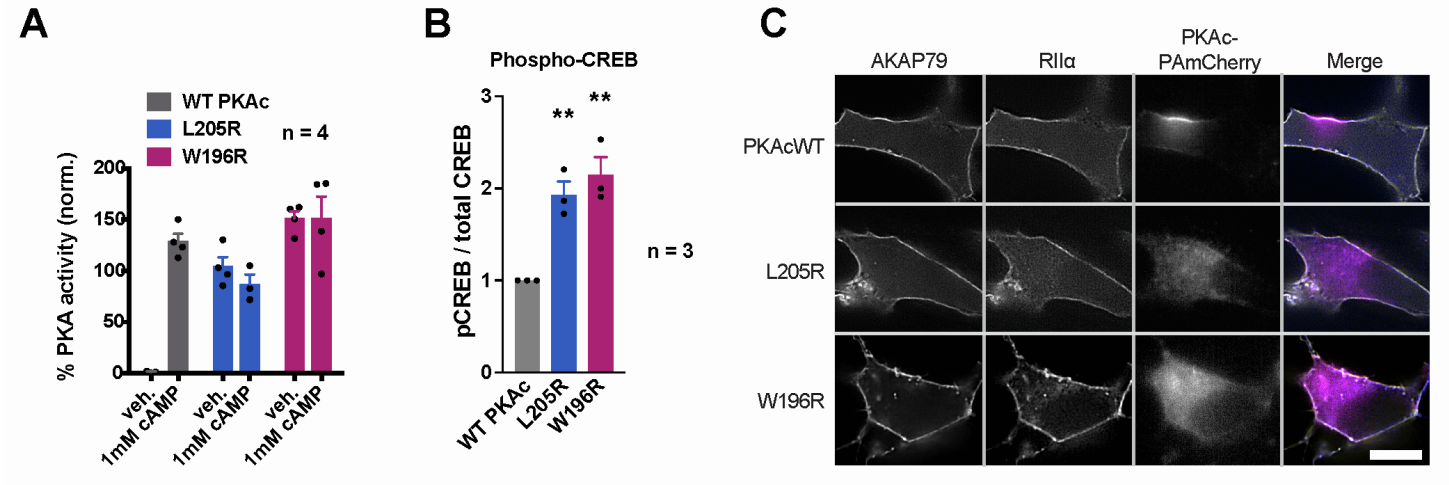
Cell Reports, Volume *40*

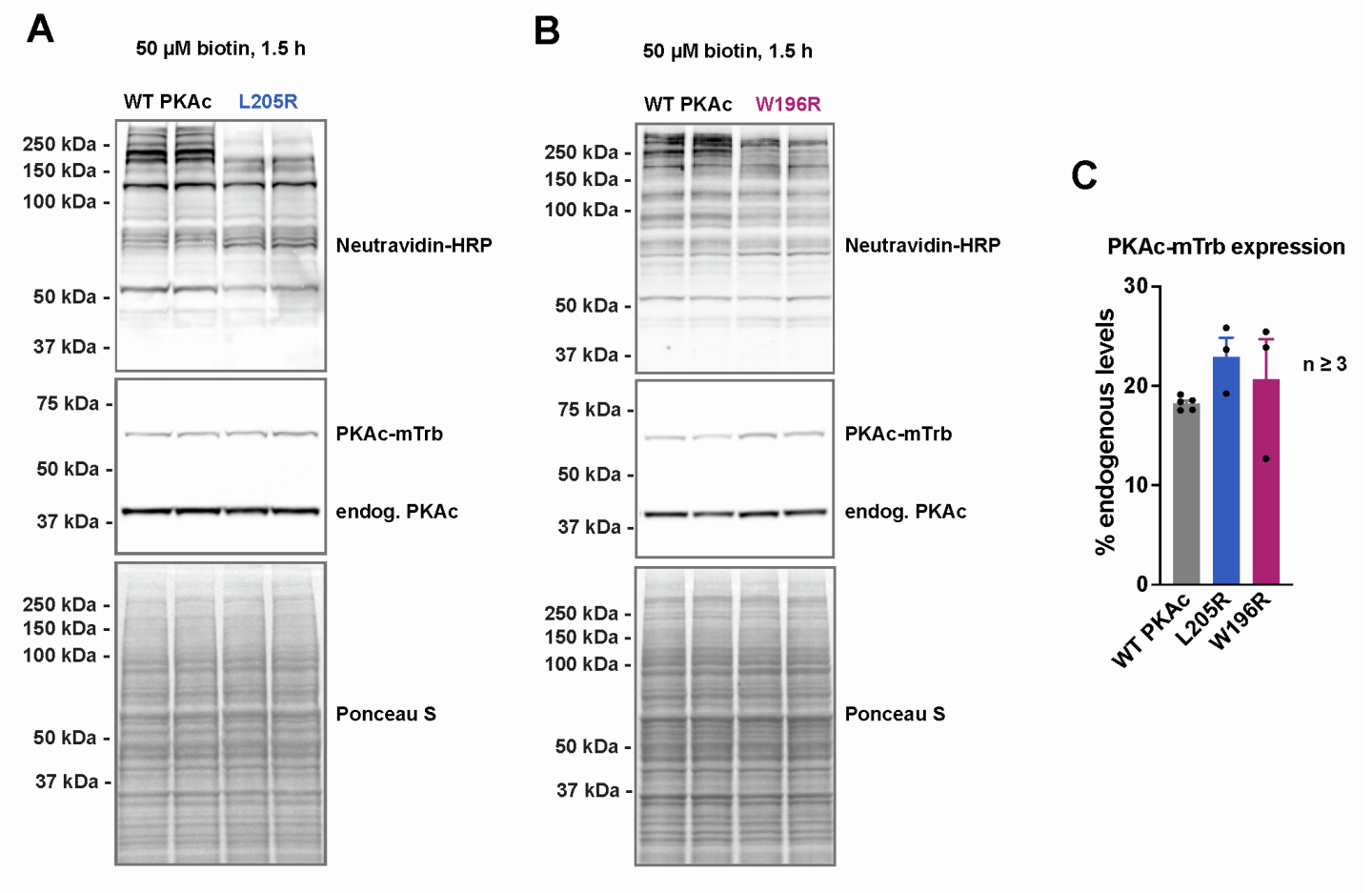
Supplemental information

Mislocalization of protein kinase A drives pathology in Cushing's syndrome

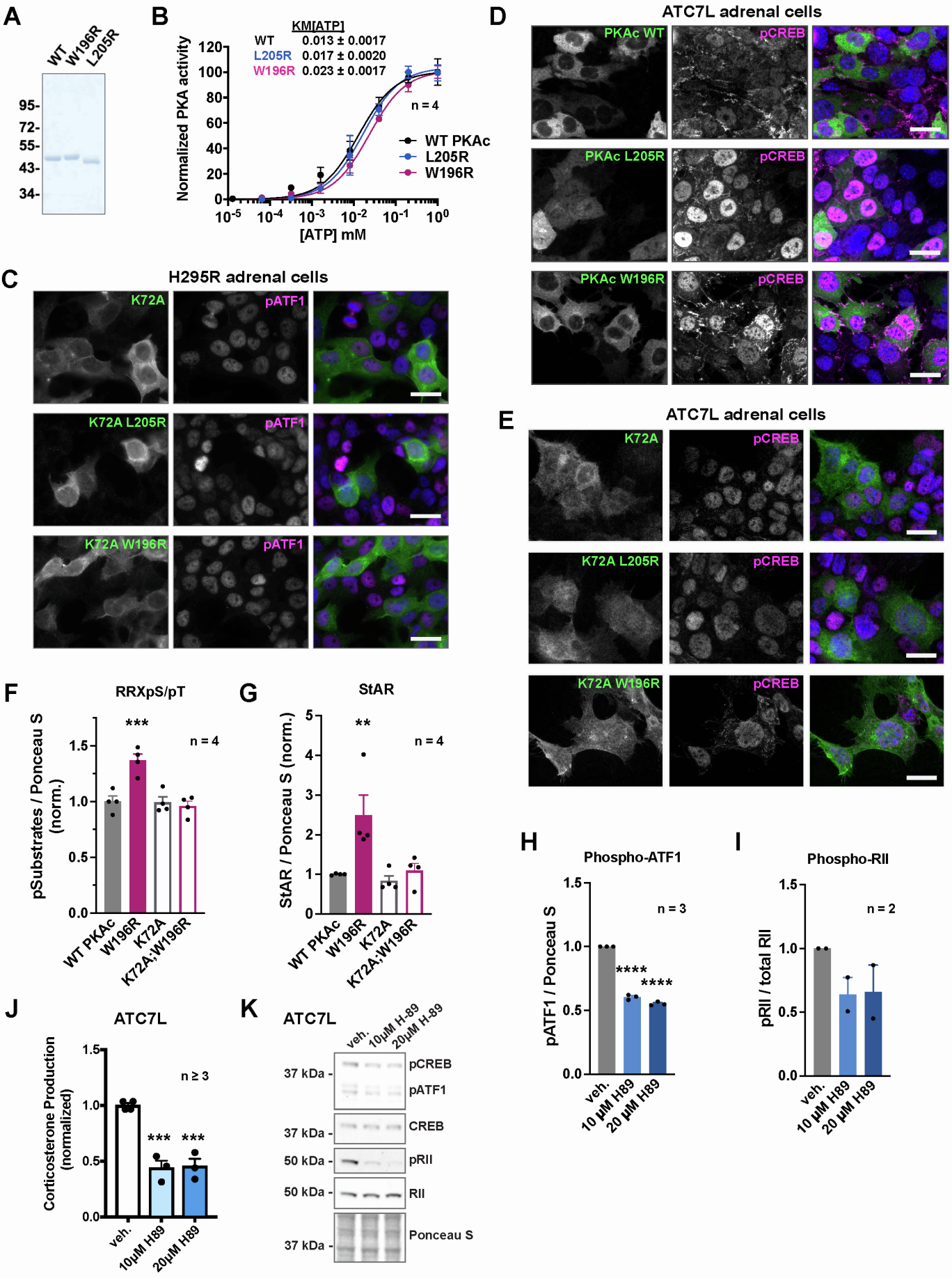
Mitchell H. Omar, Dominic P. Byrne, Kiana N. Jones, Tyler M. Lakey, Kerrie B. Collins, Kyung-Soon Lee, Leonard A. Daly, Katherine A. Forbush, Ho-Tak Lau, Martin Golkowski, G. Stanley McKnight, David T. Breault, Anne-Marie Lefrançois-Martinez, Antoine Martinez, Claire E. Eyers, Geoffrey S. Baird, Shao-En Ong, F. Donelson Smith, Patrick A. Eyers, and John D. Scott



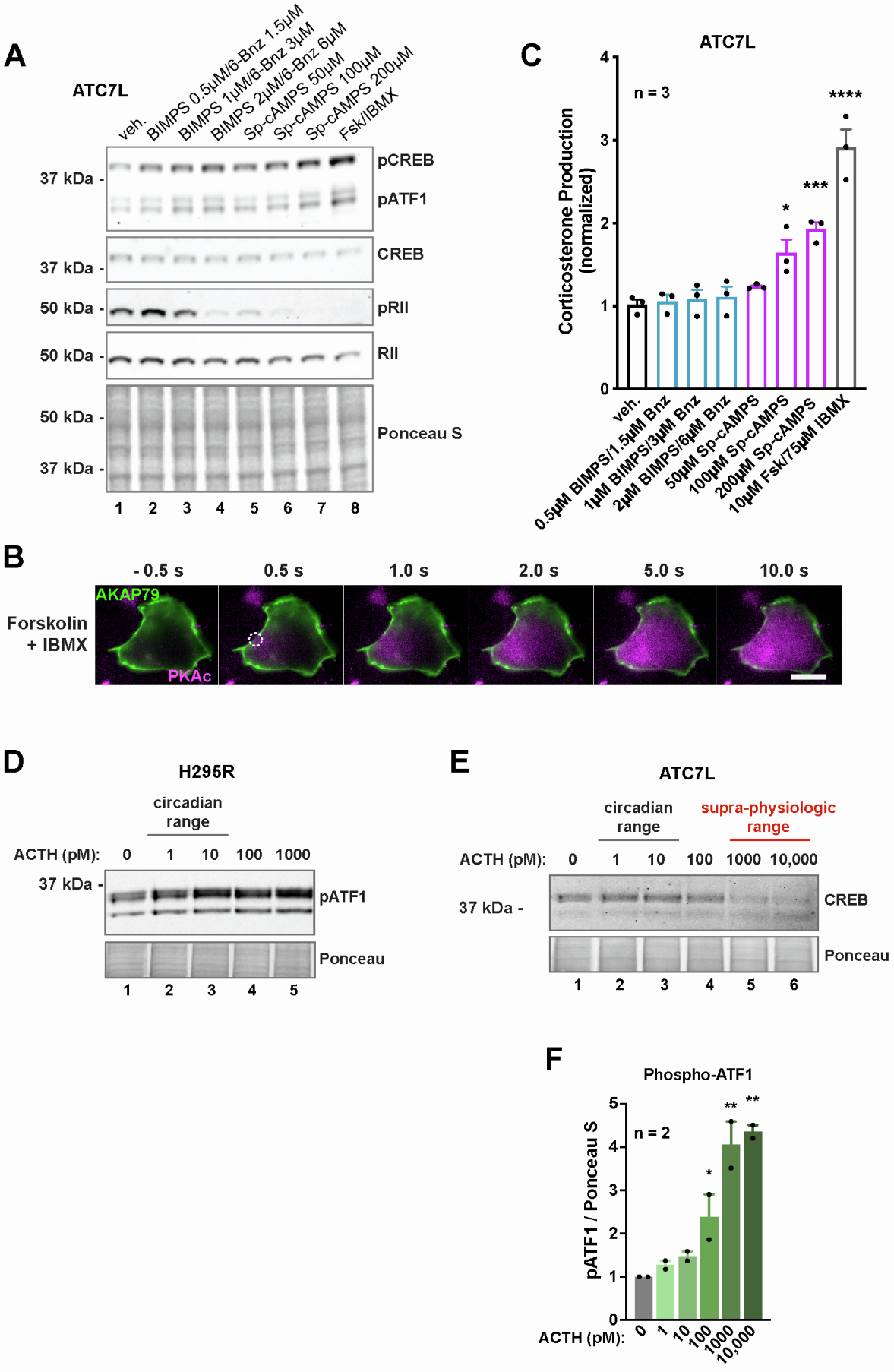
