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**Supplementary information**

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**Somatic mutations of *GNA11* and *GNAQ* in *CTNNB1*-mutant aldosterone-producing adenomas presenting in puberty, pregnancy or menopause**

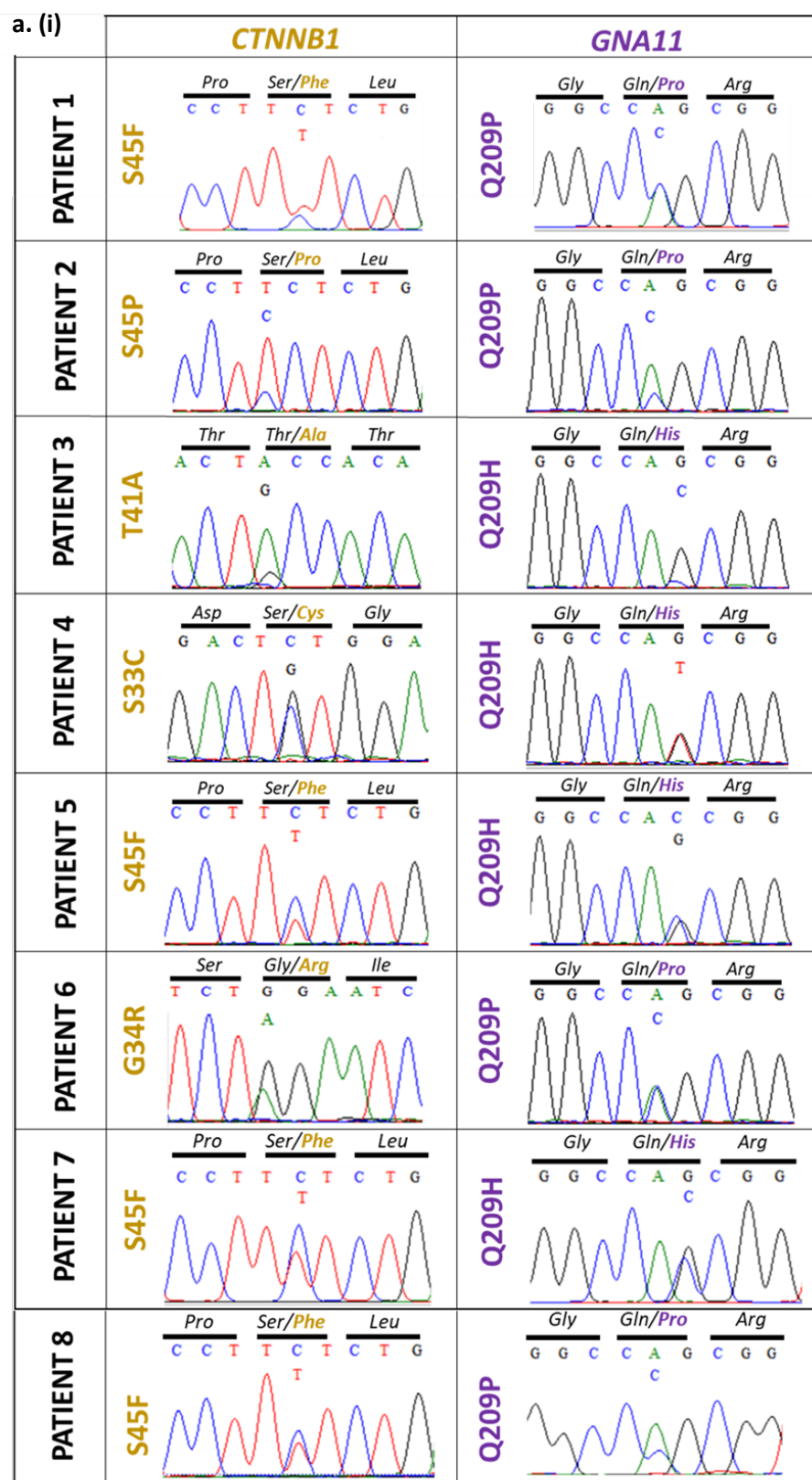
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In the format provided by the  
authors and unedited

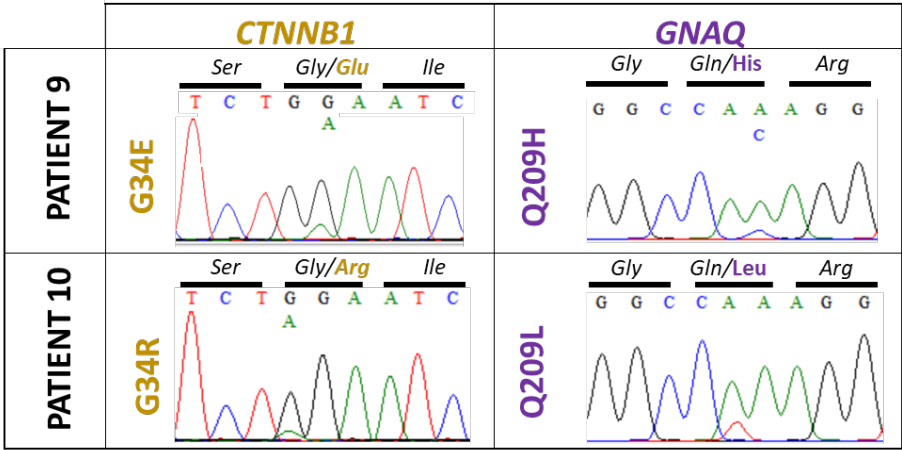
# Supplementary Figure 1

**Sanger Sequencing of *GNA11/Q* somatic mutations in *CTNNB1* mutant APAs from PA patients in the UK/Irish discovery cohort (a) and in the French replication cohort (b).**

(a) In the UK/Irish cohort, *GNA11* (i) and *GNAQ* (ii) were mutated in 10/10 *CTNNB1*-mutant APAs. Somatic mutations of *CTNNB1* and *GNA11* in patient 1-4 were first detected by WES. Patient 5-10 were either investigated because of onset presentation of primary aldosteronism during periods of high LH/HCG or because of high expression of *LHCGR* or *TMEM132E* in the APA.

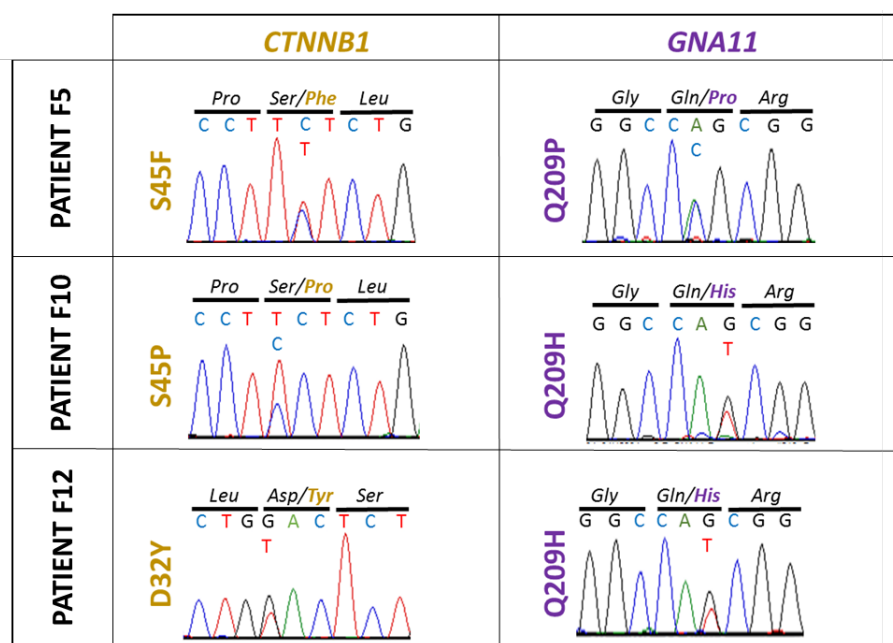


a. (ii)

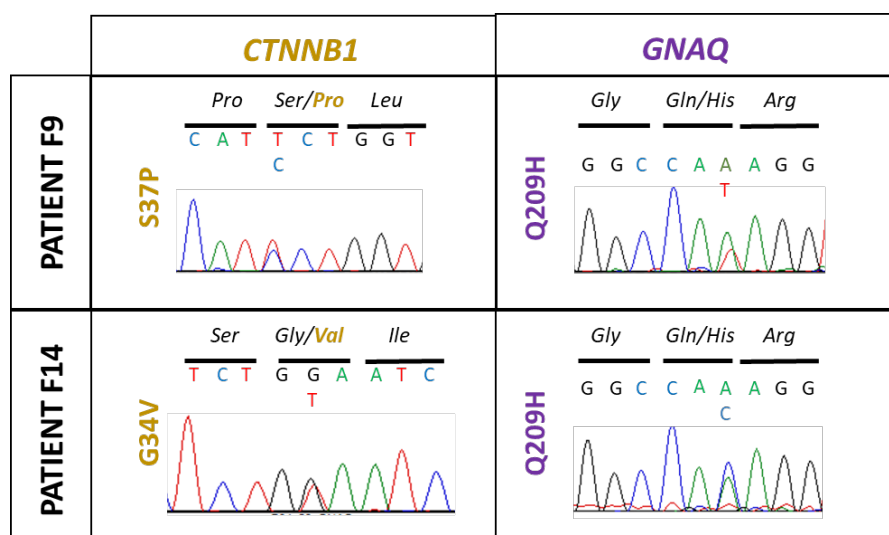


(b) In the French cohort, *GNA11* (i) and *GNAQ* (ii) were mutated in 5/14 *CTNNB1*-mutant APAs. 13 *CTNNB1* mutant APAs were detected through screening 198 primary aldosteronism patients for *CTNNB1* mutations whereas 1 was recognized through clinical presentation during the period of this study. For part of the patients included in the French cohort, the genetic screening of known aldosterone-driving mutations in ion-channel/transporter genes was previously performed. <sup>73</sup>

b. (i)



(ii)



## Supplementary Figure 2

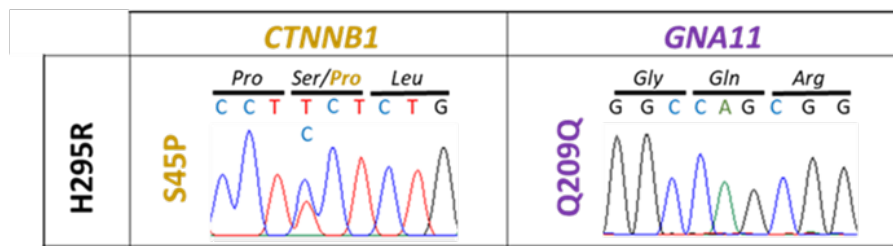
### Effect of *GNA11*/Q Q209 mutation in human adrenocortical cells.

- (a) Sanger sequencing of *CTNNB1* and *GNA11* in the immortal adrenocortical cell line H295R. H295R cells harbour an activating S45P mutation in *CTNNB1* while wild-type for *GNA11*.
- (b) *GNA11* and *GNAQ* Q209 mutations that were transfected into H295R cells (**Figure 1**). *GNA11* Q209L and Q209P are known pathogenic and likely pathogenic missense variant rs1057519742 at chromosome 19:3118944 (GRCh38). Whereas Q209H *GNA11* and *GNAQ* are known somatic cancer mutations COSM1717778 (FATHMM prediction score of 0.94, >0.7 = pathogenic) at chromosome 19:3118945 (GRCh38) and COSM1666995 at chromosome 9:77794571 (GRCh38). WT, wild-type.
- (c) Effect of *GNA11* Q209 mutations on aldosterone secretion in H295R cells stimulated with 10nM of angiotensin II (Ang II) (n=10 wells examined over 4 independent experiments). Results expressed as fold-change from wild-type transfected cells treated with vehicle control (Vehicle).
- (d) Effect of *GNA11* Q209 mutations on cortisol secretion (n=6-8 wells examined over 3 independent experiments). Transfection of *GNA11* Q209P and Q209H mutation increased H295R cells' cortisol secretion compared to *GNA11* wild-type transfected cells ( $p=0.002$  by one-way Kruskal-Wallis test,  $\chi^2(4)=16.45$ ).
- (e) *GNA11* Q209 mutations increase aldosterone/cortisol ratio (n=20 wells examined over 3 independent experiments,  $p=2 \times 10^{-14}$  by one-way Kruskal-Wallis test,  $\chi^2(4)=70.59$ ). Results calculated from data presented in **Figure 1B** and **Supp. Figure 2D**.
- (f) *GNA11* Q209 mutations increase aldosterone secretion in vehicle control (Vehicle) and in the presence of the  $\beta$ -catenin inhibitor ICG-001 (n= 3-6 wells examined over 2 independent experiments). The ICG-001 concentration used is >3x the expected IC50 (3  $\mu$ M). In the presence of 10  $\mu$ M ICG-001, the normally adhering H295R cells were observed to have increased roundness and decreased flatness.
- (g) Transfection of *GNA11* Q209P mutation and/or  $\Delta 45$  *CTNNB1* mutation increases *CYP11B2* mRNA expression in cells cultured from APA 392T (genotyped in **Supp. Table 2**) compared to vector control (n=11-12 wells examined over 4 independent experiments,  $p=0.00002$  by one-way Kruskal-Wallis test,  $\chi^2(3)=24.28$ ).

