolab) and analyzed using the ImageJ software
128 (<http://rsbweb.nih.gov/ij>). Endogenous PKA activity was subtracted and values were
129 normalized to the value of the stimulated wild-type.

130

131 **Co-immunoprecipitation**

132 Cells were lysed with a buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%
133 Triton X-100, 20 mM Tris-HCl, pH 7.5, supplemented with cComplete Mini protease inhibitor
134 cocktail (Roche). Lysates were centrifuged at 20,000 x *g* for 10 min at 4 °C. Supernatants
135 were transferred to a new vial and incubated with protein-A sepharose for 2 h at 4 °C under
136 continuous rotation. Protein-A sepharose was preincubated with a mouse monoclonal anti-
137 FLAG antibody (Sigma-Aldrich, # F3165). Preincubations were performed overnight at 4 °C
138 under continuous rotation. After the incubation of protein-A sepharose with the cell lysates,
139 samples were washed 5 times with lysis buffer. Proteins were recovered from protein-A
140 sepharose by addition of Laemmli buffer, followed by incubation at 95 °C for 3 min. After
141 Western blot analysis and quantification, the amount of C α was normalized to that in the
142 wild-type sample.

143

144 **Histone extraction**

145 Histones were acid-extracted from cells and CPA samples as described by Shechter et al.
146 (21). Briefly, harvested cells or Douncer-homogenized tissue samples were lysed in a
147 hypotonic lysis buffer (150 mM Tris/HCl, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, pH 8.0)
148 supplemented with 0.5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 0.1 mM phenylmethylsulfonyl
149 fluoride (PMSF), the cComplete Mini protease inhibitor cocktail (Roche) and a phosphatase
150 inhibitor cocktail (Roche). Lysates were then acid-extracted with H₂SO₄ for 30 min followed
151 by precipitation with trichloroacetic acid and resuspension in H₂O.

152

153 **Western blot analysis**

154 Samples were mixed with Laemmli buffer and incubated at 95 °C for 3 min. Proteins were
155 separated by electrophoresis on either a 10% or 15% (for histone H1.4) SDS polyacrylamide
156 gel and electro-transferred to a PVDF membrane (Merck Millipore). Membranes were
157 blocked with Tris-buffered saline supplemented with 1% Tween and 5% skim milk powder or
158 bovine serum albumin (antibodies detecting phosphoproteins) for 1 hour at RT, and
159 incubated overnight at 4 °C with the indicated primary antibody, followed by incubation with
160 horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. PKA C α subunits
161 were detected with a rabbit polyclonal antibody (Cell Signaling #4782, 1:7,000 dilution).
162 FLAG-tagged R subunits were detected using a rabbit monoclonal anti-FLAG antibody
163 (Sigma-Aldrich, #F7425, 1:4,000 dilution). PKA RI α subunits were detected using a mouse
164 monoclonal antibody (BD Transduction Lab., #610609, 1:1,000 dilution) and RII β subunits
165 with a mouse monoclonal antibody (BD Transduction Lab., #610625, 1:2,000 dilution).
166 Phosphorylated PKA substrates were detected using a rabbit polyclonal antibody (Cell
167 Signaling, #9621, 1:1,000 dilution). A rabbit polyclonal antibody (ThermoFisher, #PA5-
168 31908, 1:2,000 dilution) and a rabbit polyclonal antibody (ThermoFisher, #PA5-31907,
169 1:1,000 dilution) was used for total histone H1.4 and Ser36 phosphorylated histone H1.4,
170 respectively.

171

172 **Substrate specificity of PKA C α mutants *in silico***

173 The *in silico* prediction was done using the Kinase Substrate Prediction v 2.0 algorithm (22).
174 This algorithm predicts substrate specificity based on the primary amino acid sequence of
175 more than 488 human protein kinase catalytic domains and 10,000 known kinase–substrate
176 phosphosite pairs. The algorithm computes a specificity matrix for a given kinase, or mutant
177 thereof, where values indicate the relative preference for each amino acid at each position
178 around the phosphoacceptor site. Only relevant changes are reported.

179

180 **Substrate specificity of PKA C α mutants *in vitro***

181 Mutant and wild-type samples were labelled with heavy and light amino acids as described
182 in cell culture and transfection. Before preparing total cell lysates, the cells were stimulated
183 with 10 μ M forskolin for 30 min. Protein concentration of the total cell lysates were
184 determined using the BCA assay. Before processing the samples for phosphoproteomics, an
185 input sample for proteome analysis was taken, which was digested with trypsin and analysed
186 by nano LC-MS/MS. For phosphoproteomics experiments, no more than 20 mg protein were
187 used. Substrate specificity was assessed using the PTMScan Phospho-PKA Substrate Motif
188 (RRXS*/T*) Kit (Cell Signaling, #5565) following the manufacturer's instructions – see
189 Supplementary Data (23) for details. The dried peptides were stored at -20°C until the nano
190 LC-MS/MS analysis.

191

192 **Nano LC-MS/MS analysis**

193 Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS)
194 analyses were performed on an Orbitrap Fusion system (Thermo Scientific) equipped with
195 an EASY-Spray Ion Source and coupled to an EASY-nLC 1000 (Thermo Scientific).
196 Peptides, resuspended in 2% acetonitrile and 0.1% formic acid, were loaded on a trapping
197 column (2 cm x 75 μ m ID, PepMap C18, 3 μ m particles, 100 Å pore size) and separated on
198 an EASY-Spray column (50 cm x 75 μ m ID, PepMap C18, 2 μ m particles, 100 Å pore size)
199 with a 140-min linear gradient from 3% to 45% acetonitrile and 0.1 % formic acid.

200 Both MS and MS/MS scans were acquired on the Orbitrap analyzer with a resolution of
201 60,000 for MS scans and 15,000 for MS/MS scans. HCD fragmentation with 35% normalized
202 collision energy was applied. A Top Speed data-dependent MS/MS method with a fixed
203 cycle time of 3 s was used. Dynamic exclusion was applied with a repeat count of 1 and an
204 exclusion duration of 60 s; singly charged precursors were excluded from selection.
205 Minimum signal threshold for precursor selection was set to 5×10^4 . Predictive AGC was

206 used with AGC target values of 2×10^5 for MS scans and 5×10^4 for MS/MS scans. EASY-IC
207 was used for internal calibration.