Supplemental figure S1. PKAc-L205R and PKAc-W196R are constitutively active and aberrantly mobile. Relates to figure 1. A) Activity of PKAc variants in the presence of RII +/- cAMP. n = 4. B) Quantitation from figure 1I of phospho-CREB divided by total CREB. **p≤0.01, corrected for multiple comparisons with Holm-Sidak method after 1-way ANOVA. n = 3. C) 3-channel representative images of photoactivation cells demonstrating equivalent expression and colocalization of AKAP79 and RII. Scale bar = 10 μm.



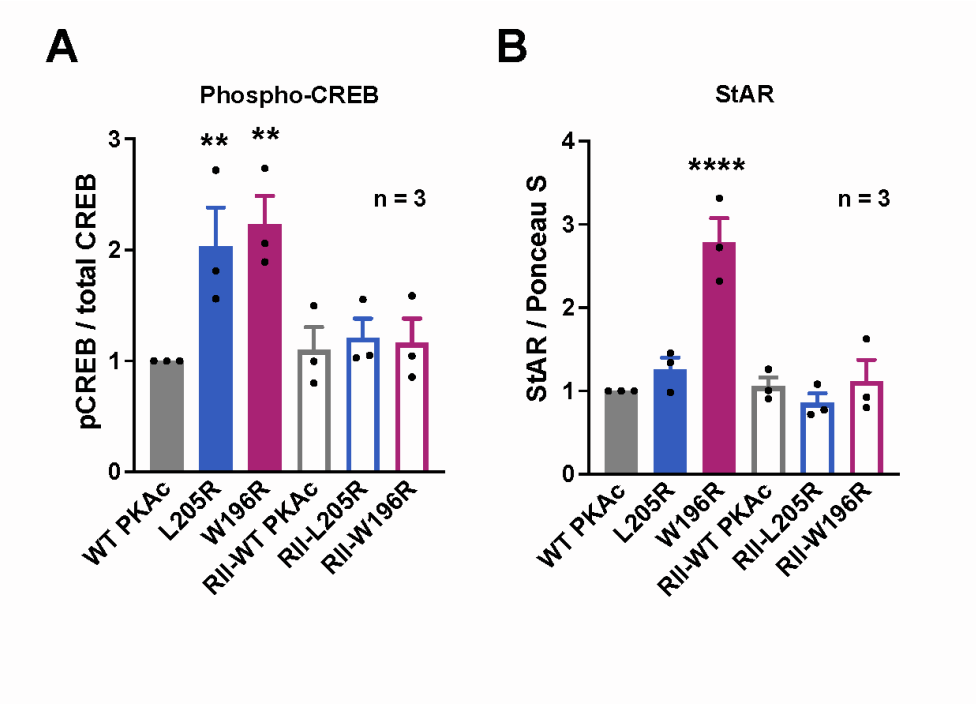