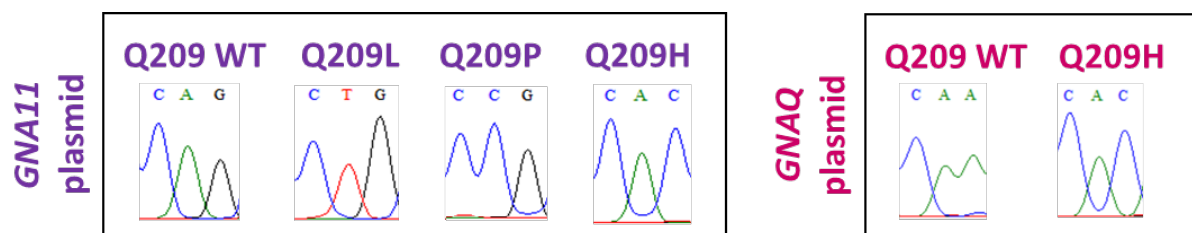
Results are represented as mean $\pm$ s.e.m (**c-f**)/Geomean $\pm$ s.d. (**g**), and normalised when needed by the cell number as estimated by protein (**c-d, f**), or by 18S RNA (**g**).

n.s., not significant. *P*-values of Dunn's multiple comparisons test are as indicated in **d, e** and **g**. *P*-values indicated in **c** and **f** are according to two-tailed Student's *t*-test. The exact sample number (*n*) are as indicated below the x-axis.

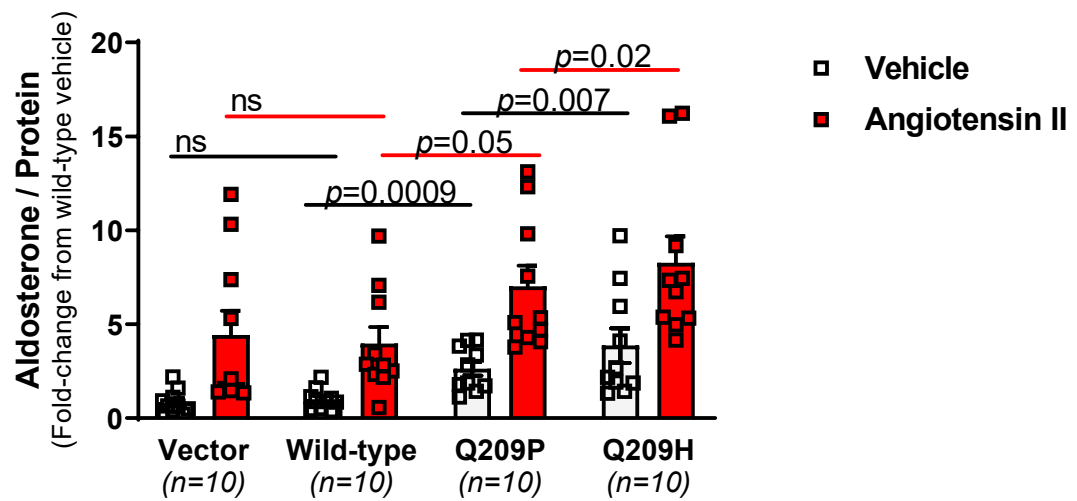
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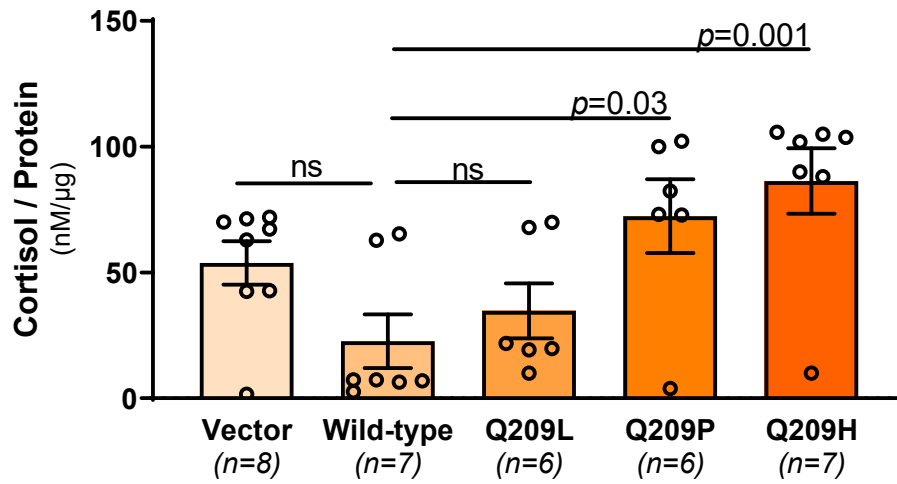
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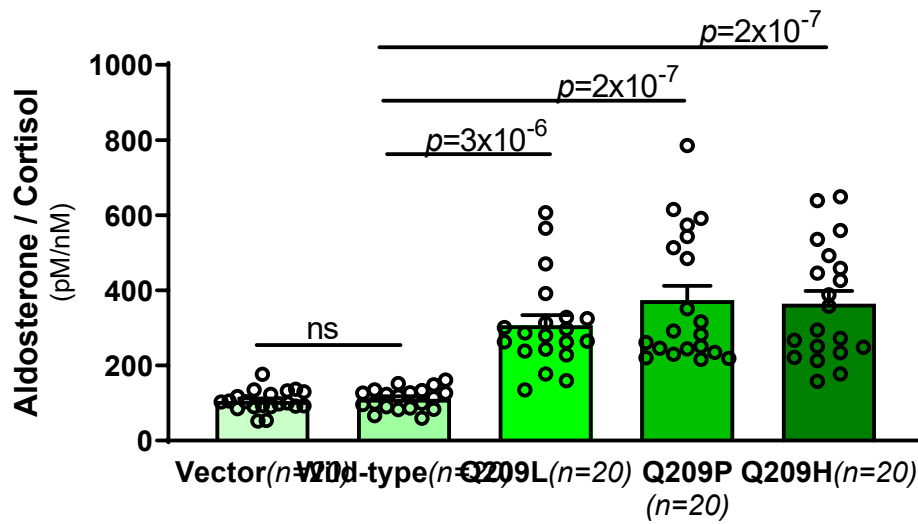
c.



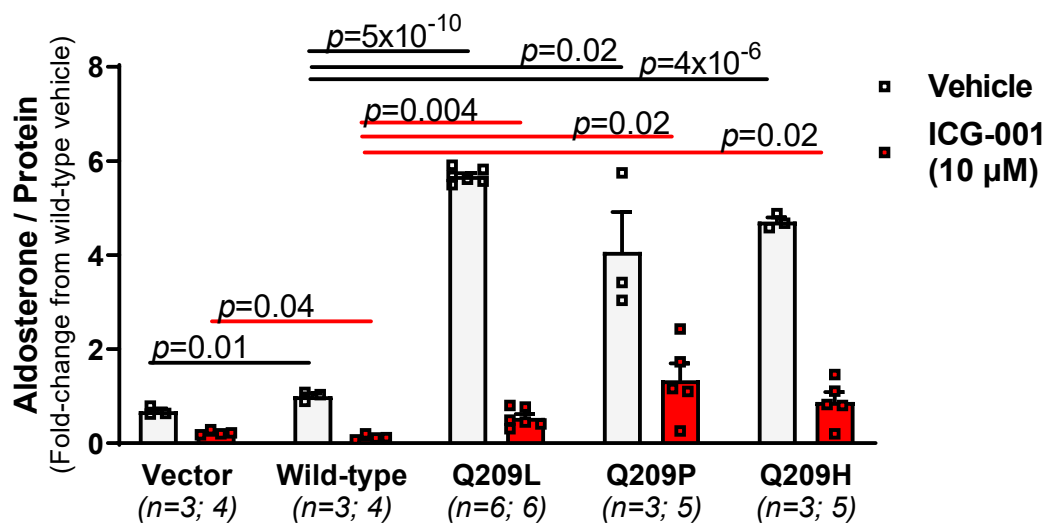
d.



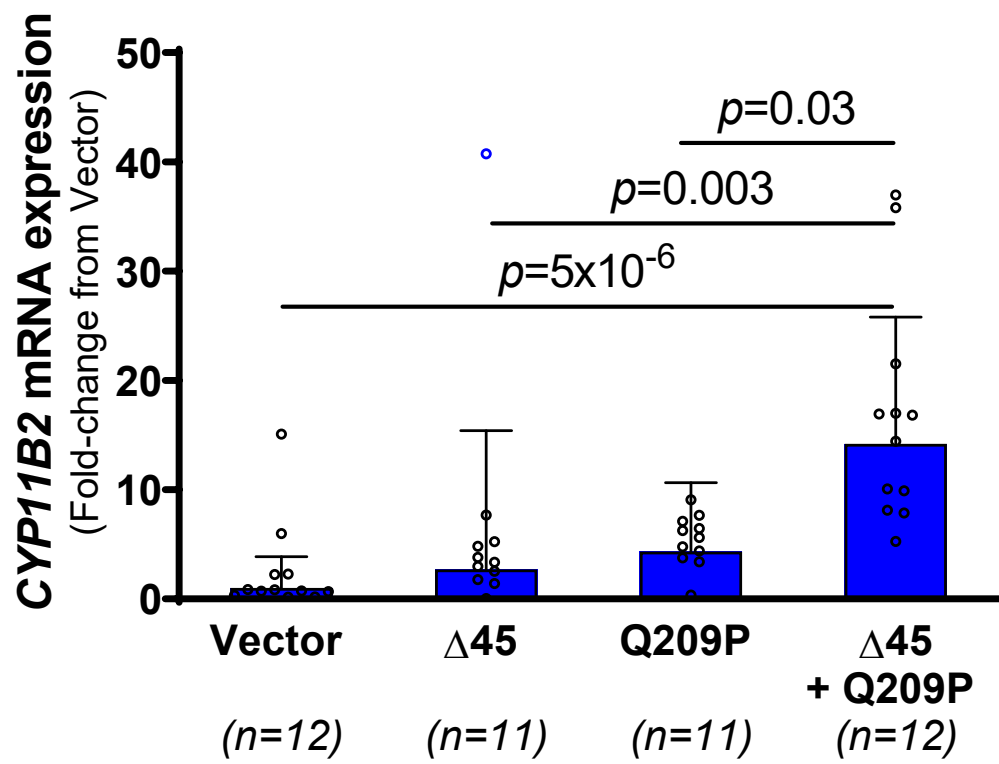
e.



f.



g.





### Supplementary Figure 3

#### Luteinizing hormone–chorionic gonadotropin receptor (LHCGR) protein is intensely expressed in double mutant APA cells.

