Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Teo AED, Garg S, Shaikh LH, et al. Pregnancy, primary aldosteronism, and adrenal *CTNNB1* mutations. N Engl J Med 2015;373:1429-36. DOI: 10.1056/NEJMoa1504869

Supplementary Appendix

Unmasking of primary aldosteronism by pregnancy in women with somatic *CTNNB1* mutations of the adrenal

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METHODS

Exome sequencing

Exome sequencing was performed by BGI Shenzhen as in *Azizan et al.* [1]. Ten ZG–like APAs (≥50% compact ZG–like cells with low expression of *CYP17A1*) and three ZF–like APAs with somatic *KCNJ5* mutations (as sensitivity controls) were sequenced. Patient 1 was Sample ID #10, the only adenoma that did not harbour mutations in *ATP1A1* or *CACNA1D* [1].

Sanger sequencing

The mutation in Patient 1's APA was validated by Sanger sequencing of exon 3 of the *CTNNB1* gene in gDNA, using specific primers (F: 5'-CATTCTGCTTTTCTTGGCTGTC-3', R: 5'-GCTATTACTCTCTTTTCTTCACC-3'). These were also used to detect the *CTNNB1* mutations in Patients 2 and 3. For these three patients, *CTNNB1* ORF from cDNA was also sequenced using two sets of specific primers (Fragment 1F: 5'-CCATACAACTGTTTTGAAAATCC-3' and 1R: 5'-GTACGCACAAGAGCCTCTATAC-3'; Fragment 2F: 5'-CTATTGTAGAAGCTGGTGGAATG-3' and 2R: 5'-CAGCTAAAGGATGATTTACAGGTC-3'). For full coverage of the ORF, both fragments were also sequenced using an additional reverse primer (For Fragment 1: 5'-CACTGCCATTTTAGCTCCTTCTTG-3'; For Fragment 2: 5'- TTCCTTGTCCTGAGCAAGTTCACA-3')

On sequencing exon 3 of *CTNNB1* gDNA, we did not detect mutations in nine control APAs (four ZG-like, five ZF-like) from patients who were not pregnant at the time of presentation. Table S1 shows the patient details and histological characteristics for all nine control APAs.

Table S1. Patient and histological characteristics for the nine control APAs with no *CTNNB1* mutation detected [2,3].
It has been previously established that ZG-like APAs appeared to contain a majority of compact cells with a low cytoplasm-to-nucleus ratio, whereas ZF-like APAs had a majority of clear lipid-laden cells [2].

Sample ID	Sex	Age at adrenalectomy	APA diameter (mm)	% of compact (ZG-like) cells
ADR032	M	49	18	80
ADR093	M	50	35	60
ADR113	F	35	14	60
ADR115	M	41	16	80
ADR049	F	66	5	0
ADR054	F	33	13	0
ADR059	F	37	10	0
ADR074	M	53	5	10
ADR096	M	48	10	10

Construction of Wild-Type and Mutant Vectors

Wild-type *CTNNB1* was PCR-amplified from HEK293T cells and cloned into eukaryotic expression vector pEGFP-C1 with an N-terminus GFP tag. Mutants were generated by site-directed mutagenesis (Q5 Site-Directed Mutagenesis Kit, New England Biolabs, Austria) and mutations were confirmed by Sanger sequencing.

TCF/LEF Luciferase Reporter Assay

Canonical Wnt signaling was measured using the Cignal TCF/LEF Reporter (luc) Kit (CCS-018L; SABiosciences, USA) which consists of the firefly luciferase reporter gene along with a constitutively expressing *Renilla* luciferase construct (40:1). HEK293T cells were plated at a density of 5×10^4 cells per well in a 96-well plate. Following 24h incubation at 37°C in 5% CO_2, using Polyethylenimine (PEI), cells were co-transfected with Cignal reporter and either mutant CTNNB1 plasmids (S33C/S45F/G34R), wild-type CTNNB1 or empty vector. Wnt signaling activity was determined by cell extracts, quantifying both firefly and *Renilla* luciferase reporters using Dual-Glo Luciferase Assay (E2940; Promega, USA) 48h after transfection according to manufacturer's instructions. All results are represented as Luciferase/*Renilla* ratios.