Supplemental figure S2. PKAc-miniTurbo variants are expressed at moderate levels and equally biotinylate proteins. Relates to figure 2. A & B) Representative western blots for L205R (A) and W196R (B) variants of PKAc-miniTurbo expressed in H295R cells and incubated with 50 µM biotin for 1.5 h. C) Quantitation of PKAc-miniTurbo expression as compared to endogenous PKAc. No significant differences by 1-way ANOVA. n ≥ 3.


A
WT
W196R
L205R
95-
72-
55-
43-
34-
B
KM[ATP]
WT 0.013 ± 0.0017
L205R 0.017 ± 0.0020
W196R 0.023 ± 0.0017
n = 4
WT PKAc
L205R
W196R
Normalized PKA activity
[ATP] mM
C
H295R adrenal cells
K72A
pATF1
K72A L205R
K72A W196R
D
ATC7L adrenal cells
PKAc WT
pCREB
PKAc L205R
PKAc W196R
E
ATC7L adrenal cells
K72A
pCREB
K72A L205R
K72A W196R
F
RRXpS/pT
pSubstrates / Ponceau S (norm.)
n = 4
WT PKAc
W196R
K72A
K72A;W196R
G
StAR
StAR / Ponceau S (norm.)
n = 4
H
Phospho-ATF1
pATF1 / Ponceau S
n = 3
veh.
10 μM H89
20 μM H89
I
Phospho-RII
pRII / total RII
n = 2
J
ATC7L
Corticosterone Production (normalized)
n ≥ 3
K
ATC7L
veh.
10μM H-89
20μM H-89
37 kDa
pCREB
pATF1
CREB
50 kDa
pRII
RII
Ponceau S

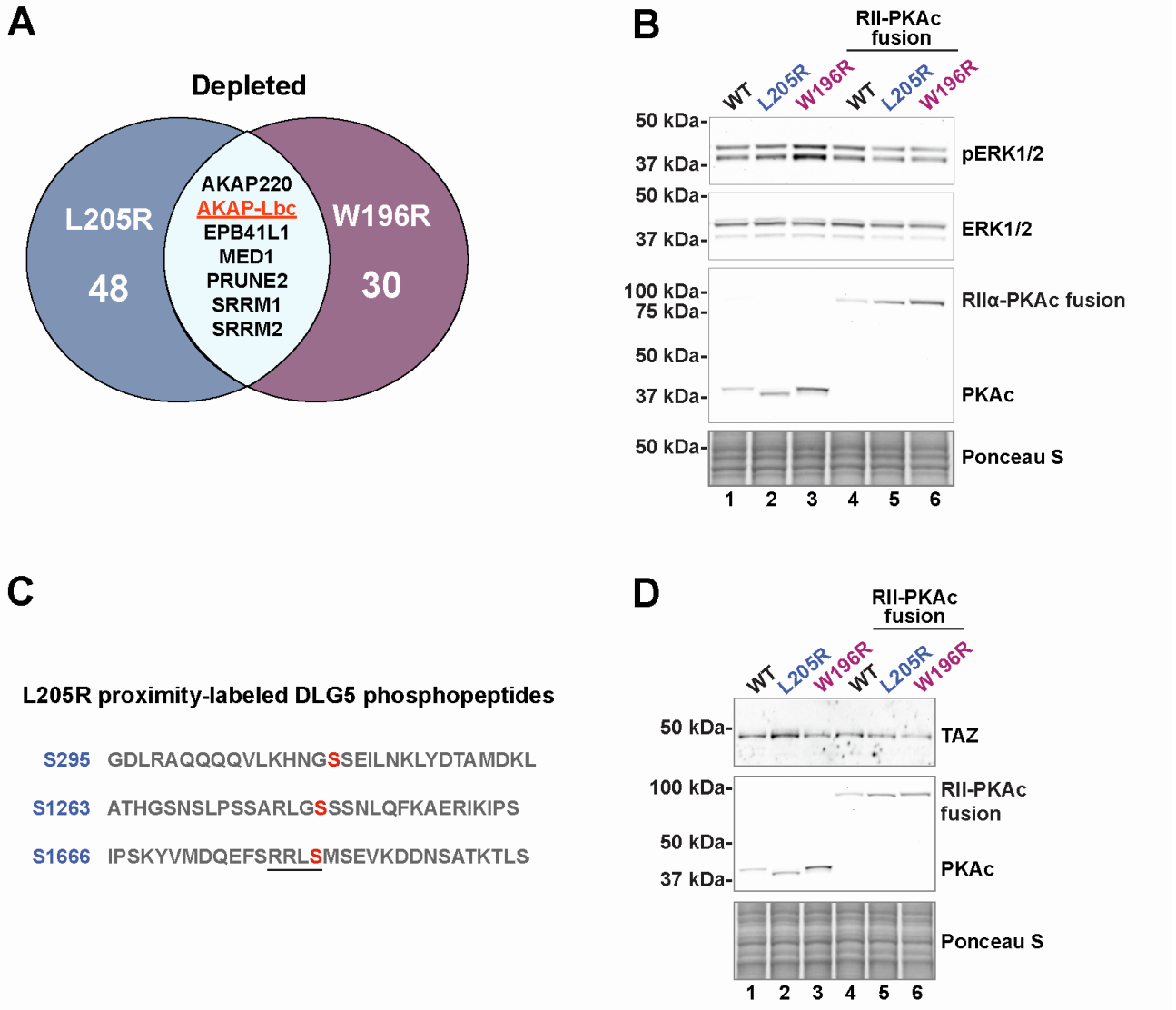
Supplemental figure S3. Cushing's mutants aberrantly phosphorylate substrates. Relates to figure 3. A) SDS-PAGE and Coomassie blue staining of purified recombinant WT and mutant PKAc variants. B) KM[ATP] determination for recombinant