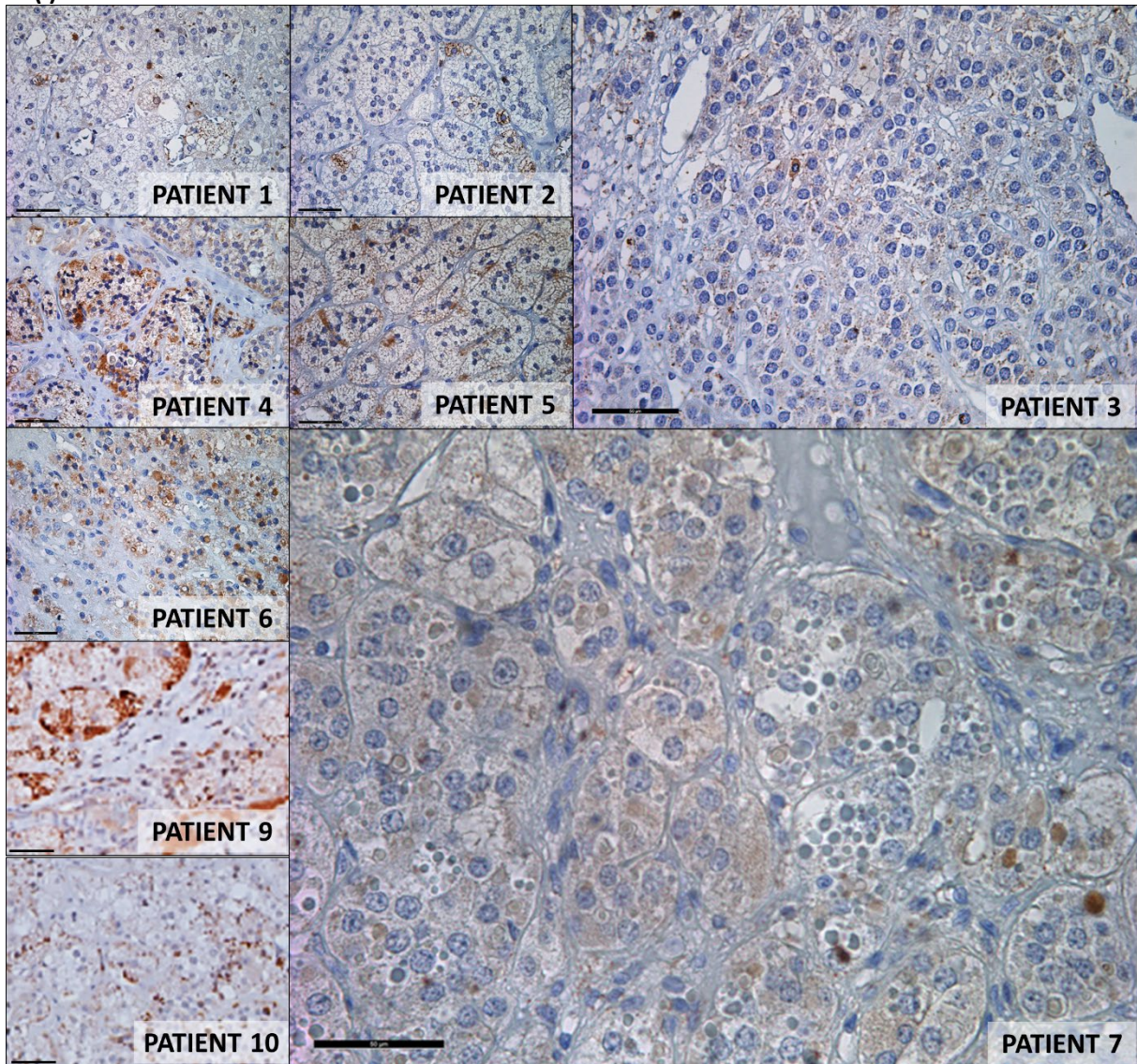
- (a) Immunohistochemistry (IHC) of LHCGR using the primary antibody anti-LHCGR (NLS1436, Novus Biologicals, UK; 1:200; also presented in **Figure 3**) confirmed variable protein expression of LHCGR in double mutant APAs from the UK/Irish cohort (i). Variable protein expression of LHCGR in APA from patient 10 (ii) was validated by qPCR (iii). The adrenal medulla of patient 10 and the adrenal medullary gene *PNMT* were used as controls while CYP11B2 IHC staining and qPCR of CYP11B2 mRNA expression were used to confirm APA sample selection.
- (b) Positive staining of LHCGR in the adrenal medulla (i) was validated by high LHCGR mRNA in adrenal medulla collected by laser capture microdissection (ii).
- (c) Variable intracellular sites of expression of LHCGR in APAs corroborated with different expected subcellular localizations (derived from database annotations, automatic text mining of the biomedical literature, and sequence-based predictions as reported in COMPARTMENTS, a subcellular localization database).<sup>74</sup> Images representative of 17 adrenals.
- (d) Different subcellular expression of LHCGR found in adjacent ZG. The APA is demarcated by CYP11B2 positivity while the adjacent ZG is demarcated by lack of CYP11B1. Pictured is IHC staining of FFPE adrenal section from patient 7. APA, aldosterone-producing adenoma; ZR, zona reticularis; ZF, zona fasciculata; ZG, zona glomerulosa; C, Capsule. Images representative of 7 adrenals.
- (e) LHCGR was detected in the cell membranes of OVCAR3 cells (positive control) but not H295R cells (negative control). Wheat Germ Agglutinin conjugated to Alexa Fluor™ 647 (WGA, Invitrogen, USA, 5µg/ml) was used as a cell membrane marker. LHCGR protein expression was visualized using either the 1° antibody rabbit anti-LHCGR (#1, NLS1436, Novus Biologicals, UK; 1:200) or the 1° antibody mouse anti-LHCGR used in **Supp. Figure 2e-g** (#2, NBP2-52504, Novus Biologicals, UK; 1:100). 2° antibodies used were goat anti-rabbit Alexa Fluor™ 568 (#1, A-11011, Invitrogen, USA, 1:1000), or goat anti-mouse Alexa Fluor™ 568 (#2, A-11004, Invitrogen, USA, 1:1000). Images representative of 3 independent experiments.
- (f-g) LHCGR immunofluorescence is increased in Q209 *GNA11* mutant transfected 351T and 392T primary APA cells (genotyped in **Supp. Table 2**) with high *CTNNB1* expression, compared to *GNA11* (GFP-tagged) and *CTNNB1* wild-type or single mutant transfected cells. No change in LHCGR expression was identified in H295R cells (which harbour the pathogenic *CTNNB1* mutation S45P) transfected with wild type or Q209P *GNA11*. LHCGR protein expression was visualized using 1° #1 2° #1 antibodies used in **Figure 2e**. *CTNNB1* transfection was visualized using the primary antibody mouse anti-CTNNB1 (#610154, BD transduction Lab, USA; 1:100) and either (f) goat anti-mouse Alexa Fluor™ 647 (A-21235, Invitrogen, USA, 1:1000) or (g) goat anti-mouse Alexa Fluor™ 405 (A-31553, Invitrogen, USA, 1:1000). Images representative of 3 independent experiments.
- (h) Validation of increased LHCGR protein expression in *GNA11* mutant and *CTNNB1* mutant/ high *CTNNB1* expression cells was demonstrated using alternative primary antibodies to those used in (f-g). LHCGR protein expression was visualized using the 1° #2 antibody used in (e) and the anti-mouse secondary used in (f) or that used in (g). *CTNNB1* transfection was visualised using the primary antibody rabbit anti-CTNNB1 (#71-2700, Thermo Fisher Scientific, USA; 1:100) and the secondary antibody goat anti-rabbit used in (e). (**Supp. Figure 2h** 392T, images representative of 5 cells; **Supp. Figure 2h** 351T, images representative of 8 cells)
- (i) Double mutant co-transfected cells (ii) cultured from APA 357T (genotyped in **Supp. Table 2**) had higher intense staining of LHCGR compared to single mutant only (i). Arrows ( ↓ ) highlight

suspected non-steroid producing cell (e.g. fibroblast) in primary human adrenal cultures. Antibodies used are the same as in (e). Images representative of 2 independent experiments.

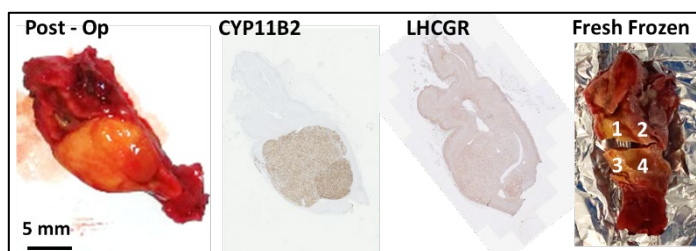
- (j) Correlation of Corrected Total Cell Fluorescence of LHCGR and CTNNB1 in GFP positive cells transfected with either Vector control or GNA11 Q209P. The coefficient of determination,  $R^2$ , for the linear regression model is as shown.

Scale bars: (a.(i), c.) = 50  $\mu$ m, (a.(ii)) = 5 mm, (b, d) = 100  $\mu$ m, (e-h) = 20  $\mu$ m.

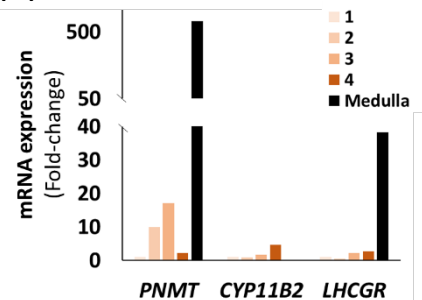
**a. (i)**



**(ii)**



**(iii)**

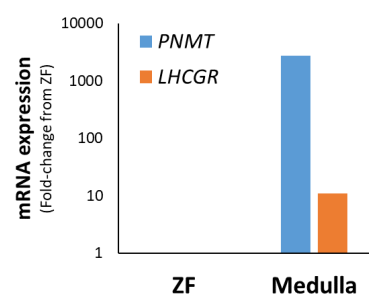




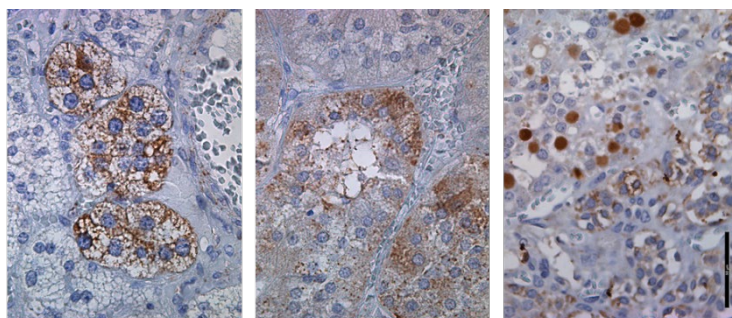
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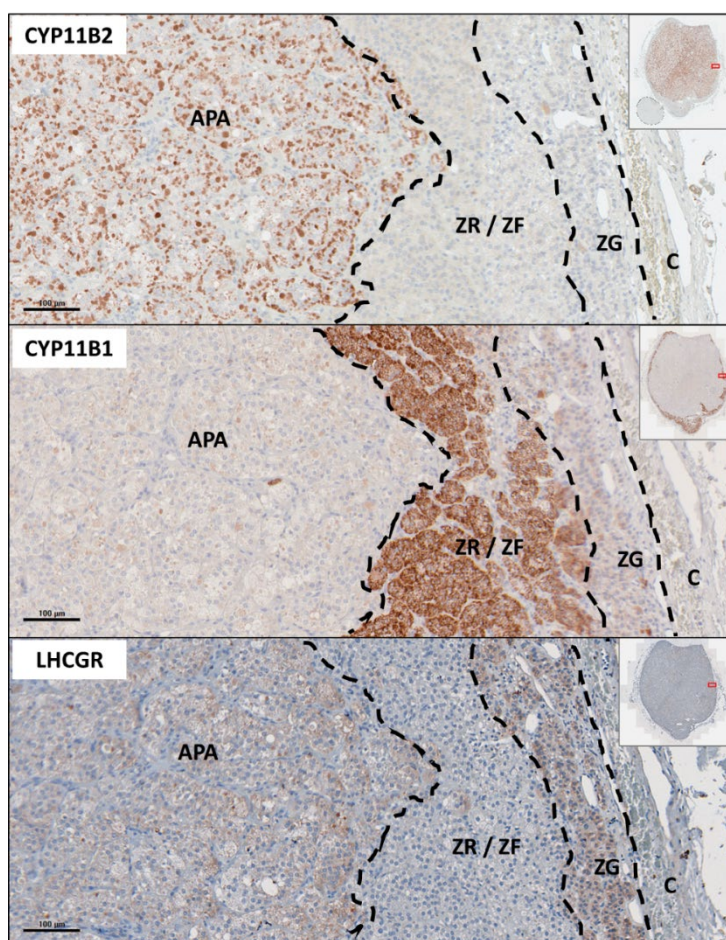
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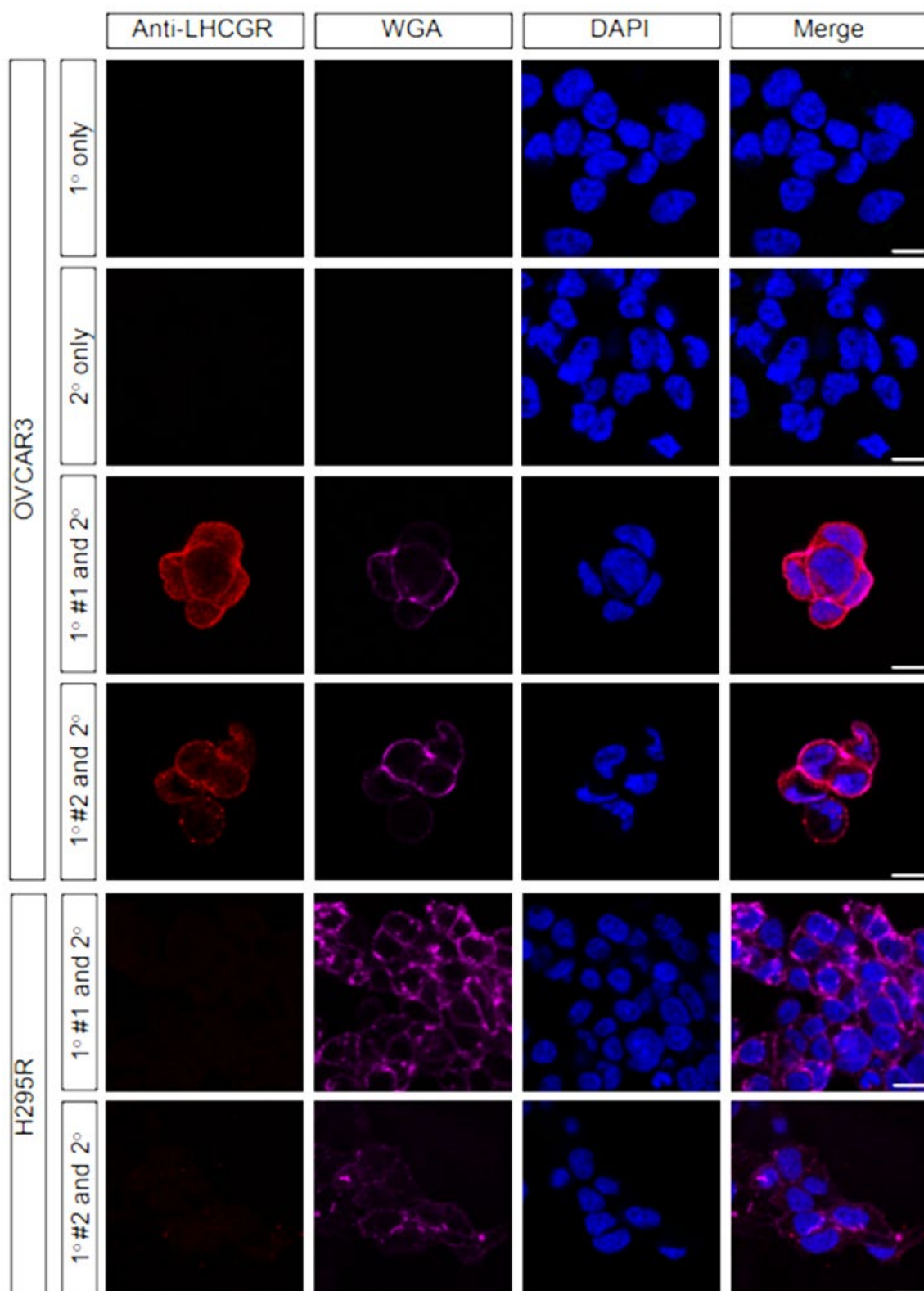
c.



d.