Western Blot

Western blotting was performed using total protein extracts from HEK293T cells transfected with wild-type, mutant or empty vectors. Proteins were separated by electrophoresis on 4-12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, immunoblotted with anti-β-catenin (610154, 1:2000 dilution; BD Transduction Laboratories, USA) or anti-Non-phospho (active) β-catenin (8814, 1:5000 dilution; Cell Signaling, USA) [4,5]. Fluorescent secondary antibodies were used and bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). Anti-GAPDH (G8795, 1:1500 dilution; Sigma-Aldrich, UK) was used as an internal control for protein expression.

Microarray Assay

Microarray assay was performed on 42 RNA samples (14 trios of ZF, ZG and APA from patients with Conn's syndrome) by Genomics Corelab, Cambridge [3]. Histologically, 7 of the APAs were ZF-like with *KCNJ5* mutations while the remaining 7 (including Patient 1) were ZG-like and wild-type for *KCNJ5*. The Affymetrix Human Genome U133 Plus 2.0 Array was used, measuring 22,148 genes through 33,297 probes. Quality control, data processing and analysis were performed using Affymetrix GeneChip Command Console Software and Partek GenomicSuite 6.5 (Partek Inc., USA). Gene expressions were represented as the summarized log-signal of the Robust Multichip Average (RMA) with quantile normalisation and median polish for probe set summarisation. Genes were considered differentially expressed where the fold change (FC) >2 and false discovery rate p-value < 0.05.

qPCR Analysis of Gene Expression

Adrenal tissue stored in RNALater was extracted with TRIzol (both from Ambion, USA). Total DNA-free RNA was then isolated using the PureLink® RNA Mini Kit with the PureLink® DNase Set (Life Technologies, USA) according to manufacturer's instructions. Reverse transcription was performed using the Reverse Transcriptase System (Promega, USA) with a 1:1 mixture of random hexamer and oligo-dT primers according to manufacturer's instructions. mRNA expression of genes of interest was quantified using commercially available TaqMan gene expression probes (Life Technologies, USA) for *LHCGR* (Hs00896336), *GnRHR* (Hs00171248) and *GATA4* (Hs00171403). Results were analysed using the $2^{-\Delta\Delta CT}$ method and the housekeeping 18S rRNA (Life Technologies, USA) was used for normalisation.

Tissue Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded adrenal sections (4 μm) using a chromogen based detection system, 3,3'-diaminobenzidine (DAB). Commercial antibodies to β-catenin (610154, 1:150 dilution; BD Transduction Laboratories, USA) and LHCGR (L6792, 1:100 dilution; Sigma, UK) were used on slides of adrenals from the three patients. Slides were counter-stained with Mayer's Haematoxylin solution (MHS1; Sigma, UK). Negative controls, in which primary antibodies were omitted, resulted in absence of staining.

Human testis sections were used as positive control for LHCGR. Images were captured using a standard bright-field microscope, U-TV1-X digital camera and the CellD software (Olympus Ltd, UK).

Immunohistochemistry for *CYP11B2* and *CYP11B1* were performed as previously described [1].

Cell Immunofluorescent Staining

Immunofluorescent staining was performed on transfected ZG-like APA cells to co-localise LHCGR protein with cells transfected with mutant-*CTNNB1*.

ZG-like APA cells were from human adrenal tissues of patients who underwent adrenalectomy after being diagnosed with Conn's syndrome, and were obtained from Cambridge University Hospitals' Human Research Tissue Bank post-surgery at Addenbrooke's Hospital, Cambridge, United Kingdom. All tissues were obtained with approval from the Cambridgeshire 2 Research Ethics Committee and written informed consent from each patient prior to surgery. APAs were identified and macroscopically dissected by histopathologists from the Tissue Bank. After a two-hour digestion with collagenase, cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) /Nutrient F-12 Ham supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 0.1 mg/ml streptomycin, 0.4 mM L-glutamine and ITS (insulin–transferrin–sodium selenite media) at 37 °C in 5% CO^2.