208

209 **Raw data processing and database search**

210 For raw-data processing, database searches and SILAC quantification, the MaxQuant
211 software version 1.5.6.5 was used (24). The search was performed against the human
212 UniProt reference proteome database (download date: 2016-12-09) plus a small database
213 containing known immunoglobulin chains. Additionally, a database containing common
214 contaminants (included in MaxQuant) was used. The search was performed with tryptic
215 cleavage specificity and 3 allowed miscleavages. Results were filtered to a false-discovery
216 rate (FDR) <1% on protein, peptide and phosphosite level and for modified peptides a
217 minimum score of 40 and a minimum delta score of 6 were required. In addition to default
218 settings, protein N-terminal acetylation, Gln to pyro-Glu formation (N-term. Q), oxidation (M)
219 and phosphorylation (STY) were included as variable modifications; carbamidomethyl (C)
220 was set as fixed modification. For SILAC quantification, light (Arg0/Lys0) and heavy
221 (Arg10/Lys8) labeling were selected, with max. 4 labeled amino acids per peptide allowed;
222 matching between runs was disabled.

223 For further data analysis in R, the MaxQuant "Phospho (STY) Sites" table was processed,
224 and only phosphosites with a localization probability >0.75 and a PEP < 0.05 have been
225 included. For each experiment, the distribution of log₂-transformed heavy/light SILAC ratios
226 were normalized to the first mode of the distribution. Data from replicate experiments were
227 combined, and Student's t-test as well as limma p-values were calculated and corrected for
228 multiple-hypothesis testing (Benjamini-Hochberg algorithm, FDR). In addition, boxplot
229 outliers with values outside of the 1.5x and/or the 3x interquartile range (IQR) of the 1st or
230 3rd quartile (Q1 or Q3) were labeled as significant or highly significant, only if a replicate
231 notch, defined as $1.58 \cdot \text{IQR} / \sqrt{\text{number of experiments}}$, was not overlapping with the
232 median of all such notches.

233

234 **Motif logo**

235 For calculation of position weight matrices from the phosphoproteomics data, the web tool
236 plogo (<http://plogo.uconn.edu/>) (25) was used. Values were calculated relative to the
237 background amino acid frequencies in the human proteome. Motif logos were then
238 generated using the Weblogo software version 3.5.0 (26).

239

240 **Structures**

241 Protein structures were generated using PyMol version 1.7.4.5. PDB entries 3TNP (9) and
242 3TNQ (9) were used as templates for the structures of the PKA C α -RII β holoenzyme and
243 the C α subunit in complex with a phosphorylated substrate, respectively.

244

245 **Statistical analyses**

246 Statistical analyses were performed using the Prism 6 software (GraphPad). Values are
247 given as mean \pm s.e.m. Differences between two groups were assessed by two-tailed
248 Student's t-test. Differences among three or more groups were assessed by two-way
249 analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Differences were
250 considered significant for P values < 0.05 .

251 **Results**

252 ***PRKACA* mutations have variable effects on R subunit binding**

253 Through genetic screening of a large series of CPAs from patients with Cushing's syndrome,
254 we have identified a total of 7 *PRKACA* mutations, including the most frequent L206R, with
255 an overall prevalence in CPAs of about 40% (10-12). The results of all available studies to
256 date are summarized in Table 1. With the exception of E32V, all mutations identified so far
257 lie at a hot spot at the interface with the R subunit (Fig. 1). Since our previous data indicated
258 that the two initially identified mutations (L206R and 199_200insW) interfere with the
259 formation of a stable complex with the inhibitory RII β subunit (13), we first evaluated the
260 interaction of all mutations identified so far with both RI α and RII β – the two main R subunit
261 isoforms expressed in adrenocortical cells – by co-immunoprecipitation (Fig. 2 and
262 Supplementary Data, Fig. S1 (23)).

263 As expected, we observed a robust co-immunoprecipitation of exogenously expressed wild-
264 type C α with both RI α and RII β (Fig. 2 and Supplementary Data, Fig. S1 (23)). In the case
265 of four mutants (199_200insW, L206R, 200_201_insV and S213R+insIILR), we detected
266 only faint signals in the presence of either RI α or RII β , indicating that the interaction with
267 both R subunit isoforms was largely lost, consistent with our previous observations (13).
268 Unexpectedly, however, two mutants (W197R and E32V) displayed normal binding to both
269 RI α and RII β (Fig. 2, arrows). Of note, a previous report indicates that the W197R mutant
270 cannot bind to R subunits unless they are stripped off of cAMP, a condition that is not
271 occurring in intact cells (27). The d244-248+E249Q mutant had an interesting behavior as it
272 showed normal binding to RII β but a complete lack of binding to RI α (Fig 2, arrow). With the
273 exception of S213R+insIILR, all C α mutants dissociated virtually completely from R subunits
274 upon addition of cAMP (Fig. 2 and Supplementary Data, Fig. S1 (23)).

275

276 ***PRKACA* mutations have variable effects on PKA activity**

277 Next, we investigated the activity of the $C\alpha$ mutants against the widely used synthetic
278 peptide substrate kemptide. In lysates obtained from HEK293A cells co-transfected with
279 wild-type or mutant $C\alpha$ subunits and either $R1\alpha$ or $R11\beta$, we observed a high variability among
280 the investigated $C\alpha$ mutants. A first group of mutants (199_200insW, L206R, W197R, d244-
281 248+E249Q and S213R+insIILR) was characterized by increased basal activity against
282 kemptide at least in one condition (i.e. with $R1\alpha$ or $R11\beta$) (Fig. 3). The E32V mutant was
283 apparently behaving like wild-type $C\alpha$ (Fig. 3). The 200_201insV mutant was characterized
284 by a strongly reduced maximal activity (measured in the presence of cAMP) against
285 kemptide compared to wild-type $C\alpha$ (Fig. 3).