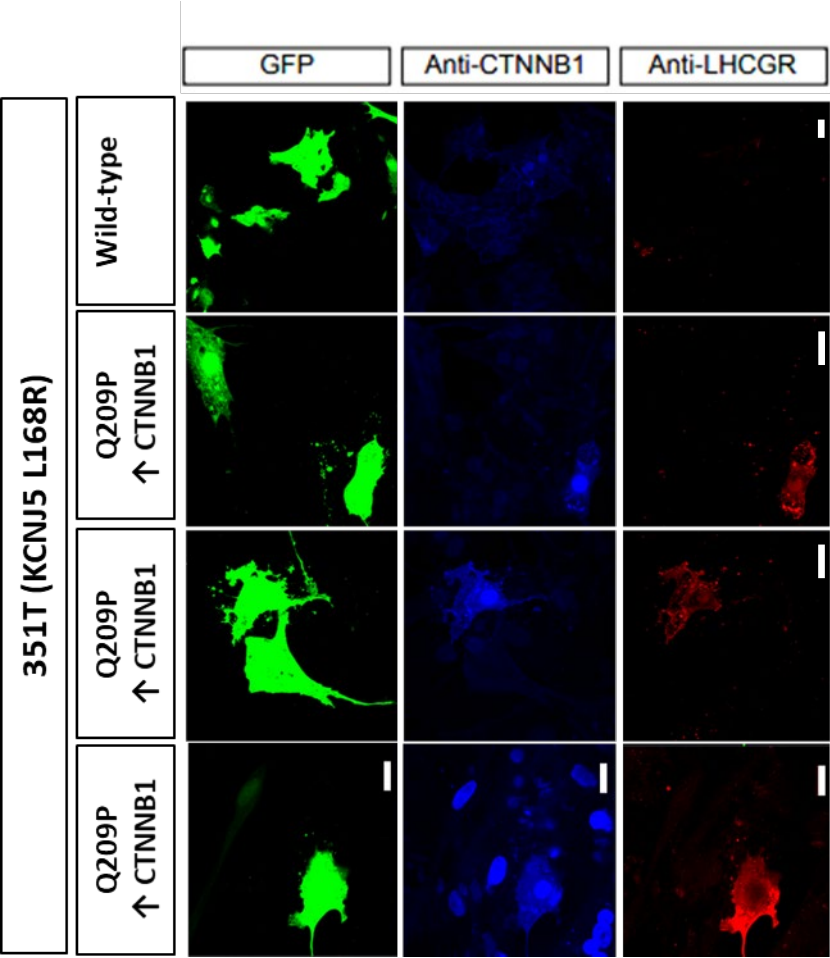
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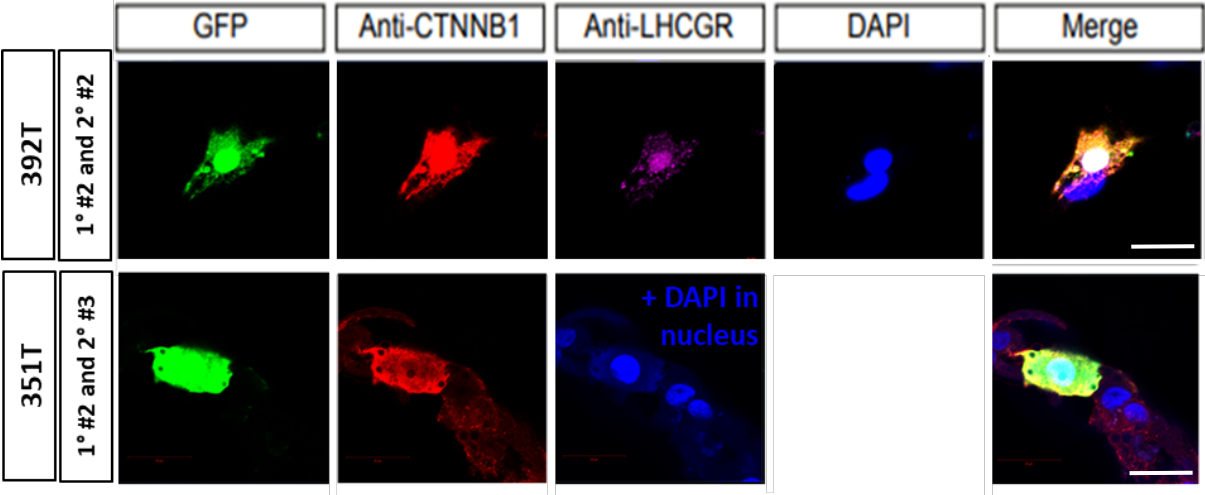




g.

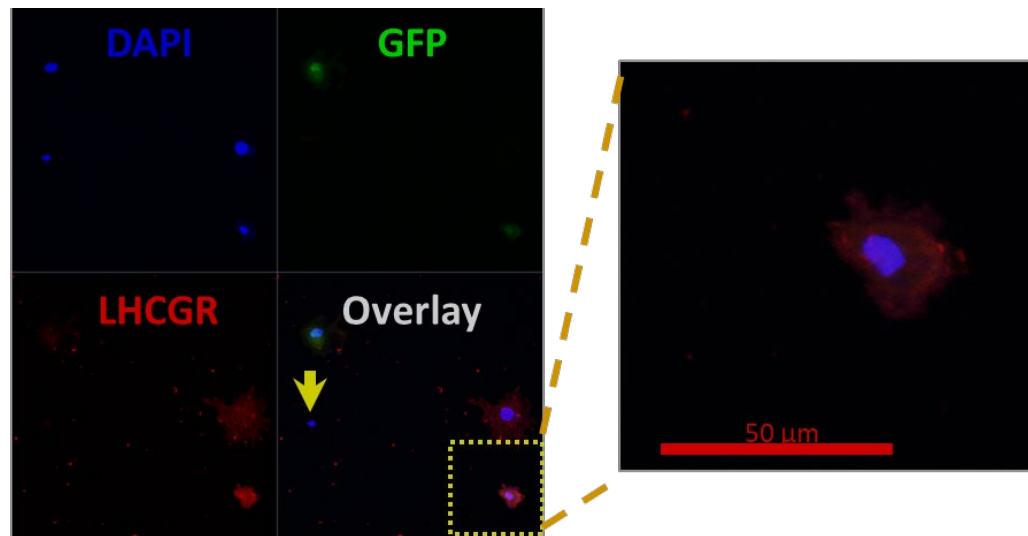


h. Double mutation (*CTNNB1* Δ45 + *GNA11* Q209P) transfection

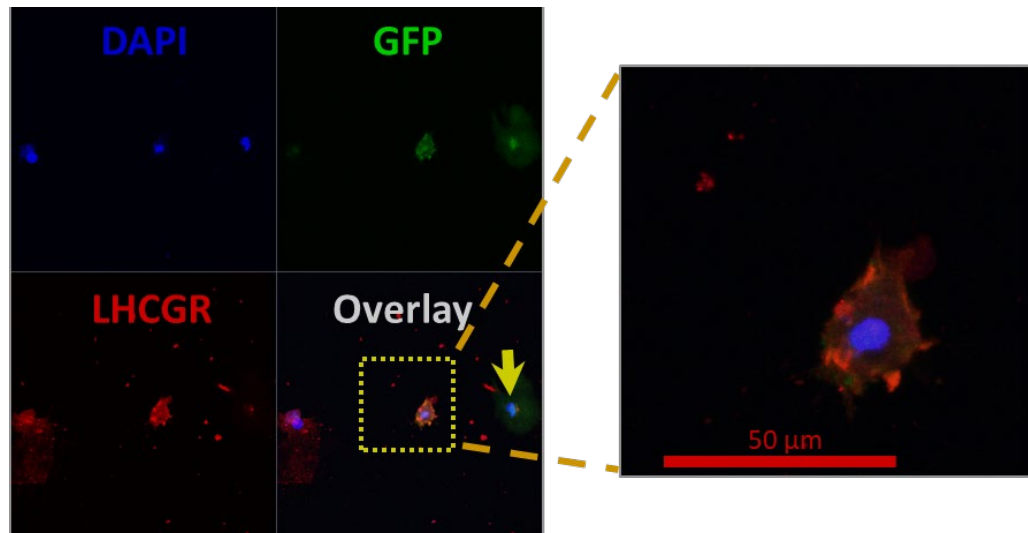


i.

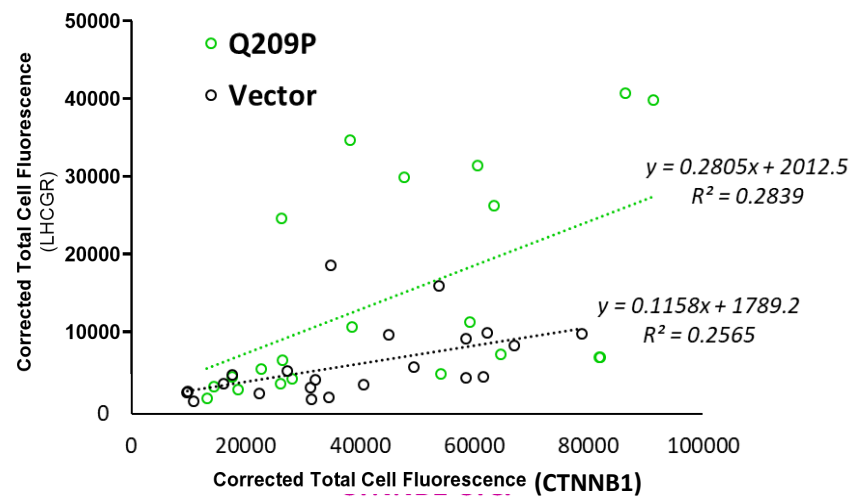
[i] Single mutation (*CTNNB1* WT + *GNA11* Q209H) transfection in 357T



[ii] Double mutation (*CTNNB1*  $\Delta$ 45 + *GNA11* Q209H) transfection in 357T



j.