These primary APA cells were plated on coverslips at a density of 2.5×10^4 cells per well in a 12-well plate. Following 24h incubation at 37°C in 5% CO_2, cells were transfected with either mutant CTNNB1 (S33C), wild-type *CTNNB1* or empty vector, using the Lipofectamine 3000

system (Invitrogen, USA) according to the manufacturer's instructions. 72h post-transfection, cells were fixed with 4% paraformaldehyde and incubated with blocking solution (3% BSA in PBS) followed by overnight incubation with an antibody to LHCGR (TA328868, 1:25 dilution; OriGene, USA) diluted in blocking solution at 4°C. They were then incubated with secondary antibody conjugated to Alexa 568 (A10042, 1:300 dilution; Life Technologies, USA) in blocking solution. After washing with PBS, coverslips were mounted on slides using Vectashield containing DAPI (Vector Labs, UK) and visualised with a Zeiss LSM510 Meta confocal microscope with the Zen 2011 software.

As a positive control, LHCGR immunofluorescent staining was carried out on a human ovarian carcinoma cell-line, OVCAR-3.

Statistical Analysis

All data are presented as mean ± S.E.M unless stated otherwise, and compared using the two-tailed Student t-test. Statistical analysis was performed using standard statistical software. P-values lower than 0.05 were considered statistically significant.