286 Unexpectedly, a different picture was observed when PKA activity was evaluated against
287 endogenous substrates in lysates of HEK293A cells transfected with the different $C\alpha$
288 mutants and analyzed by Western blot analysis with an anti-phospho-PKA substrate
289 antibody (Supplementary Data, Fig. S2 (23)). In this assay, $R11\beta$ but not $R1\alpha$ co-expression
290 was capable of buffering the activity of exogenous wild-type or mutant $C\alpha$ subunits,
291 consistent with previous observations (9). Therefore, we focused on the results obtained in
292 the presence of $R11\beta$. In cells co-transfected with $R11\beta$, two $C\alpha$ mutants (199_200insW and
293 L206R) showed strongly increased basal activity compared to wild-type $C\alpha$ (Supplementary
294 Data, Fig. S2 (23)). The remaining mutants were characterized by a variable degree of
295 increased basal activity compared to wild-type $C\alpha$ (Supplementary Data, Fig. S2 (23)).
296 Notably, in this assay, the 200_201insV mutant, which was characterized by a strongly
297 reduced maximal activity in the kemptide assay, could be stimulated by addition of the
298 adenylyl cyclase activator forskolin to a similar although slightly lower extent compared to
299 wild-type $C\alpha$ (Supplementary Data, Fig. S2 (23)). At a closer look, this analysis also
300 revealed potentially different phosphorylation patterns for the $C\alpha$ mutants compared to wild-
301 type $C\alpha$ as well as among the $C\alpha$ mutants (Supplementary Data, Fig. S2A, arrows (23)).
302 Altogether, these data suggested the possibility that the investigated *PRKACA* mutations

303 might alter the relative preference of PKA for its substrates, so that investigating a single
304 synthetic substrate does not provide a satisfactory description of the activity of C α mutants
305 against endogenous substrates.

306

307 ***In silico* analysis of *PRKACA* mutations predicts changes in substrate specificity**

308 Our results suggested the possibility that *PRKACA* mutations might alter the preference of
309 PKA for its substrates and, thus, cause a relative change in substrate specificity. This
310 hypothesis was consistent with the location of most *PRKACA* mutations in a region of C α
311 that is critical for substrate binding and recognition (27,28) (Fig. 1B). To further explore this
312 possibility, we performed an *in silico* analysis using a protein kinase substrate prediction
313 algorithm (22). The algorithm predicted relevant changes in substrate specificity for four
314 mutants: 199_200insW, L206R, d244-248+E249Q and 200_201insV (Fig. 1B and
315 Supplementary Data, Fig. S3 (23)). The largest change was predicted for the d244-
316 248+E249Q mutant. For the S213R+insIILR mutant, the algorithm predicted only minor
317 effects on substrate specificity, whereas no changes were predicted for the two remaining
318 mutants (E32V, W197R).

319

320 **Phosphoproteomics reveals major changes in substrate specificity caused by** 321 ***PRKACA* mutations**

322 Motivated by the results of the Western blot analysis and the *in silico* prediction, we used a
323 quantitative phosphoproteomics approach to comprehensively analyze and quantitatively
324 compare the substrate repertoires of wild-type and mutant PKA C α in mammalian cells. The
325 three PKA C α mutations predicted to have the largest effects on substrate specificity were
326 analyzed (Fig. 4). These included the frequent L206R mutation, the d244-248+E249Q
327 mutation, which was predicted to have the strongest effect on substrate specificity, and the
328 200_201insV mutation, which shared prediction with the adjacent 199_200insW mutation. All
329 cell lysates were obtained after 30-min stimulation with the adenylyl cyclase activator

330 forskolin to allow comparison under conditions of maximal PKA activity. The
331 phosphoproteomics analysis identified 463 sites phosphorylated by the wild-type C α subunit.
332 A direct quantitative comparison between wild-type and mutant C α subunits demonstrated
333 that the investigated *PRKACA* mutations caused major changes in the pattern of PKA
334 substrate phosphorylation (Fig. 4). These changes involved 62, 116 and 75 substrates that
335 were either hyper- or hypophosphorylated by the L206R, d244-248+E249Q and
336 200_201insV mutants, respectively, compared to wild-type (Fig. 4 and Supplementary Data,
337 Dataset S1 (23)). Our data also allowed us to compute sequence logos of the region
338 encompassing the phosphoacceptor site, where the size of the letters, each corresponding
339 to a given amino acid, indicates their relative frequency at a given position (Fig. 4). Overall,
340 the analysis showed relevant changes in the preferred amino acids surrounding the
341 RR/KXpS/T phosphorylation consensus sequence, including an increased preference for
342 amino acids with small or hydrophobic, non-aromatic side chains (glycine, alanine, valine,
343 methionine) at position +1 from the phosphoacceptor site, at the expense of phenylalanine
344 and other amino acids (Fig. 4). Moreover, we observed an increased preference for leucine
345 at position +1 from the phosphoacceptor site and an increased preference for acidic
346 residues in the residues downstream of the phosphoacceptor site for the L206R mutant,
347 consistent with a recent report by Lubner *et al.* in *E. coli* (29). Additionally, the L206R mutant
348 was characterized by a loss of preference for arginine residues at positions -7 to -4 relative
349 to the phosphoacceptor site and an increased preference for glutamic acid and serine at
350 several positions. For the d244-248+E249Q mutant, we found an increased preference for
351 leucine at position -1 from the phosphoacceptor site and a reduced preference for acidic
352 residues downstream of the phosphoacceptor site. The latter trend was mirrored in the
353 hypophosphorylated peptides, where acidic residues were overrepresented downstream of
354 the phosphoacceptor site. Interestingly, both mutants (L206R and d244-248+E249Q)
355 showed an asymmetric effect on substrate phosphorylation with a higher number of peptides
356 that were hyperphosphorylated (40 and 93, respectively) compared to a relatively lower

357 number of hypophosphorylated ones (22 and 23, respectively). Therefore, both mutations
358 mainly seemed to increase the phosphorylation of poor substrates of the wild-type enzyme,
359 while causing only smaller decreases in the phosphorylation of other substrates. For the
360 200_201insV mutant, we observed an increased preference for leucine and alanine at
361 position -1, accompanied by a loss of preference for acidic residues downstream of the
362 phosphoacceptor site and an increased preference for proline at position +3. The loss of
363 preference for peptides containing acidic residues at downstream positions was reflected by
364 their relative overrepresentation in the corresponding hypophosphorylated peptides (left
365 panel). In contrast to the other two mutants, the 200_201insV mutant was characterized by a
366 rather symmetric distribution with 35 hypo- and 40 hyperphosphorylated peptides,
367 suggesting that comparable numbers of substrates were either hyper- or
368 hypophosphorylated. In general, the phosphorylation patterns differed among the three C α
369 mutants. However, four substrates were consistently found to be hyperphosphorylated by all
370 three mutants compared to wild type. These were the citron rho-interacting kinase (CIT; Ser
371 480), the mitochondrial import receptor subunit TOM34 (Ser 93), histone H1.2 (Ser 36) and
372 histone H1.4 (Ser 36) (Fig. 4). H1.2, H1.4, TOM34 and CIT were also found to be present
373 and phosphorylated at the same sites in lysates of the cortisol-producing adrenocortical cell
374 line NCI-H295R (Supplementary Data, Dataset S1 (23)).