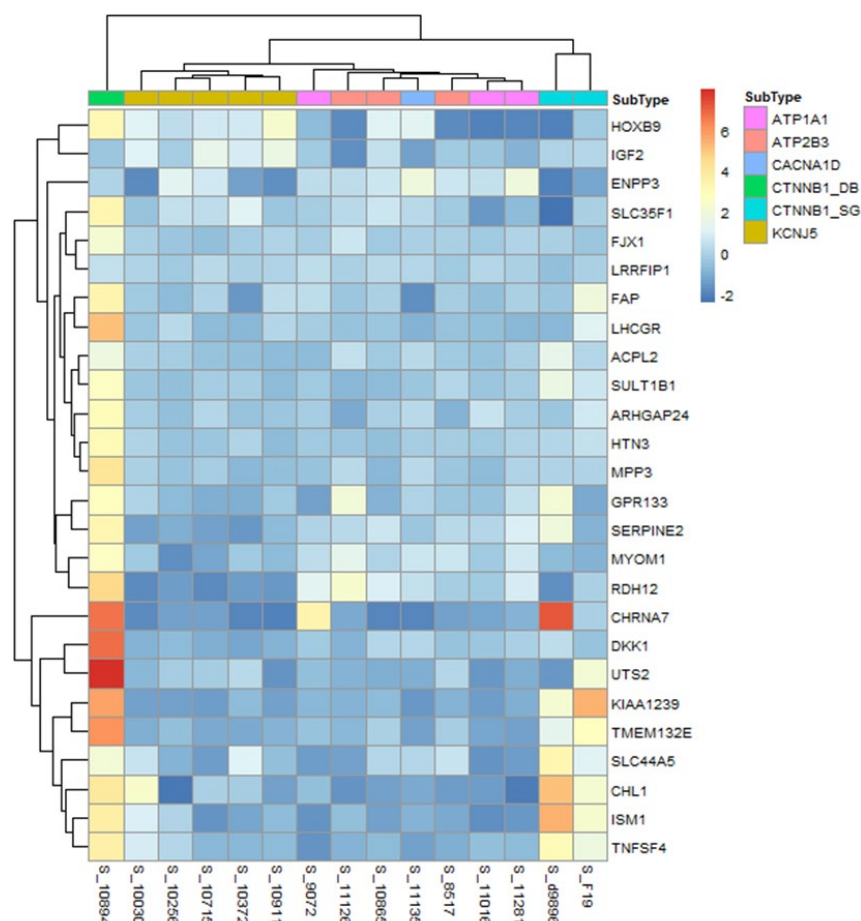


## Supplementary Figure 4

### In silico comparison of gene expression between APAs with double- and single-mutation in the Swedish Cohort.

Each column represents an APA whose genotype-subtype (coded by coloured box beneath brackets) and transcriptome was established by RNAseq.<sup>19</sup> Each row represents a gene whose expression was upregulated >20-fold, in the microarray which compared the transcriptome of our first *CTNNB1*-mutant APA with 13 other APAs, and identified LHCGR as the most upregulated gene.<sup>26</sup> Rows (genes) and columns (individual APAs) are hierarchically clustered. The unsupervised cluster analysis of samples, using the whole transcriptome, clustered the expression profiles of APAs by genotype, as indicated by the bracketing above the heat map. As previously reported, the *CTNNB1*-mutant APAs clustered separately from all other APAs, but the green-coded *CTNNB1*-mutant APA (*CTNNB1\_DB*) lies at the opposite end of the tree, with a several-hundred fold elevation in LHCGR expression, to the two turquoise-coded *CTNNB1*-mutant APAs (*CTNNB1\_SG*). Further analysis of the RNAseq showed the green-coded APA to have an additional mutation of *GNAQ*, p.Gln209His.

*CTNNB1\_DB*, *CTNNB1* and *GNAQ* double mutant APA. *CTNNB1\_SG*, *CTNNB1* single mutant APA.





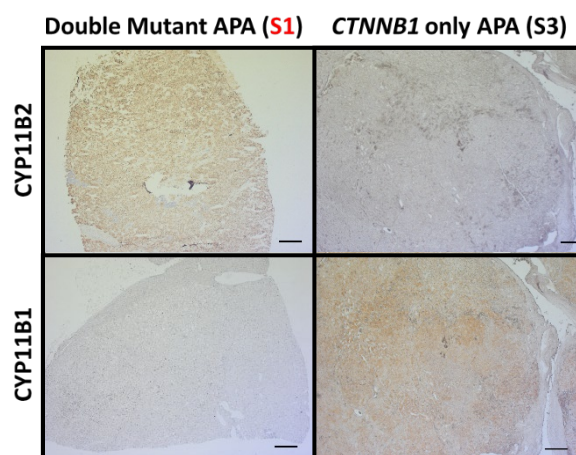
## Supplementary Figure 5

***CTNNB1* and *GNA11/Q* double mutant APA have a low CYP11B1/CYP11B2 ratio and a hyperplastic ZG in the adjacent adrenal gland that is negative for CYP11B2.**

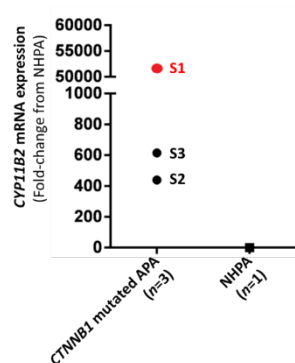
- (a) Immunohistochemistry of CYP11B2 and CYP11B1 in the Swedish cohort using the primary antibody anti-CYP11B2 #MABS1251, clone 41-17B (1:2500; Sigma-Aldrich, USA) and anti-CYP11B1 #MABS502, clone 80-7 (1:100; Sigma-Aldrich, USA). H-score immunohistochemistry quantification of the CYP11B2 and CYP11B1 protein expression was performed on the double mutant APA (S1) and the *CTNNB1* only mutant APA (S3). The double mutant APA (S1) had a H-score for CYP11B2 of 277.5 and a H-score of CYP11B1 of 0.22, while the single mutant (*CTNNB1* only) APA (S3) had a H-score for CYP11B2 of 28.7 and a H-score for CYP11B1 of 291.2. Scale bar = 0.5 mm.
- (b) CYP11B2 mRNA expression (i) in *CTNNB1* mutant APAs correlated with amount of aldosterone present in APA lysate (ii) in the Swedish cohort.
- (c) P1-P7 are FFPE sections of the adjacent adrenal glands of patients 1-7 from the discovery cohort that had double mutant APAs stained for CYP11B2 (1:100) and CYP11B1 (1:100) using custom made antibodies, or anti-LHCGR #NLS1436 (1:200; Novus Biological, USA). All 7 adrenals contained ZG hyperplasia that were CYP11B2 negative but LHCGR positive. ZG was identified based on lack of CYP11B1 expression in the adrenal cortex subcapsular. **ZG**, zona glomerulosa; **ZF**, zona fasciculata; **APA**, aldosterone-producing adenoma. Scale bar = 100  $\mu$ m.

Red symbol, double mutant. Blue symbol, *KCNJ5* mutant.

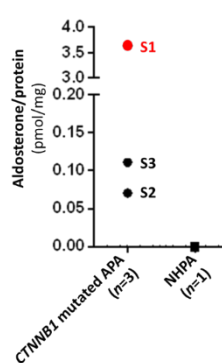
a.



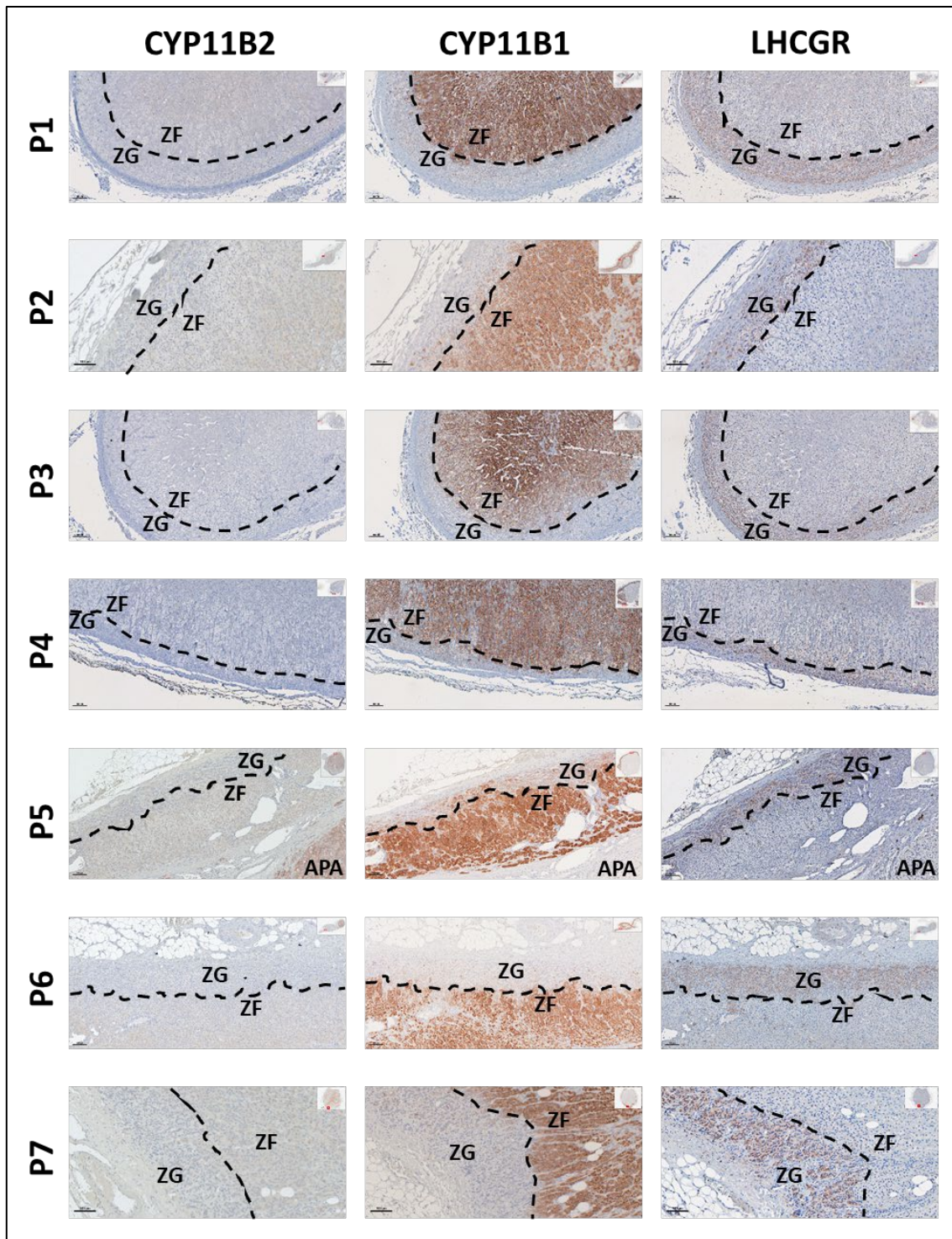
b. (i)



(ii)



c.