WT and mutant PKAc. Data shown is PKA-catalyzed peptide phosphorylation (pmol phosphate/min/ng protein) with increasing concentrations of ATP. Normalized to the maximum rate of phosphorylation for each protein. KM[ATP] values (± SE) were calculated from four independent experiments. C) H295R adrenal cells expressing kinase dead (K72A) versions of V5-tagged PKAc variants stained with V5 and pCREB antibodies. Scale bars = 20 μm. D & E) ATC7L mouse adrenal cells expressing V5-tagged WT or mutant PKAc (D) or kinase dead (K72A) versions of these (E) were stained with V5 and pCREB antibodies. Scale bars = 20 μm. F & G) Quantitation of phospho-RRXS/T (F) and StAR (G) immunoblots (figure 3H). **p≤0.01, ***p≤0.001, corrected for multiple comparisons with Holm-Sidak method after 1-way ANOVA. n = 4. H & I) Quantitation of phospho-ATF1 (H) and phospho-RII (I) immunoblots (figure 3J). ****p≤0.0001, corrected for multiple comparisons with Holm-Sidak method after 1-way ANOVA. J) Corticosterone measurements from ATC7L cells after 1 h incubation with vehicle or the PKA inhibitor H89. ***p-value ≤ 0.001. Corrected for multiple comparisons using Sidak method. n ≥ 3. K) Immunoblot of ATC7L lysates after 1 h incubation with vehicle or H89. Representative of 3 experimental replicates.


A
ATC7L
veh.