375 To rule out that the observed changes were due to changes in protein levels rather than
376 phosphorylation, we performed an additional proteomics experiment on the same cell
377 lysates, which allowed us to directly compare the relative amounts of proteins at the
378 proteome level between wild-type and mutant samples, including, importantly, H1.2, H1.4
379 and TOMM34 (Supplementary Data, Fig. S4, Dataset S2 (23)). The results indicate that the
380 levels of most proteins, including H1.2, H1.4 and TOMM34, were unchanged between wild-
381 type and mutant samples.

382 Whereas hyperphosphorylation of CIT and TOM34 might mediate some effects of *PRKACA*
383 mutations, we concentrated our attention on H1 because phosphorylation by PKA of histone

384 H1.4 on Ser36 – and possibly of the highly homologous Ser36 site on H1.2 – is required for
385 mitosis (20).

386

387 ***PRKACA* mutations cause histone H1.4 hyperphosphorylation *in vitro* and in CPAs**

388 The conserved Ser36 phosphorylation site on histone H1 isoforms has been implicated in
389 chromatin condensation and mitosis (20). This has been better studied for histone H1.4,
390 which has been shown to be phosphorylated by PKA on Ser36 during mitosis, an event that
391 has been demonstrated to induce histone H1.4 dissociation from chromatin and be required
392 for mitosis (20). Because of the potential relevance of histone H1 Ser36 phosphorylation for
393 the mechanism of action of *PRKACA* mutations, we first investigated histone H1.4
394 phosphorylation in HEK293A cells transfected with C α subunit mutants. In HEK293A cells,
395 overexpression of the L206R mutant caused a strong increase (12-fold under basal
396 conditions; 3-fold in the presence of forskolin) in histone H1.4 Ser36 phosphorylation
397 compared to overexpression of wild-type C α (Fig. 5 and Supplementary Data, Fig. S5 (23)).
398 Modestly increased levels of histone H1.4 Ser36 phosphorylation were also observed with
399 the d244-248+E249Q and 200_201insV mutants compared with wild-type C α , even though
400 these differences did not reach statistical significance (Fig. 5 and Supplementary Data, Fig.
401 S5 (23)).

402 Furthermore, we analyzed histone H1.4 Ser36 phosphorylation in human CPA tumor
403 samples of patients with Cushing's syndrome with or without *PRKACA* mutations (summary
404 of clinical data in Supplementary Data, Table S1 (23)). Importantly, a 9-fold increase in
405 histone H1.4 Ser36 phosphorylation was observed in CPAs carrying the L206R mutation
406 compared with CPAs without *PRKACA* mutations (1.9 ± 0.26 vs. 0.21 ± 0.29 , $p=0.009$) (Fig. 6).
407 These results indicated that in patients with adrenal Cushing's syndrome, at least the by far
408 most frequent L206R mutation is associated with a strong increase of H1.4 phosphorylation
409 in the patients' tumor tissue.

410 **Discussion**

411 Our results indicate that *PRKACA* mutations found in CPAs alter PKA preference for its
412 targets, inducing hyperphosphorylation of several PKA substrates, including most notably
413 histone H1.4 at Ser36, which has been suggested to play an essential role in mitosis (20).
414 Thus, our study reveals another key mechanism that, together with increased PKA basal
415 activity, might explain the development of CPAs in the presence of somatic *PRKACA*
416 mutations (Supplementary Data, Fig. S6 (23)).

417 Whereas our previous study on the two *PRKACA* mutations initially identified in adrenal
418 Cushing's syndrome (L206R and 199_200insW) showed that these mutations increase
419 basal PKA activity by preventing the binding of R subunits, the present results obtained on a
420 larger group of *PRKACA* mutations indicate that this is not always the case (see
421 Supplementary Data, Fig. S7 (23) for a summary of the present results), and that a relative
422 change in PKA substrate specificity likely contributes to the mechanism of action of *PRKACA*
423 mutations.

424 Our phosphoproteomics analysis revealed four PKA substrates that were
425 hyperphosphorylated by all three PKA C α mutants analyzed, and, thus, are potentially
426 involved in the pathogenesis of CPAs due to *PRKACA* mutations. These included CIT (Ser
427 480), TOM34 (Ser 93), histone H1.2 (Ser 36) and histone H1.4 (Ser 36). Importantly, all four
428 substrates were also identified to be expressed and phosphorylated on the same sites in
429 lysates of NCI-H295R cells, which are widely used as a model of differentiated, cortisol-
430 producing adrenocortical cells (Supplementary Data, Dataset S1 (23)). Of these, histone
431 H1.4 and the highly homologous histone H1.2 appeared particularly intriguing because H1
432 histones play an important role in promoting, regulating and maintaining chromatin
433 organization and, thus, are deeply involved in the regulation of gene transcription and
434 mitosis (20,30-32). H1 histones possess a conserved serine residue at position 36 (Ser36),
435 which has been mainly investigated for histone H1.4. Interestingly, histone H1.4 has been
436 shown to be strongly phosphorylated on Ser36 by PKA at the first stage of mitosis

437 (prophase), to then rapidly revert to normal, low phosphorylation levels during cytokinesis
438 (20). Phosphorylation of histone H1.4 on Ser36 has been shown to promote histone H1.4
439 dissociation from mitogenic DNA and induce changes in chromatin organization (20).
440 Moreover, it has been shown that silencing H1.4 inhibits mitosis, an effect that cannot be
441 rescued by a phosphorylation-deficient H1.4 mutant (S36A) (20). These findings indicate that
442 the PKA-dependent phosphorylation of histone H1.4 at Ser36 plays an important role in
443 promoting mitosis, presumably by inducing changes in chromatin structure that are required
444 for the realization of the mitogenic program (20). Whereas other changes in the PKA
445 phosphorylation pattern caused by *PRKACA* mutations might also play a role in the
446 pathogenesis of CPAs, it is noteworthy that two of the four hyperphosphorylated sites shared
447 by the three tested $C\alpha$ mutants are Ser36 on histone H1.4 and the corresponding Ser36 on
448 histone H1.2, which likely plays a similar role. Our finding that histone H1.4 is highly
449 hyperphosphorylated in CPAs carrying the L206R mutation – but not in those with wild-type
450 PKA – supports a role of H1.4 hyperphosphorylation in the pathogenesis of CPAs due to
451 *PRKACA* mutations. Further investigations, such as studies on chromatin organization by
452 DAPI staining and H1.4–chromatin association by immunofluorescence (20) or chromatin
453 immunoprecipitation will be required to further clarify the impact of the increased H1.4
454 (Ser36) phosphorylation on chromatin organization in *PRKACA* mutated CPA tissue.