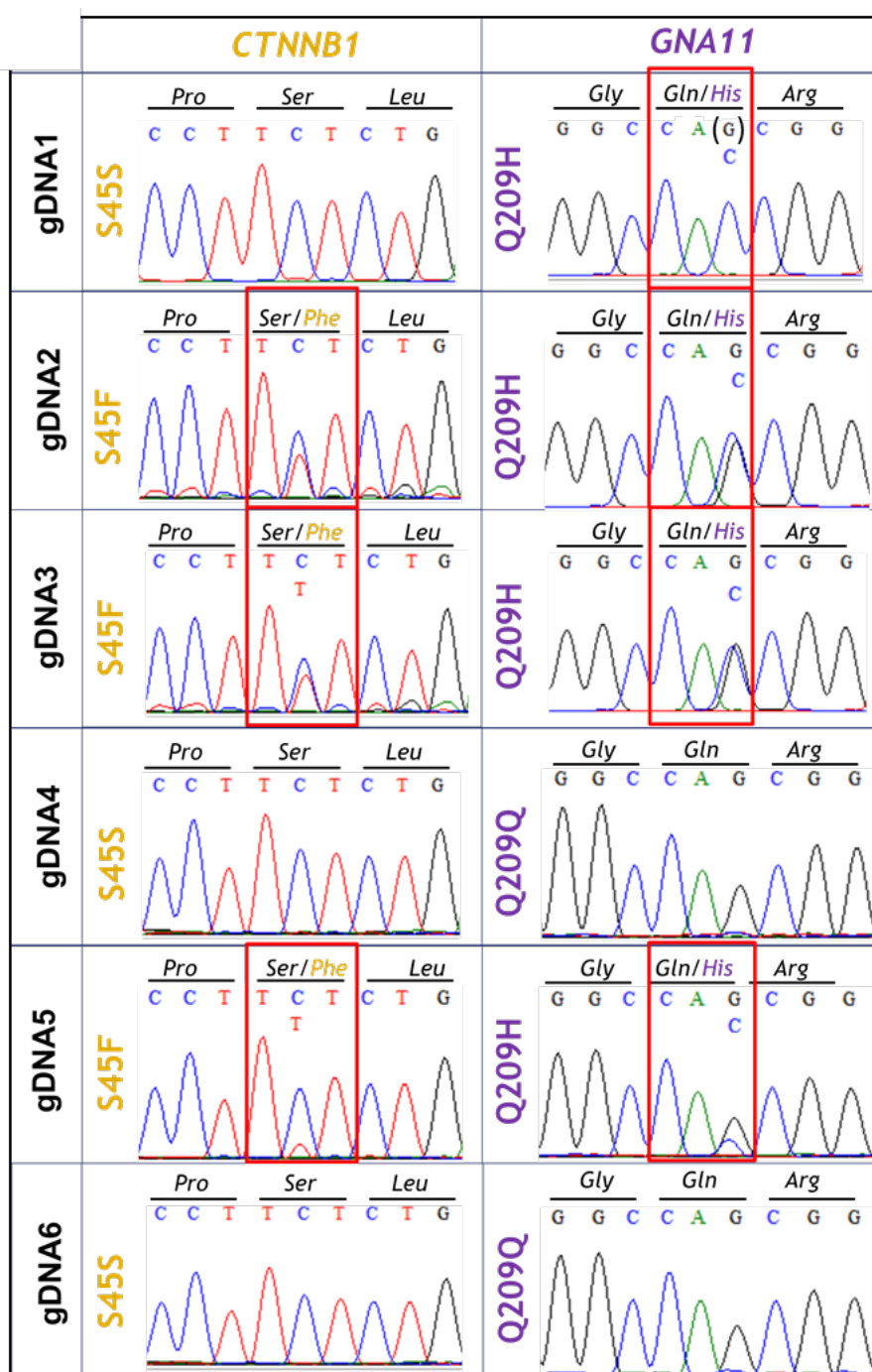


## Supplementary Figure 6

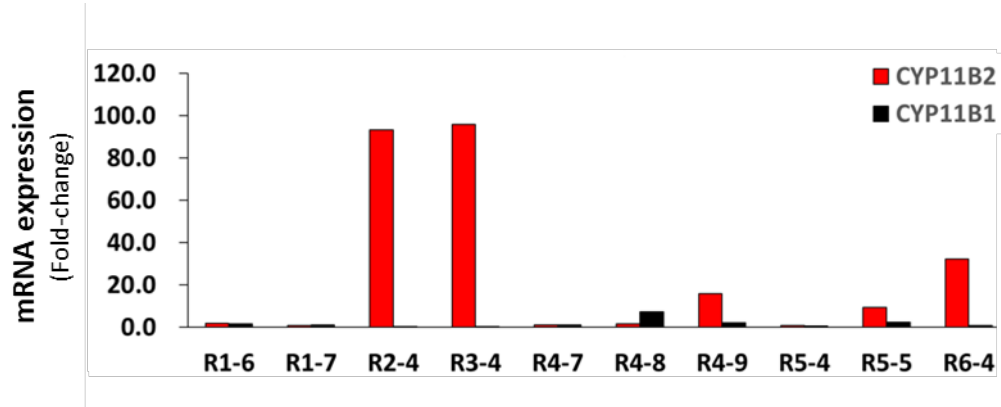
### ***CTNNB1* and *GNA11* genotypes of fresh frozen adrenal tissue samples from Patient 7 (a) and LCM samples from FFPE adrenal tissue sections of Patient 1 (b).**

- (a) (i) gDNA extracted from 6 different regions across the fresh frozen adrenal tissue of Patient 7 (mapping illustrated in **Figure 6a**) were genotyped for *CTNNB1* and *GNA11* mutations. gDNA from regions 2, 3 and 5 harbor the same heterozygous *CTNNB1* S45F and *GNA11* Q209H as the APA (**Supp. Figure 1a**). R1 gDNA harbors no *CTNNB1* somatic mutations in exon 3 but a homozygous *GNA11* Q209H somatic mutation.
- (ii) mRNA expression of CYP11B2 and CYP11B1 in fresh frozen tissue sampled from regions 1-6. The low CYP11B2 levels in regions 1, 4, and 5 (R1-6, R1-7, R4-7, R4-8, R4-9, R5-4, R5-5) and relatively higher expression of CYP11B1 compared to regions 2, 3, and 6 (R2-4, R3-4, R6-4) supports sampling of adjacent adrenal gland.
- (iii) The gene expression profile of the six hallmark differential expressed genes (DEG) related to double mutant APAs in R1, R2 and R3 cDNA (extracted from the same samples as R1, R2 and R3 gDNA) confirms separation of the sample in cDNA1 from the APA and suggests cDNA2 and cDNA3 were tumour samples. The finding of a *GNA11* mutation in the adjacent adrenal gland led to the precise analysis of the different adrenal zone genotyping through targeted sequencing of scraped FFPE adrenal samples and laser capture microdissected adrenal samples (**Extended data Figure 2, Figure 6d-f, and Supp. Figure 6b**).
- (b) (i) *CTNNB1* and *GNA11* genotype in the adjacent adrenal zones of Patient 1 (S45F *CTNNB1* and Q209P *GNA11* mutant APA) acquired by laser capture microdissection (LCM). *GNA11* mutations were detected in 2/8 zona glomerulosa (ZG) gDNA samples (the electropherograms of mutant ZGs, ZG1 and ZG6, and location of sample in adrenal section of Patient 1 are illustrated in Figure 6e).
- (ii) Single samples of ZF, ZR and medulla detected no mutations in R183/Q209 *GNA11* or exon 3 of *CTNNB1*. gDNA from the patient's skin was also wild-type.

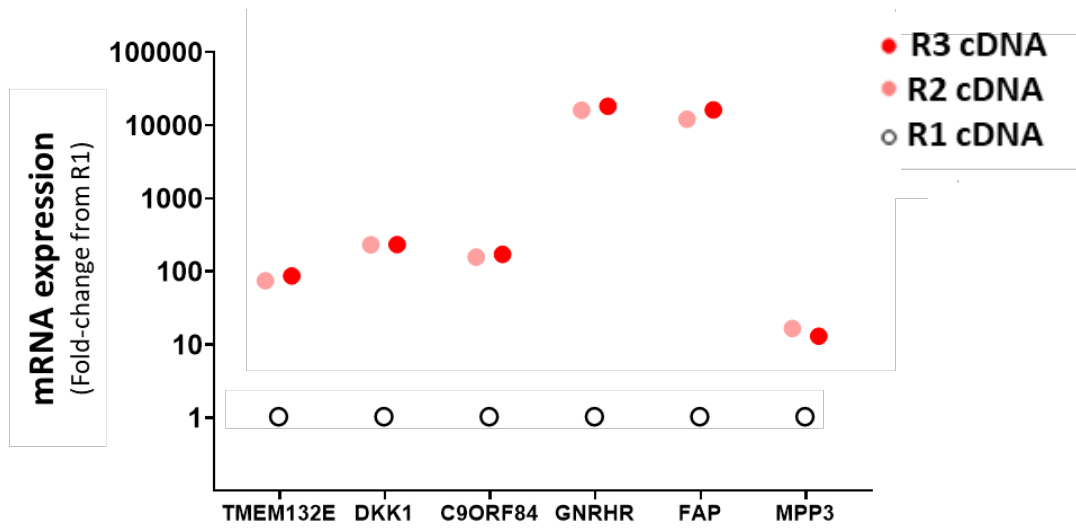
a. (i)



(ii)

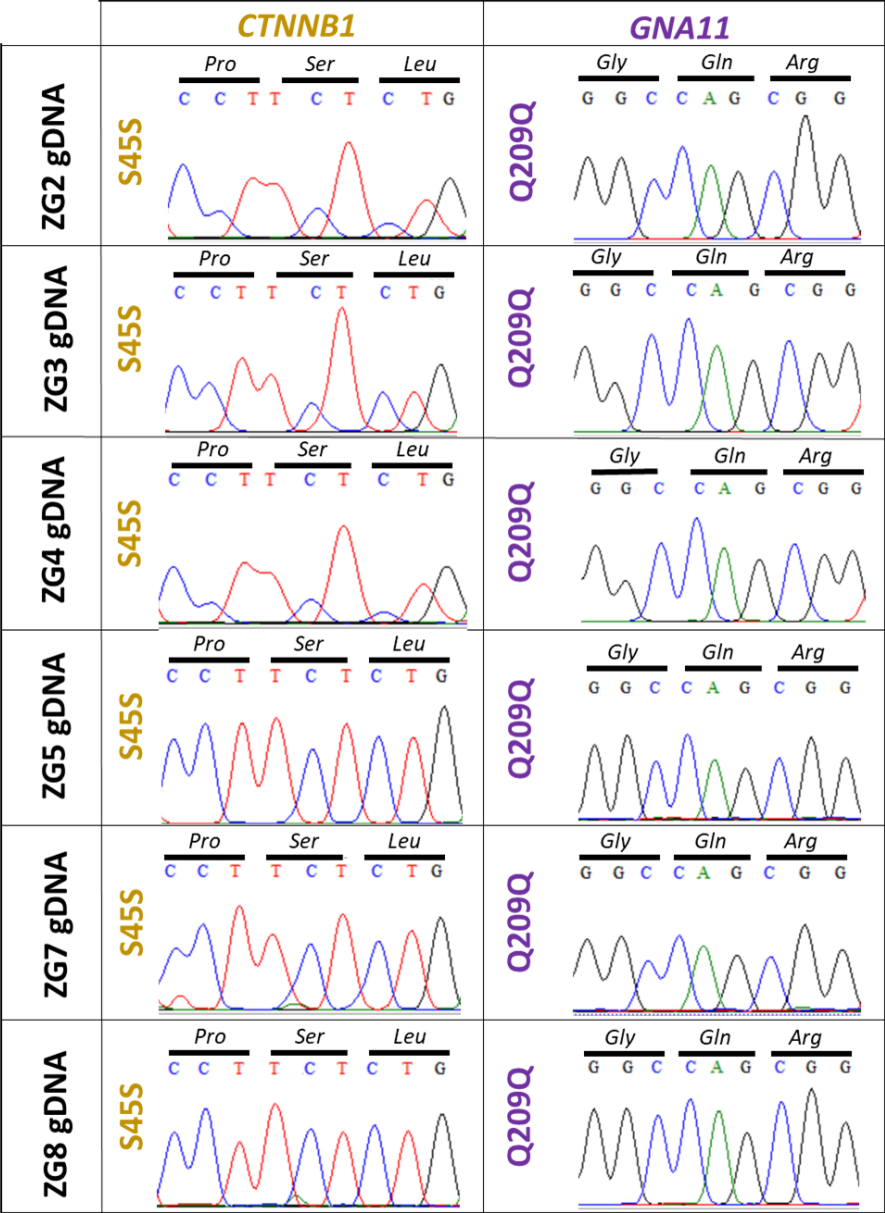


(iii)

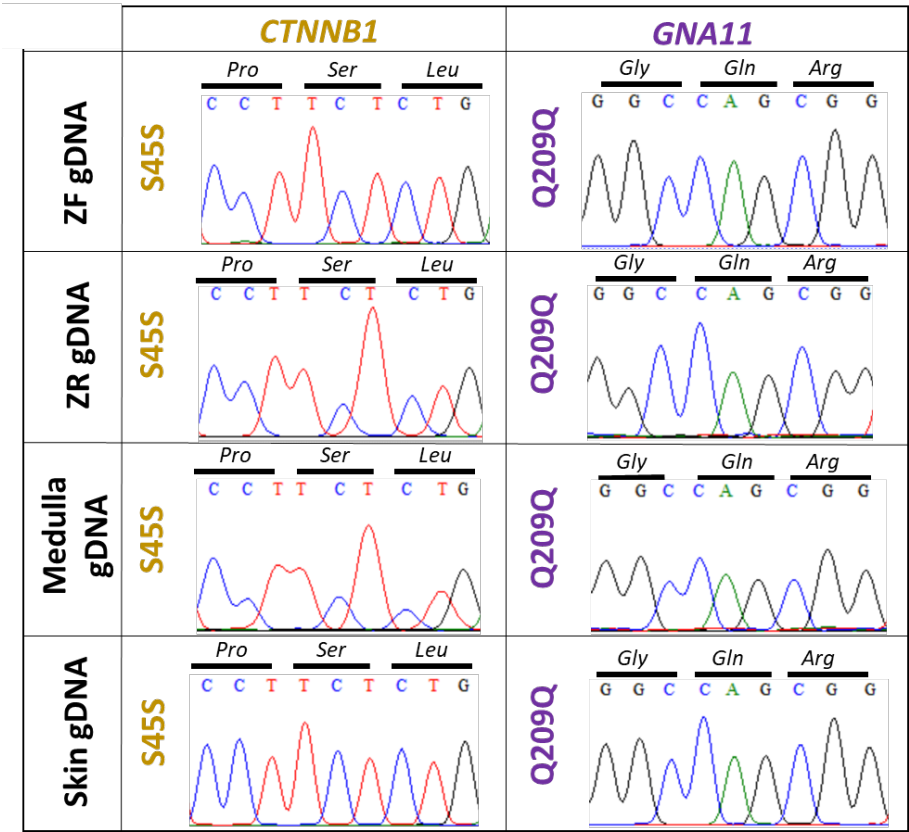




b. (i)



b. (ii)



**Supplementary Table 1**

**Genes with recurrent pathogenic somatic mutation detected through whole exome sequencing (WES) of tumour and germline DNA of 41 APAs.**

A second gene with recurrent pathogenic mutation, Q209 *GNA11* somatic mutations, was found in APAs with *CTNNB1* mutations. *ATP1A1*, *ATP2B3*, and *CACNA1D* mutant APAs predominantly occurred in males while *CTNNB1* and *KCNJ5* mutant APAs predominantly occurred in females.