BIMPS 0.5μM/6-Bnz 1.5μM
BIMPS 1μM/6-Bnz 3μM
BIMPS 2μM/6-Bnz 6μM
Sp-cAMPS 50μM
Sp-cAMPS 100μM
Sp-cAMPS 200μM
Fsk/IBMX
37 kDa -
pCREB
pATF1
37 kDa -
CREB
50 kDa -
pRII
50 kDa -
RII
50 kDa -
37 kDa -
Ponceau S
1 2 3 4 5 6 7 8
C
ATC7L
n = 3
Corticosterone Production (normalized)
0 1 2 3 4
****
***
*
veh.
0.5μM BIMPS/1.5μM Bnz
1μM BIMPS/3μM Bnz
2μM BIMPS/6μM Bnz
50μM Sp-cAMPS
100μM Sp-cAMPS
200μM Sp-cAMPS
10μM Fsk/75μM IBMX
B
- 0.5 s
0.5 s
1.0 s
2.0 s
5.0 s
10.0 s
Forskolin + IBMX
AKAP79
PKAc
D
H295R
circadian range
ACTH (pM): 0 1 10 100 1000
37 kDa -
pATF1
Ponceau
1 2 3 4 5
E
ATC7L
circadian range
supra-physiologic range
ACTH (pM): 0 1 10 100 1000 10,000
37 kDa -
CREB
Ponceau
1 2 3 4 5 6
F
Phospho-ATF1
n = 2
pATF1 / Ponceau S
0 1 2 3 4 5
**
**
*
ACTH (pM): 0 1 10 100 1000 10,000

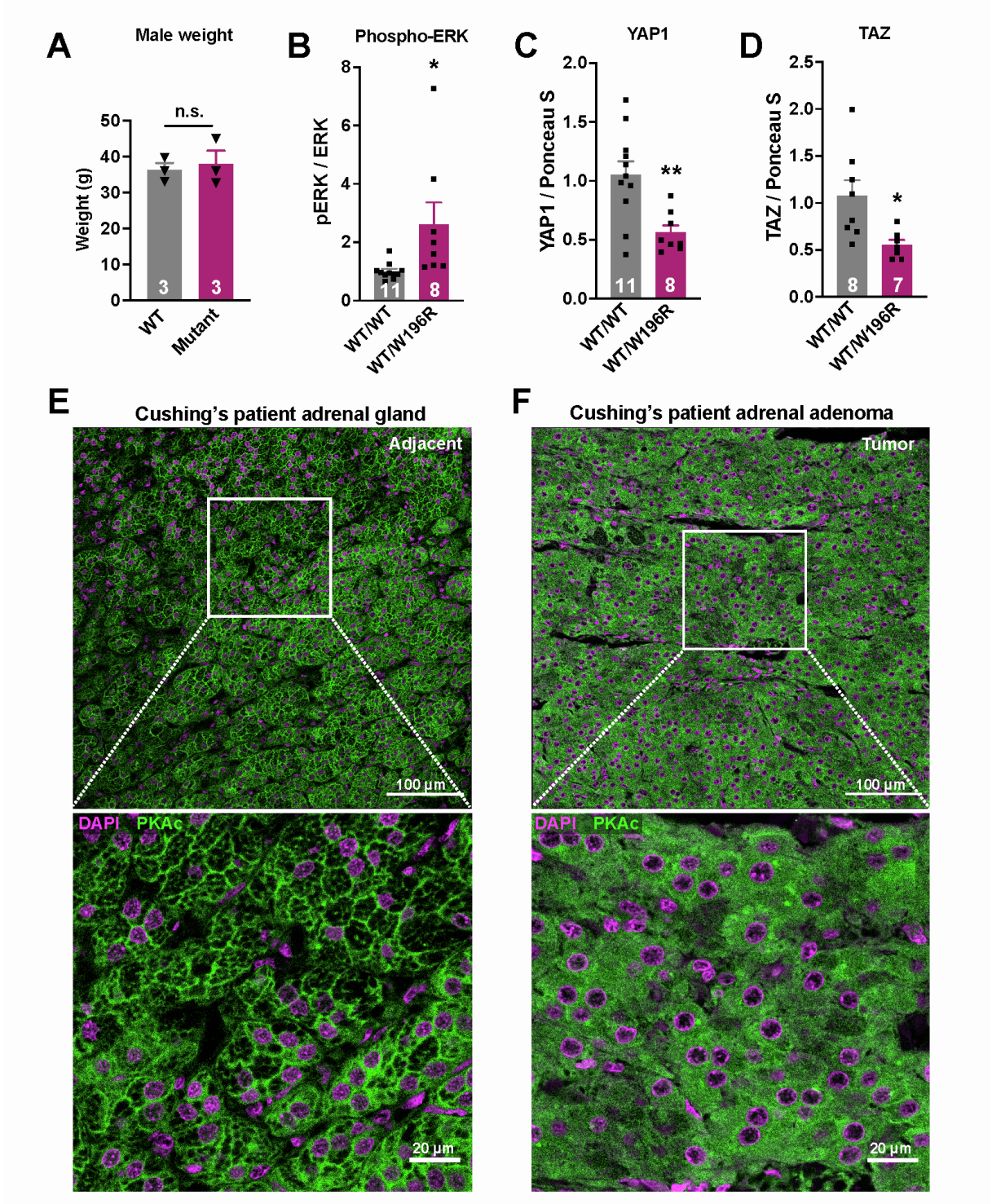
Supplemental figure S4. Moderate activation does not displace PKAc-WT in adrenal cells. Relates to figure 4. A) Immunoblot of ATC7L lysates treated for 1 h with vehicle or increasing concentrations of PKA-activating drugs probed for pCREB/pATF1, total CREB, pRII, and RII. Representative of 3 experimental replicates. B) Photoactivation microscopy timecourse of H295R cell expressing AKAP79-YFP, RII-iRFP, and WT-PKAc tagged with photoactivatable mCherry in the presence of 10 μM forskolin and 75 μM IBMX. White circle indicates region of photoactivation. Scale bar = 10 μm. C) Corticosterone measurements from ATC7L cells after 1 h with vehicle or increasing concentrations of PKA-activating drugs. n = 3. D) Representative immunoblot of H295R cells after treatment for 1 h with increasing concentrations of ACTH. E) Corresponds to figure 4K. Total CREB and Ponceau S immunoblot of ATC7L cells after treatment for 1 h with increasing concentrations of ACTH. F) Quantification of figure 4K phospho-ATF1 signal divided by Ponceau S. *$p<0.05$, **$p\leq0.01$, corrected for multiple comparisons with Holm-Sidak after 1-way ANOVA. n = 2 full dosage curves.