455 In addition to hyperphosphorylation of histone H1, that of CIT and of TOM34 – which, to the
456 best of our knowledge, have not been previously reported to be phosphorylated by PKA –
457 might also play a role in the mechanism of action of *PRKACA* mutations. CIT is a
458 serine/threonine-protein kinase that has been shown to be required for cytokinesis (33).
459 Phosphorylation of Ser480 might potentially influence the activity of CIT and, thus, affect
460 cytokinesis. TOM34 is involved in mitochondrial protein import (34). Interestingly, yeast
461 TOM70 has been shown to be phosphorylated by PKA on Ser174 (35), which is homologous
462 to Ser93 in human TOM34. In yeast, TOM70 phosphorylation by PKA inhibits its receptor
463 activity, which results in reduced mitochondrial import of metabolite carriers (35).

464 Phosphorylation of TOM34 at Ser93 could have a similar effect. Future studies will be
465 required to evaluate the role of CIT and TOM34 in the pathogenesis of CPAs.

466 The finding that *PRKACA* mutations cause histone H1.4 phosphorylation might help
467 explaining why these mutations lead to the development of CPAs, while normal cAMP/PKA
468 signaling such as resulting from physiological stimulation by the adrenocorticotrophic
469 hormone (ACTH) has no or at most only mild proliferative effects on adrenocortical cells
470 (36,37). Intriguingly, this may suggest that whereas an increase in PKA activity might be
471 sufficient to induce excess cortisol production in the presence of *PRKACA* mutations, a
472 change in PKA substrate preference – and the resulting hyperphosphorylation of histone
473 H1.4 and possibly other targets involved in the control of cell replication – might be required
474 to stimulate cell proliferation.

475 Finally, our findings indicate that, given the important changes in substrate specificity caused
476 by *PRKACA* mutations, it might be possible to develop selective orthosteric PKA inhibitors
477 that bind only to the mutant C α subunits and, thus, selectively inhibit the mutated PKA in
478 CPAs without affecting the function of wild-type PKA in normal cells.

479 In summary, our findings indicate that *PRKACA* mutations found in CPAs lead to a change in
480 the pattern of PKA phosphorylation, which might play an important role in their mechanism of
481 action. This not only reveals a novel key mechanism potentially involved in the pathogenesis
482 of CPAs and Cushing's syndrome, but might also allow the development of selective PKA
483 inhibitors for therapeutic purposes that block mutated but not wild-type PKA. Moreover,
484 these findings provide a highly illustrative example of how mutations altering substrate
485 specificity of a protein kinase might cause a human disease, which might also have
486 implications for other conditions.

487

488 **Author Contribution**

489 D.C. designed research with contributions from M.F. and A.S. K.B., I.W. and J.W. performed
490 research. K.B., I.W. and J.W. analyzed data. M.F., C.L.R. and S.S. provided tumor samples
491 together with clinical and genetic data. K.B. and D.C. wrote the paper with contributions from
492 M.F., A.S., I.W., C.L.R. and S.S.

493

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622

Figure legends

Fig. 1. Somatic *PRKACA* mutations identified in adrenal Cushing's Syndrome. (A)

Location of the identified *PRKACA* mutations in the crystal structure of the PKA holoenzyme. Note the clustering of the *PRKACA* mutations at the interface with the R subunit. The structure of the RII β :C α ₂ holoenzyme (Protein data bank/PDB entry 3TNP) was used as template. (B) Enlarged view of the PKA C α -RII β interface. Shown are the positions of residues affected by *PRKACA* mutations relative to the inhibitory sequence of RII β in the PKA RII β :C α ₂ holoenzyme. The inhibitory sequence of RII β interacts with active site cleft of C α and acts as a PKA substrate. Note the phosphorylated serine (S) residue within the canonical consensus for PKA phosphorylation (RRXS) in the inhibitory sequence. Most residues affected by *PRKACA* mutations participate directly or indirectly in substrate recognition. *, Residues affected by *PRKACA* mutations with largest predicted effects on substrate specificity based on *in silico* analysis. PDB entry 3TNQ was used as template.

Fig. 2. Effect of *PRKACA* mutations on the association of C α with R subunits.

HEK293A cells were co-transfected with FLAG-tagged RII β or RI α and either wild-type (WT) or mutant C α subunits. The association between C α and R subunits in the absence or presence of cAMP (200 μ M) was analyzed by co-immunoprecipitation. Samples were immunoprecipitated (IP) with an anti-FLAG antibody, followed by Western blot analysis with an antibody against C α . Western blot images were quantified by densitometric analysis. Data are mean \pm s.e.m. of three independent experiments. Arrows, mutants with conserved binding to R subunits. Differences are statistically significant by two-way ANOVA. *P<0.05, **P<0.01 and ****P<0.0001 vs. WT basal by Bonferroni's post hoc test. Representative Western blot images are shown in Supplementary Data, Fig. 1 (23).

Fig. 3. Effect of *PRKACA* mutations on PKA activity against a synthetic substrate.

HEK293A cells were co-transfected with RI α or RII β and either wild-type (WT) or mutant C α subunits. PKA activity in cell lysates was then measured against an artificial peptide substrate (kemptide) in the presence or absence of cAMP (40 μ M). The PKA activity measured in cells transfected with the empty expression vector (pcDNA) was subtracted. Data are mean \pm s.e.m. of three independent experiments. Data are statistically significant by two-way ANOVA. *P<0.05, ***P<0.001 vs. WT basal and ##P<0.01, ###P<0.001 vs. WT stimulated with cAMP by Bonferroni's post hoc test.

Fig. 4. *PRKACA* mutations affect substrate specificity as revealed by phosphoproteomics analysis.