RESULTS

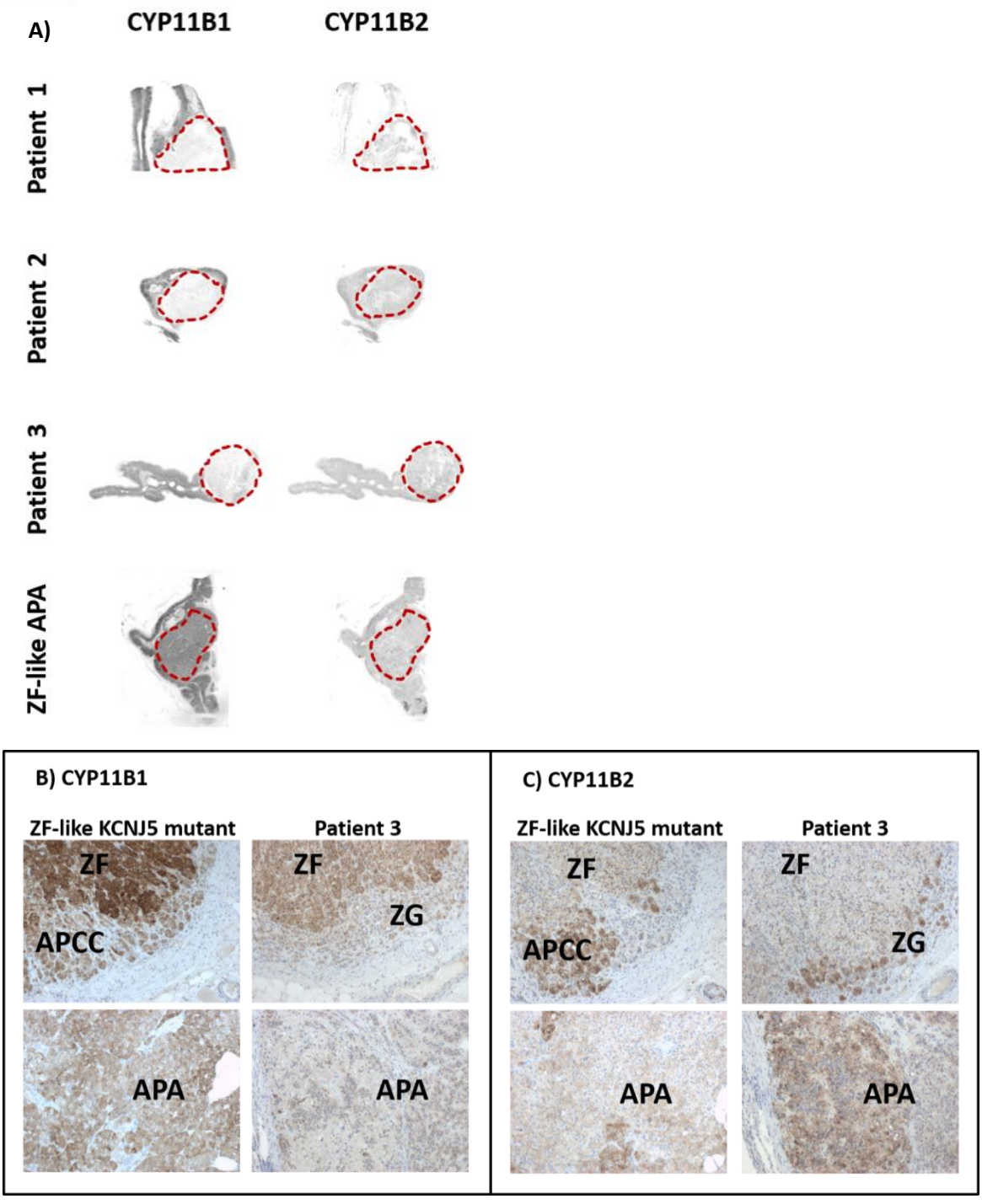


Figure S1. A) Immunohistochemistry of the enzyme responsible for cortisol synthesis (CYP11B1) and the aldosterone synthase enzyme (CYP11B2) on the three *CTNNB1*-mutated APAs with corresponding adjacent adrenal tissue, compared to a ZF-like APA. Immunohistochemistry of CYP11B1 (B) and CYP11B2 (C) in the normal adrenal and APA of Patient 3, compared to a ZF-like KCNJ5 mutant.

Dashed lines encompass the APA.

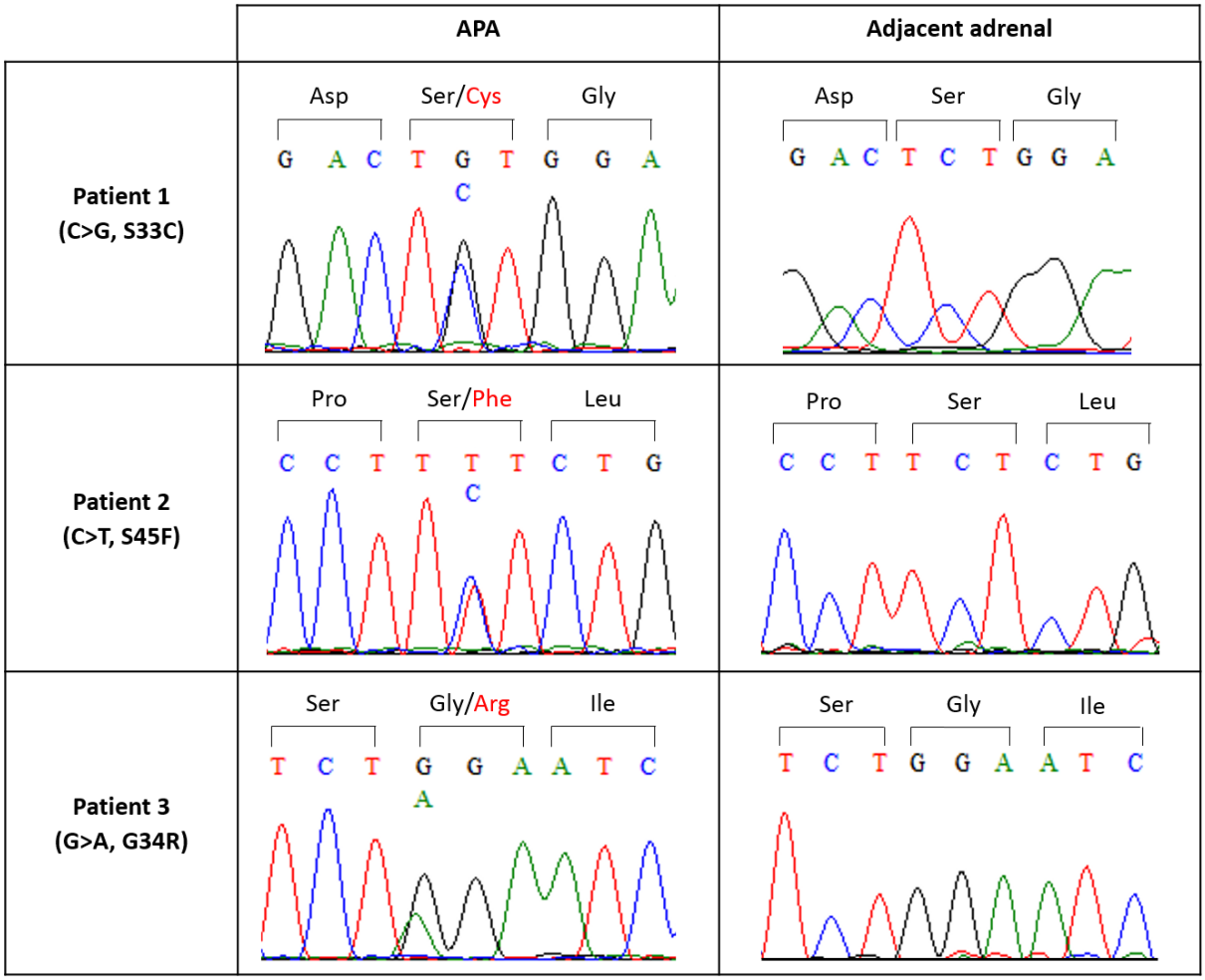
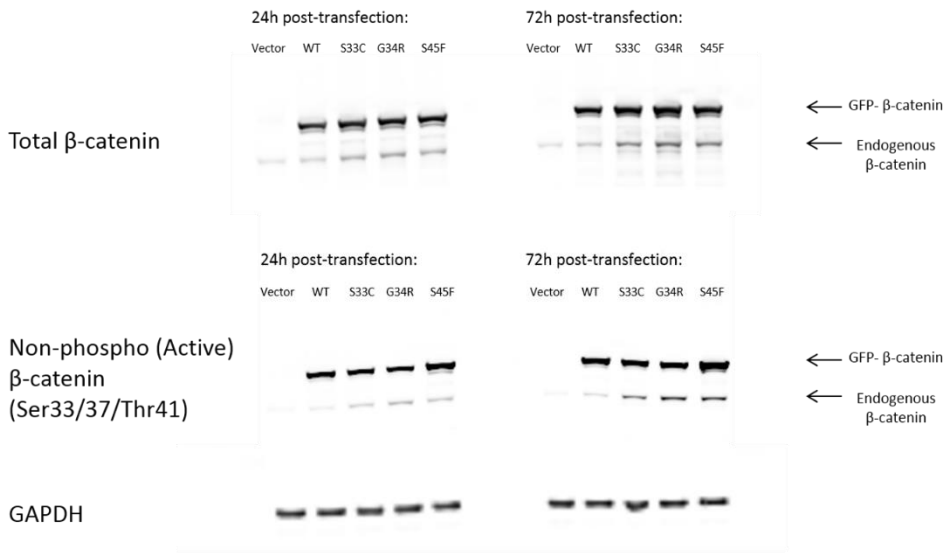