Supplemental figure S5. Fusion of Cushing's mutants to RII corrects stress hormone production. Relates to figure 5. Quantitation of immunoblots represented in figure 5F. **p≤0.01, ****p≤0.0001, corrected for multiple comparisons with Holm-Sidak after 1-way ANOVA. n = 3.



Supplemental figure S6. Proximity phosphoproteomics identifies aberrant signaling by PKAc mutants. Relates to figure 6. A) Venn diagram of significantly depleted phosphopeptides in both mutant conditions. B) Immunoblot of ATC7L cells lysates. Conditions expressing separate RII and PKAc proteins demonstrated increased ERK phosphorylation in the W196R condition. This effect was rescued by fusion of RII and PKAc. C) Discs large 5 phosphopeptides identified in PKAc-L205R proximity phosphoproteomic screen. Red indicates the phosphorylated residue. Underlined region is a conforming PKA substrate recognition motif. D) Immunoblot of ATC7L cells expressing separate (lanes 1-3) or fused (lanes 4-6) RII and PKAc variants.



Supplemental figure S7. A Cushing's mouse model and patient tissue display signaling defects. Relates to figure 7. A) Weight of 1-year male mutant mice (red) compared to littermate controls (gray); n = 3. Not significantly different by students t-test. B-D) Corresponds to figure 7J. Quantitation of adrenal gland immunoblot signals from multiple mice. *p<0.05, **p≤0.01, students t-test. n ≥ 7 mice. E & F) Immunofluorescence in resected tissue from an additional Cushing's patient. PKAc (green) and DAPI (magenta) were stained in normal adjacent adrenal tissue (E) and adenoma (F). Scale bars as indicated.