HEK293A cells were co-transfected with RII β and either wild-type (WT) or mutant C α subunits and labeled with light amino acids (WT) or heavy amino acids (mutant). Protein lysates from these cells were then proteolytically fragmented and the resulting phosphopeptides were immunoprecipitated using a phospho-PKA substrate antibody recognizing the RR/KXpS/T consensus. Phosphopeptides were separated and detected by nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS). Depicted are scatter plots showing the abundance of the detected phosphopeptides in the mutant relative to wild-type samples of three independent experiments. The sequence logos report, for each mutant, the relative amino acid preferences around the consensus motif (RR/KXpS/T) for both hypo- (left) and hyperphosphorylated (right) sequences, calculated from the phosphopeptides. Values are expressed as log₁₀-odds of the significance of overrepresentation versus the significance of underrepresentation, which are calculated using the binomial probability of residue frequencies.

Fig. 5. Effect of *PRKACA* mutations on histone H1.4 phosphorylation in HEK293A cells. HEK293A cells were co-transfected with wild-type (WT) or mutant PKA C α and the

R11 β subunit. Histone H1.4 phosphorylation was probed by Western blot analysis with an antibody specifically recognizing H1.4 phosphorylated at Ser36. A Western blot analysis for total histone H.1.4 was used as loading control. Western blot images were quantified by densitometric analysis. The adenylyl cyclase activator forskolin (10 μ M) was used to induce maximal PKA activity. Data are mean \pm s.e.m of three independent experiments. Differences are statistically significant by two-way ANOVA. *** P<0.001 vs. WT basal and # P<0.05 vs. WT forskolin by Bonferroni's post hoc test.

Fig. 6. Effect of *PRKACA* mutations on histone H1.4 phosphorylation in tumor (CPA) samples from patients with adrenal Cushing's syndrome. Histones were extracted from CPAs with or without *PRKACA* mutations and analyzed by Western blot analysis with an antibody specifically recognizing H1.4 phosphorylated at Ser36. A Western blot analysis for total histone H1.4 was used as loading control. Western blot images were quantified by densitometric analysis. Quantified data are mean \pm s.e.m of 3 and 4 CPAs carrying the L206R mutation or with *PRKACA* wild-type (WT), respectively. Differences are statistically significant by unpaired t-test (** P=0.009).

Table 1. List of somatic *PRKACA* mutations identified so far in CPAs of patients with overt Cushing's syndrome. Bold, studies by our group.

Tables

Mutation	Frequency (n mutated/total)	Reference
L206R	36% (21/59)	(10)
	60% (33/55)	(16)
	67% (84/126)	(14)
	35% (13/37)	(15)
	23% (3/13)	(17)
	34% (22/64)	(11)
	31% (11/35)	(18)
199_200insW	2% (1/59)	(10)
200_201insV	5% (3/64)	(11)
S213R+insIIIR	2% (1/64)	(11)
W197R	3% (1/39)	(12)
d244-248+E249Q	3% (1/39)	(12)
E32V	3% (1/39)	(12)