| Sample              | Age | Gender | Ethnicity         | Gene           | Mutation          | Gene-2       | Mutation-2   |
|---------------------|-----|--------|-------------------|----------------|-------------------|--------------|--------------|
| 176T                | 64  | Male   | Caucasian         | <i>ATP1A1</i>  | <i>L104R</i>      |              |              |
| 182T                | 41  | Male   | Caucasian         | <i>ATP1A1</i>  | <i>L104R</i>      |              |              |
| 187T                | 33  | Male   | Caucasian         | <i>ATP1A1</i>  | <i>L104R</i>      |              |              |
| 200T                | 59  | Male   | Caucasian         | <i>ATP1A1</i>  | <i>960_964del</i> |              |              |
| 228T                | 50  | Male   | Caucasian         | <i>ATP1A1</i>  | <i>L104R</i>      |              |              |
| 141T                | 53  | Male   | Caucasian         | <i>ATP2B3</i>  | <i>del423-424</i> |              |              |
| 148T                | 54  | Male   | Caucasian         | <i>ATP2B3</i>  | <i>del423-424</i> |              |              |
| 156T                | 66  | Male   | Caucasian         | <i>ATP2B3</i>  | <i>del423-424</i> |              |              |
| 192T                | 63  | Female | Caucasian         | <i>ATP2B3</i>  | <i>del423-424</i> |              |              |
| 181T                | 47  | Male   | Caucasian         | <i>CACNA1D</i> | <i>G403R</i>      |              |              |
| 194T                | 55  | Male   | Caucasian         | <i>CACNA1D</i> | <i>V1338M</i>     |              |              |
| 195T                | 36  | Female | African-Caribbean | <i>CACNA1D</i> | <i>I650N</i>      |              |              |
| 196T                | 63  | Male   | Caucasian         | <i>CACNA1D</i> | <i>R990G</i>      |              |              |
| 202T                | 53  | Male   | Caucasian         | <i>CACNA1D</i> | <i>F1147L</i>     |              |              |
| 214T                | 49  | Male   | Caucasian         | <i>CACNA1D</i> | <i>F747L</i>      |              |              |
| 223T                | 65  | Male   | African-Caribbean | <i>CACNA1D</i> | <i>I750M</i>      |              |              |
| 233T                | 47  | Male   | Caucasian         | <i>CACNA1D</i> | <i>P1336R</i>     |              |              |
| 239T1               | 55  | Male   | Caucasian         | <i>CACNA1D</i> | <i>G403R-8B</i>   |              |              |
| 240T                | 57  | Male   | Caucasian         | <i>CACNA1D</i> | <i>F747L</i>      |              |              |
| 252T                | 52  | Male   | Caucasian         | <i>CACNA1D</i> | <i>V1338M</i>     |              |              |
| 14T<br>(Patient 3)  | 20  | Female | Caucasian         | <i>CTNNB1</i>  | <i>T41A</i>       | <i>GNA11</i> | <i>Q209H</i> |
| 238T<br>(Patient 2) | 35  | Female | Caucasian         | <i>CTNNB1</i>  | <i>S45P</i>       | <i>GNA11</i> | <i>Q209P</i> |
| 346T<br>(Patient 1) | 12  | Male*  | Caucasian         | <i>CTNNB1</i>  | <i>S45F</i>       | <i>GNA11</i> | <i>Q209P</i> |
| 171T                | 34  | Female | Caucasian         | <i>KCNJ5</i>   | <i>G151R</i>      |              |              |
| 185T                | 42  | Female | Caucasian         | <i>KCNJ5</i>   | <i>G151R</i>      |              |              |
| 190T                | 47  | Female | Caucasian         | <i>KCNJ5</i>   | <i>G151R</i>      |              |              |
| 191T                | 39  | Female | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 198T                | 41  | Female | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 204T                | 28  | Female | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 219T                | 49  | Female | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 221T                | 63  | Male   | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 244T                | 55  | Female | Caucasian         | <i>KCNJ5</i>   | <i>L168R</i>      |              |              |
| 30T                 | 55  | Male   | Caucasian         |                |                   |              |              |
| 136T                | 59  | Male   | Caucasian         |                |                   |              |              |
| 183T                | 56  | Male   | Caucasian         |                |                   |              |              |
| 184T                | 48  | Male   | Caucasian         |                |                   |              |              |
| 218T                | 56  | Female | African-Caribbean |                |                   |              |              |
| 227T                | 59  | Male   | Caucasian         |                |                   |              |              |
| 235T                | 34  | Male   | Caucasian         |                |                   |              |              |
| 247T                | 61  | Male   | Caucasian         |                |                   |              |              |
| 250T                | 69  | Male   | Caucasian         |                |                   |              |              |

\*Boy presenting with incipient puberty, with 6ml testes.

## Supplementary Table 2

**Genotype of *CTNNB1*, *GNA11*, *GNAQ*, *GNAS*, and *KCNJ5* in the primary adrenal cells from 3 patients' APAs used for functional experiments.**

Sample 351T, 357T, and 392T were target sequenced at known hot spots of the aldosterone-driver genes.

| Sample | Age | Gender | APA diameter (mm) | Tumour genotype |              |             |             |              |
|--------|-----|--------|-------------------|-----------------|--------------|-------------|-------------|--------------|
|        |     |        |                   | <i>CTNNB1</i>   | <i>GNA11</i> | <i>GNAQ</i> | <i>GNAS</i> | <i>KCNJ5</i> |
| 351T   | 32  | Female | 30                | WT              | WT           | WT          | WT          | L168R        |
| 357T   | 44  | Male   | 13                | WT              | WT           | WT          | WT          | WT           |
| 392T   | 32  | Female | 40                | WT              | WT           | WT          | WT          | WT           |

WT, wild-type.

### Supplementary Table 3

#### Gene-annotation enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID ) v6.8.

Analysis of 561 genes whose expression were up- or down- regulated by at least 2-fold between the double-mutant APA and the average of all other APAs in at least 2 of the following 3 publicly available studies.<sup>7, 19, 26</sup> The 3 publicly available microarray or RNA seq dataset used had been performed before the 3 double mutant APAs genotype were known: the index case from 2013 (patient 4)<sup>7</sup>; the APA from a menopausal woman (patient 6)<sup>26</sup>; and the newly diagnosed Swedish double mutant APA (S1)<sup>19</sup>. 8 terms passed the filter of more than 2-fold enrichment with a Bonferroni corrected and FDR corrected p-value <0.05 (a). Cell junction and cell adhesion are the most significant enrichment (unadjusted  $p$ -value<5x10<sup>-9</sup>; Fisher's Exact test). Genes counted in the GO terms in (a) are shown in (b).

a.

| Category         | Term                                        | Count | List Total | Population Hits | Population Total | Fold Enrichment | Unadjusted P-Value |
|------------------|---------------------------------------------|-------|------------|-----------------|------------------|-----------------|--------------------|
| UP_KEYWORDS      | Cell junction                               | 48    | 561        | 675             | 20581            | 2.61            | 4.08E-09           |
| UP_KEYWORDS      | Cell adhesion                               | 39    | 561        | 479             | 20581            | 2.99            | 4.24E-09           |
| UP_KEYWORDS      | Calcium                                     | 52    | 561        | 877             | 20581            | 2.18            | 3.01E-07           |
| GOTERM_CC_DIRECT | GO:0030054~cell junction                    | 35    | 547        | 459             | 18224            | 2.54            | 1.27E-06           |
| GOTERM_BP_DIRECT | GO:0007155~cell adhesion                    | 35    | 511        | 459             | 16792            | 2.51            | 1.67E-06           |
| UP_KEYWORDS      | Synapse                                     | 28    | 561        | 357             | 20581            | 2.88            | 1.91E-06           |
| INTERPRO         | IPR003961:Fibronectin, type III             | 21    | 540        | 207             | 18559            | 3.49            | 2.77E-06           |
| GOTERM_CC_DIRECT | GO:0009897~external side of plasma membrane | 21    | 547        | 213             | 18224            | 3.28            | 6.77E-06           |

b.

| Accession  | Term                             | Genes                                                                                                                                                                                                                                                                 |
|------------|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| GO:0030054 | cell junction                    | <i>STON2, SYT4, GRIK4, AFAP1L1, AMOTL1, AMPH, SYP, ATP2B2, GCOM1, C4ORF19, CHRNA7, CHRFAM7A, SV2C, CHRNA3, LRFN2, DTNA, SHC4, DLG1, GRID1, F11R, MAGI1, RIMBP2, SDK2, BSN, SLC6A17, HOMER1, LDLRAD3, EPHA4, TMEM47, EGFLAM, SRPX2, PLEKHA7, CHRN4, CYFIP2, UNC13A</i> |
| GO:0007155 | cell adhesion                    | <i>ITGAL, VTN, IL32, CX3CL1, DDR2, LAMB4, CD9, SRPX, ITGAX, FAT1, FAP, CD2, ROBO2, GPNMB, ENTPD1, SPP1, CYR61, THBS4, COL4A3, F11R, OLR1, MAGI1, SELL, AJAP1, SLAMF1, CTNNA3, CTNNA2, EPHA4, OMD, DSG2, FREM2, ITGA7, RELN, CHL1, NTM</i>                             |
| GO:0009897 | external side of plasma membrane | <i>IL2RB, SELL, CD3E, CTLA4, CXCL9, IL7R, ITGB1, SLAMF1, ABCG1, CD9, CD83, TNFRSF9, ACE, CCR7, ITGAX, CD69, ICOS, CD2, AMOT, CHRNA7, CHRFAM7A</i>                                                                                                                     |



**Supplementary Table 4**

**Genotyping regions of adrenals that harbour double mutant APAs. (a) Comparison of digital droplet PCR, next generation sequencing (NGS), and whole exome sequencing results. (b) Range of % of DNA positive for mutation as detected by ddPCR (c) or NGS (c) in adrenal gland adjacent to a double mutant APA. (d) No mutations were found in control adrenals in the targeted regions.**

**a.**

| Patient ID<br><br>(Sample Type) | Region ID | % of DNA positive for mutation |                         |            |               |            |                        |            |               |            |
|---------------------------------|-----------|--------------------------------|-------------------------|------------|---------------|------------|------------------------|------------|---------------|------------|
|                                 |           | ddPCR                          | NGS targeted sequencing |            |               |            | Whole Exome Sequencing |            |               |            |
|                                 |           | <i>GNA11</i>                   | <i>GNA11</i>            |            | <i>CTNNB1</i> |            | <i>GNA11</i>           |            | <i>CTNNB1</i> |            |
|                                 |           | %                              | %                       | Total Read | %             | Total Read | %                      | Total Read | %             | Total Read |
| Patient 7<br>(Fresh Frozen)     |           | Q209H (CAC)                    | Q209H (CAC)             |            | S45F (TTT)    |            | Q209H (CAC)            |            | S45F (TTT)    |            |
|                                 | R1        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R1-1      | 16%                            | 17%                     | 2096       | 15%           | 3427       | 10%*                   | 393        | 8%*           | 168        |
|                                 | R1-2      | 8%                             | 8%                      | 1839       | 6%            | 2947       | 3%*                    | 408        | 1%*           | 192        |
|                                 | R1-3      | 3%                             | 4%                      | 2235       | 2%            | 3657       | *Combined with R1-1    |            |               |            |
|                                 | R1-4      | 1%                             | 1%                      | 2080       | 1%            | 3648       | *Combined with R1-2    |            |               |            |
|                                 | R2        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R2-1      | 21%                            | 21%                     | 2172       | 17%           | 3152       | 15%*                   | 436        | 17%*          | 173        |
|                                 | R2-2      | 14%                            | 15%                     | 2362       | ND            | ND         | *Combined with R2-1    |            |               |            |
|                                 | R3        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R3-1      | 16%                            | 16%                     | 424        | 12%           | 3569       | 12%*                   | 467        | 14%*          | 162        |
|                                 | R3-2      | 14%                            | 16%                     | 2100       | 14%           | 3388       | *Combined with R3-1    |            |               |            |
|                                 | R4        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R4-1      | 5%                             | 6%                      | 1506       | 4%            | 2781       | 3%*                    | 266        | 1%*           | 147        |
|                                 | R4-2      | 4%                             | 5%                      | 1833       | 3%            | 3158       | ND                     |            |               |            |
|                                 | R4-3      | 2%                             | 4%                      | 2000       | 3%            | 3450       | ND                     |            |               |            |
|                                 | R4-4      | 2%                             | 2%                      | 1754       | 1%            | 3062       | *Combined with R4-1    |            |               |            |
|                                 | R4-5      | 0%                             | 1%                      | 1948       | 0%            | 3175       | ND                     |            |               |            |
|                                 | R4-6      | 0%                             | 0%                      | 2164       | 0%            | 3723       | ND                     |            |               |            |
|                                 | R5        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R5-1      | 3%                             | 5%                      | 1063       | 2%            | 2028       | 6%                     | 235        | 3%            | 191        |
|                                 | R5-2      | 1%                             | 2%                      | 2139       | ND            |            | ND                     |            |               |            |
|                                 | R6        |                                |                         |            |               |            |                        |            |               |            |
|                                 | R6-1      | 10%                            | 10%                     | 1735       | 8%            | 2997       | 11%*                   | 574        | 7%*           | 215        |
|                                 | R6-2      | 8%                             | 9%                      | 1949       | 4%            | 2541       | *Combined with R6-1    |            |               |            |
|                                 | R6-3      | 2%                             | 5%                      | 909        | 2%            | 2064       | 4%                     | 158        | 3%            | 154        |
| Patient 6<br>(FFPE)             |           | Q209P (CCG)                    | Q209P (CCG)             | G34R (AGA) |               | ND         |                        |            |               |            |
|                                 | R6        | 6%                             | 6%                      | 4202       | 6%            | 10837      |                        |            |               |            |
|                                 | APA       | 18%                            | 21%                     | 5004       | 19%           | 9169       |                        |            |               |            |