Figure S2. Representative traces of mutations found in *CTNNB1* (S33C, S45F, G34R) by Sanger sequencing of APAs and the adjacent adrenal.

These mutations were absent from adrenal adjacent to each APA, confirming their somatic nature.

Sequencing was carried out on both gDNA and cDNA for Patients 1 and 2, and on cDNA only for Patient 3 due to lack of good-quality gDNA (cDNA sequences shown here). 9 other APAs were also sequenced as controls, and all found to be wild-type.

A



B **Active endogenous β-catenin as a percentage (%) of total endogenous β-catenin**

24h post-transfection

Empty vector	WT	S33C	G34R	S45F
6.6	10.8	15.1	20.9	19.7

72h post-transfection

Empty vector	WT	S33C	G34R	S45F
7.4	17.0	39.3	34.6	46.9

Figure S3. (A) Immunoblots of total and active β-catenin in HEK-293T cells after 24h and 72h transfection with vector, wild-type and mutant CTNNB1 constructs. (B) Table showing proportion of total endogenous β-catenin that is active (ie non-phosphorylated at residues Ser33/37/Thr41), quantified by band intensity.

Consistent with previous literature [6,7], all three mutations impair β-catenin degradation and upregulate canonical Wnt activity as a result of elevated levels of active β-catenin.

Activation of the Wnt pathway exerts a positive feedback effect on *CTNNB1* transcription [8], and this is the likely explanation why transfection of mutant *CTNNB1* increased the endogenous wild-type protein levels.