Figures

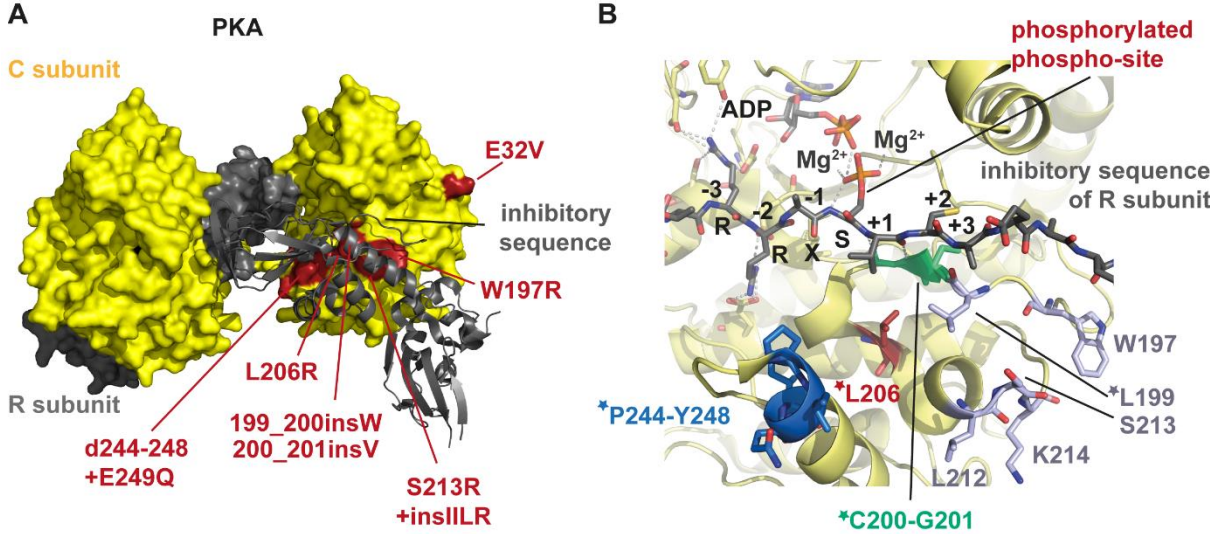


Fig. 1

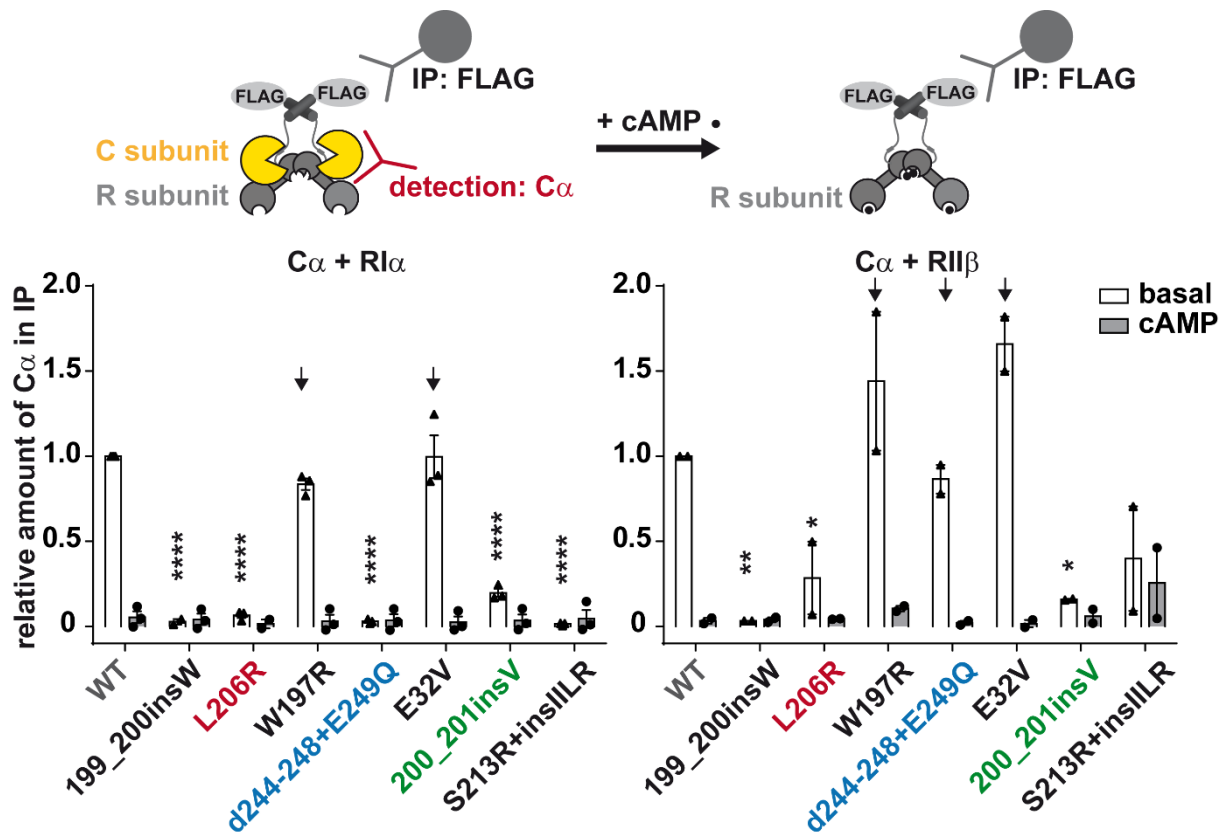


Fig. 2

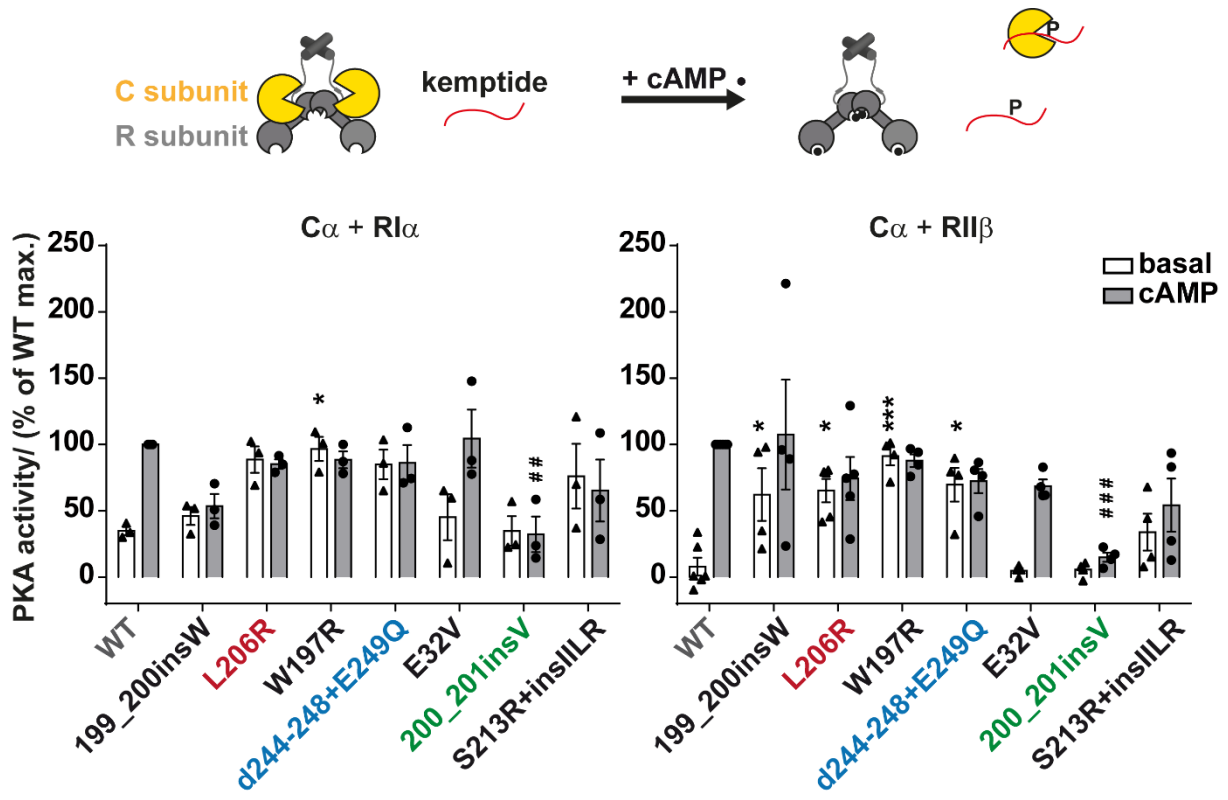