\*Whole exome sequencing combined 2 samples from the same region to increase starting template.

b.

| Patient ID<br>Mutation                      | Sample<br>type         | Number of<br>AAG<br>Regions<br>Analyzed | % of DNA positive for mutation as detected by ddPCR |                |                |                |                |
|---------------------------------------------|------------------------|-----------------------------------------|-----------------------------------------------------|----------------|----------------|----------------|----------------|
|                                             |                        |                                         | <i>GNA11</i>                                        |                |                | <i>GNAQ</i>    |                |
|                                             |                        |                                         | Q209P<br>(CCG)                                      | Q209H<br>(CAC) | Q209H<br>(CAT) | Q209H<br>(CAC) | Q209H<br>(CAT) |
| <b>Patient 1</b><br><i>GNA11</i><br>(Q209P) | FFPE                   | <b>AAG</b><br>(N1-N9)                   | 0-2%                                                | 0% (N1)<br>ND  | 0 % (N1)<br>ND | 0% (N1)<br>ND  | 0% (N1)<br>ND  |
|                                             |                        | <b>APA</b><br>(T1)                      | 42%                                                 | ND             | ND             | ND             | ND             |
| <b>Patient 2</b><br><i>GNA11</i><br>(Q209P) | Fresh<br>Frozen<br>LCM | <b>AAG</b><br>(N1)                      | 0%                                                  | ND             | ND             | ND             | ND             |
|                                             |                        | <b>ZF</b><br>(ZF1-ZF2)                  | 0%                                                  | ND             | ND             | ND             | ND             |
|                                             |                        | <b>ZG</b><br>(ZG1-ZG4)                  | 0%                                                  | ND             | ND             | ND             | ND             |
|                                             |                        | <b>Medulla</b><br>(M1)                  | 0%                                                  | ND             | ND             | ND             | ND             |
|                                             | FFPE                   | <b>AAG</b><br>(N1-N6)                   | 0%                                                  | ND             | ND             | ND             | ND             |
|                                             |                        | <b>APA</b><br>(T1-T3)                   | 19-21%                                              | ND             | ND             | ND             | ND             |
| <b>Patient 6</b><br><i>GNA11</i><br>(Q209P) | FFPE                   | <b>AAG</b><br>(N1-N6)                   | 0-6%                                                | ND             | ND             | ND             | ND             |
|                                             |                        | <b>APA</b><br>(T1)                      | 18%                                                 | ND             | ND             | ND             | ND             |
| <b>Patient 7</b><br><i>GNA11</i><br>(Q209H) | Fresh                  | <b>R1-R6</b>                            | ND                                                  | 0-21%          | ND             | ND             | ND             |
|                                             | Frozen                 | <b>AAG</b><br>(N1)                      | ND                                                  | 13%            | ND             | ND             | ND             |
|                                             | FFPE                   | <b>APA</b><br>(T1-T4)                   | ND                                                  | 27-37%         | ND             | ND             | ND             |
| <b>Patient 9</b><br><i>GNA11</i><br>(Q209H) | Fresh<br>Frozen        | <b>AAG</b><br>(N1)                      | ND                                                  | ND             | ND             | 0              | ND             |
|                                             |                        | <b>APA</b><br>(T1-4)                    | ND                                                  | ND             | ND             | 19,17          | ND             |

c.

| Patient ID<br>Mutation                            | Genotype      | Regions | % of DNA positive for mutation as detected by NGS |            |               |            |
|---------------------------------------------------|---------------|---------|---------------------------------------------------|------------|---------------|------------|
|                                                   |               |         | <i>GNA11</i>                                      |            | <i>CTNNB1</i> |            |
|                                                   |               |         | %                                                 | Total Read | %             | Total Read |
| <b>Patient 3</b><br><i>GNA11</i><br><i>CTNNB1</i> | Q209H<br>T41A | AAG 1   | 1                                                 | 1890       | 0             | 5942       |
|                                                   |               | AAG 2   | 1                                                 | 2007       | 2             | 4712       |
|                                                   |               | APA     | 36                                                | 2275       | 34            | 9140       |
| <b>Patient 8</b><br><i>GNA11</i><br><i>CTNNB1</i> | Q209P<br>S45F | AAG 1   | 0                                                 | 4958       | 0             | 11148      |
|                                                   |               | AAG 2   | 0                                                 | 4520       | 0             | 13821      |
|                                                   |               | APA     | 38                                                | 4645       | 33            | 10590      |

d.

| Tumour ID<br>(Tumour<br>Genotype)        | Sample<br>type | Number of<br>AAG<br>Regions<br>Analyzed | % of DNA positive for mutation |                |                |                |                |               |
|------------------------------------------|----------------|-----------------------------------------|--------------------------------|----------------|----------------|----------------|----------------|---------------|
|                                          |                |                                         | ddPCR, NGS, and WES*           |                |                |                |                | NGS & WES*    |
|                                          |                |                                         | <i>GNA11</i>                   |                |                | <i>GNAQ</i>    |                | <i>CTNNB1</i> |
|                                          |                |                                         | Q209P<br>(CCG)                 | Q209H<br>(CAC) | Q209H<br>(CAT) | Q209H<br>(CAC) | Q209H<br>(CAT) | Exon 3        |
| <b>313T</b><br><i>ATP1A1</i><br>(L104R)  | FFPE           | 4                                       | 0%                             | 0%             | 0%             | 0%             | 0%             | 0%            |
| <b>335T</b><br><i>CACNA1D</i><br>(R990G) | Fresh          | 6                                       | 0%                             | 0%             | 0%             | 0%             | 0%             | 0%            |
|                                          | Frozen         |                                         |                                |                |                |                |                |               |
|                                          | FFPE           | 4                                       | 0%                             | 0%             | 0%             | 0%             | 0%             | 0%            |
| <b>342T</b><br><i>KCNJ5</i><br>(G151R)   | Fresh          | 6                                       | 0%                             | 0%             | 0%             | 0%             | 0%             | 0%            |
|                                          | Frozen         |                                         |                                |                |                |                |                |               |
| <b>351T</b><br><i>KCNJ5</i><br>(L168R)   | Fresh          | 6                                       | 0%                             | 0%             | 0%             | 0%             | 0%             | 0%            |
|                                          | Frozen         |                                         |                                |                |                |                |                |               |

\*WES was only performed on Fresh Frozen samples.

**Supplementary Table 5**

**Primers used for CTNNB1, GNA11, GNAQ, GNAS and KCNJ5 sequencing in gDNA samples**

| Gene symbol   | Exon    | Forward primer 5' → 3'  | Reverse primer 5' → 3'  |
|---------------|---------|-------------------------|-------------------------|
| <i>CTNNB1</i> | 3       | CATTCTGCTTTTCTTGGCTGTC  | GCTATTACTCTCTTTTCTTCACC |
|               |         | GGAACCAGACAGAAAAGCGG    | CCACTCATAACAGGACTTGGGA  |
| <i>GNA11</i>  | 4       | GTGCTGTGTCCCTGTCCTG     | GGGCAAATGAGCCTCTCAG     |
| <i>GNA11</i>  | 5       | TCCAGCCGATGTCAGTCTG     | CGAAGGCAGAGGGAATCAGA or |
|               |         |                         | CTTGGCAGGTGGGGAAGG      |
|               |         | TGTCAGTCTGGTGTGGCAG     | TGAGGGCGACGAGAAACAT     |
|               |         | CCAGTGAGGGAAGGGTCTG     | ACCAGGGGTAGGTGATGATG    |
| <i>GNAQ</i>   | 4       | CCCTTCCGTAGACAGCTT      | CTTGTTTGAAGCCTACACATGA  |
| <i>GNAQ</i>   | 5       | TCATGATGTGTTACCCAGAATGT | TCCATTGCCTGTCTAAAGAACAC |
| <i>GNAS</i>   | 8 and 9 | GACTCTGAGCCCTCTTCCA     | GCGACCCTGATCCCTAACAA    |
| <i>KCNJ5</i>  | 2       | GCTTCATTTGGTGGCTCATT    | GAGATGACTGCGTTGTTGGA    |

**Supplementary Table 6**

**Primers used for CTNNB1, GNA11, GNAQ, GNAS and KCNJ5 sequencing in cDNA samples**

| Gene symbol   | Exon    | Forward primer 5' → 3' | Reverse primer 5' → 3'                          |
|---------------|---------|------------------------|-------------------------------------------------|
| <i>CTNNB1</i> | 3       | TGATGGAGTTGGACATGGCC   | CGAGTCATTGCATACTGTCCA                           |
|               |         | GCTGATTTGATGGAGTTGGACA | GTACCCTCTGAGCTCGAGTC                            |
| <i>GNA11</i>  | 4 and 5 | TACCTGACCGACGTTGACC    | TCACGTTCTCAAAGCAGTGG or<br>TTGAGGTTGAGCTGCAGGAT |
| <i>GNAQ</i>   | 4 and 5 | GTAGCTGACCCTGCCTACC    | AGAATAACCGAGGAGTTCTGGA                          |
| <i>GNAS</i>   | 8 and 9 | CAAGCAGGCTGACTATGTGC   | GCGGTTGGTCTGGTTGTC                              |
| <i>KCNJ5</i>  | 2       | GCTTCATTTGGTGGCTCATT   | GAGATGACTGCGTTGTTGGA                            |

**Supplementary Table 7**

**Primers used for CTNNB1 and GNA11 plasmids mutagenesis**

| Gene symbol  | Plasmid genotype | Forward primer 5' → 3' | Reverse primer 5' → 3'         |
|--------------|------------------|------------------------|--------------------------------|
| <i>GNA11</i> | Q209H            | TGGGGGGCCACCGGTCGGAGC  | CATCCACCATCCGGAAGATGATGTTCTCC  |
| <i>GNA11</i> | Q209P            | GTGGGGGGGCCCGCGGTCGGAG | ATCCACCATCCGGAAGATGATGTTCTCCAG |

# Supplementary Table 8

## Taqman Gene Expression Assays used for RT-qPCR analysis

| Gene            | Assay ID      | Amplicon Length | Properties                        |
|-----------------|---------------|-----------------|-----------------------------------|
| <i>18S</i>      | Hs99999901_s1 | 187             | Primer and probe within same exon |
| <i>C9orf84</i>  | Hs00330277_m1 | 124             | Probe spans exons                 |
| <i>CTNNB1</i>   | Hs00355045_m1 | 86              | Probe spans exons                 |
| <i>CYP11B1</i>  | Hs01596404_m1 | 137             | Probe spans exons                 |
| <i>CYP11B2</i>  | Hs01597732_m1 | 137             | Probe spans exons                 |
| <i>DKK1</i>     | Hs00183740_m1 | 68              | Probe spans exons                 |
| <i>FAP</i>      | Hs00990791_m1 | 64              | Probe spans exons                 |
| <i>GNRHR</i>    | Hs00171248_m1 | 109             | Probe spans exons                 |
| <i>GNA11</i>    | Hs01588833_m1 | 140             | Probe spans exons                 |
| <i>LHCGR</i>    | Hs00896336_m1 | 105             | Probe spans exons                 |
| <i>MPP3</i>     | Hs01115505_m1 | 74              | Probe spans exons                 |
| <i>TMEM132E</i> | Hs01368963_m1 | 68              | Probe spans exons                 |



# Supplementary Table 9

## Primers used for RT-qPCR of French Cohort

| Gene symbol    | Forward primer 5' → 3'    | Reverse primer 5' → 3' |
|----------------|---------------------------|------------------------|
| <i>18S</i>     | CCCTGCCTTTGTACACACC       | CGATCCGAGGGCCTCACTA    |
| <i>HPRT</i>    | CTCAACTTTAACTGGAAAGAATGTC | TCCTTTTCACCAGCAAGCT    |
| <i>GAPDH</i>   | TGCACCACCAACTGCTTAGC      | GGCATGGACTGTGGTCATGAG  |
| <i>CYP11B2</i> | TCCAGGTGTGTTTCAGTAGTTCC   | GAAGCCATCTCTGAGGTCTGT  |
| <i>CYP11B1</i> | ATGAAGTGCCCCAGGTTGG       | AGCAACCCAGTCTATGGCGT   |
| <i>LHCGR</i>   | TCTCTCAGATTGATTCCCTGGA    | CGGGCTCAATGTATCTCAGAT  |