Fig. 3

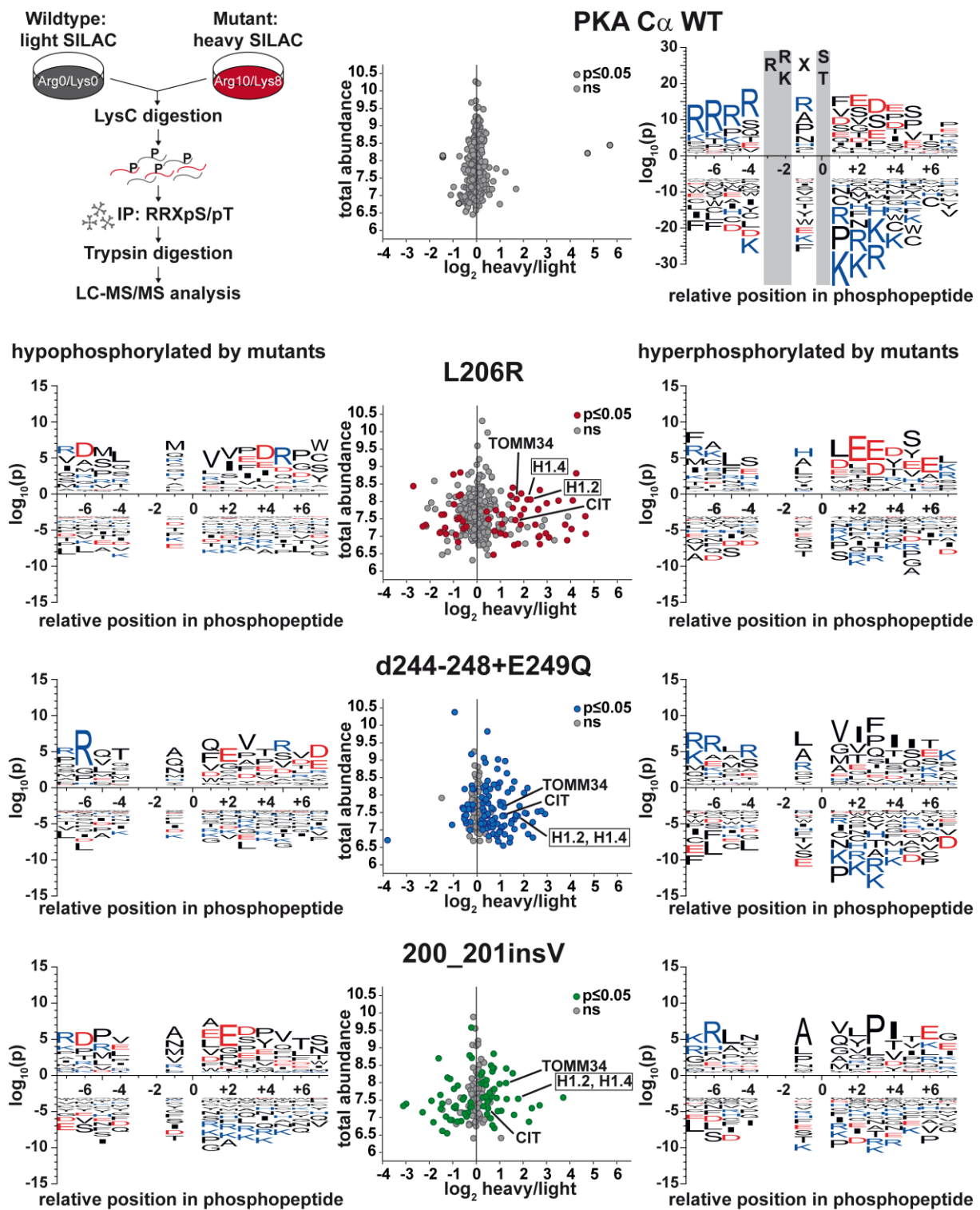


Fig. 4

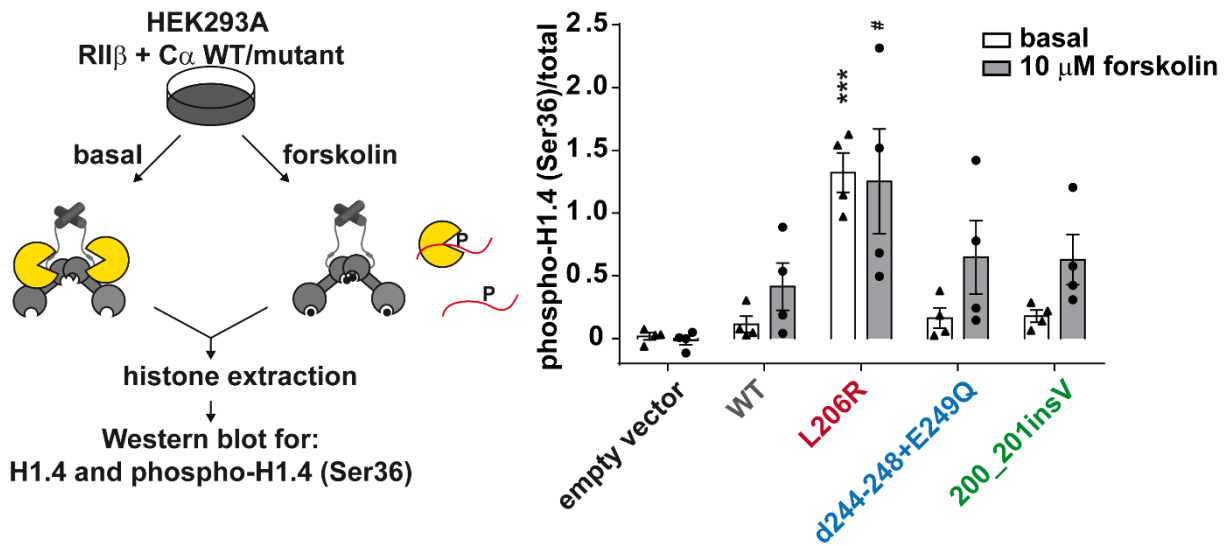


Fig. 5

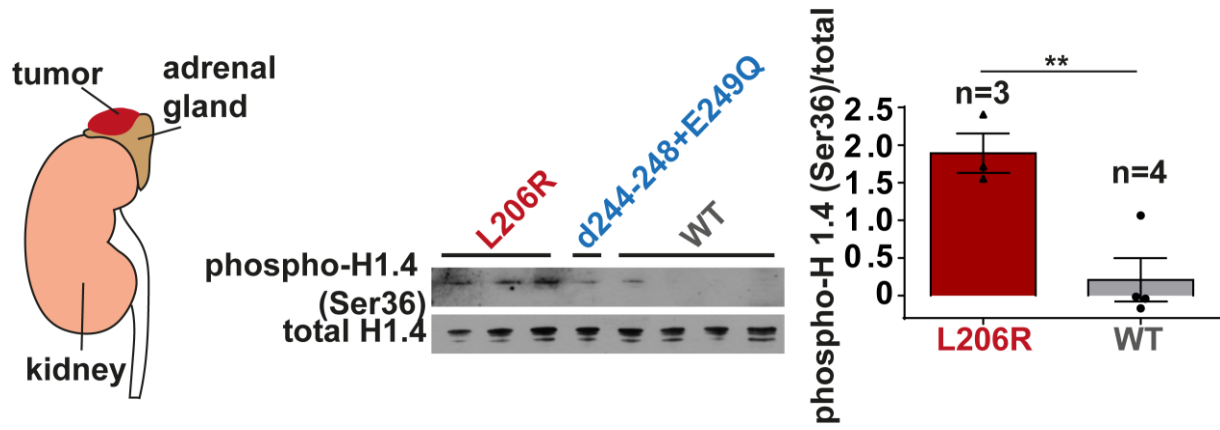


Fig. 6