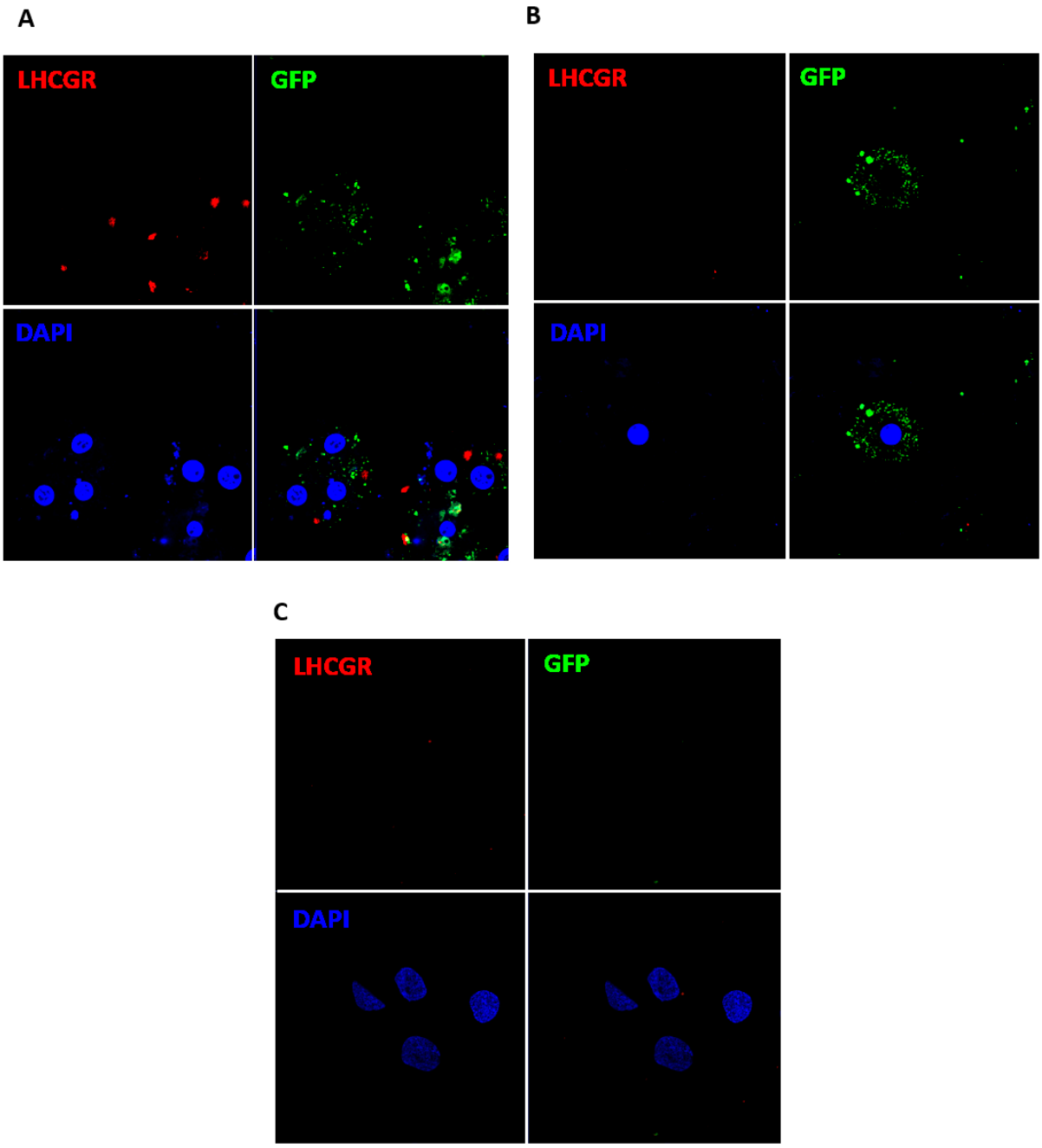


Figure S4. Localisation of LHCGR protein by immunofluorescent staining in ZG-like APA cells (A) transfected with wild type-*CTNNB1*; (B) transfected with GFP-tagged empty vector; (C) untransfected. Red, LHCGR; green, GFP; and blue, DAPI staining of nuclear DNA.

Images taken with the same microscope settings as Figure 3D.

Transfection with wild type-*CTNNB1* led to patchy areas of LHCGR expression (A), as it also results in the increased abundance of *CTNNB1* protein.

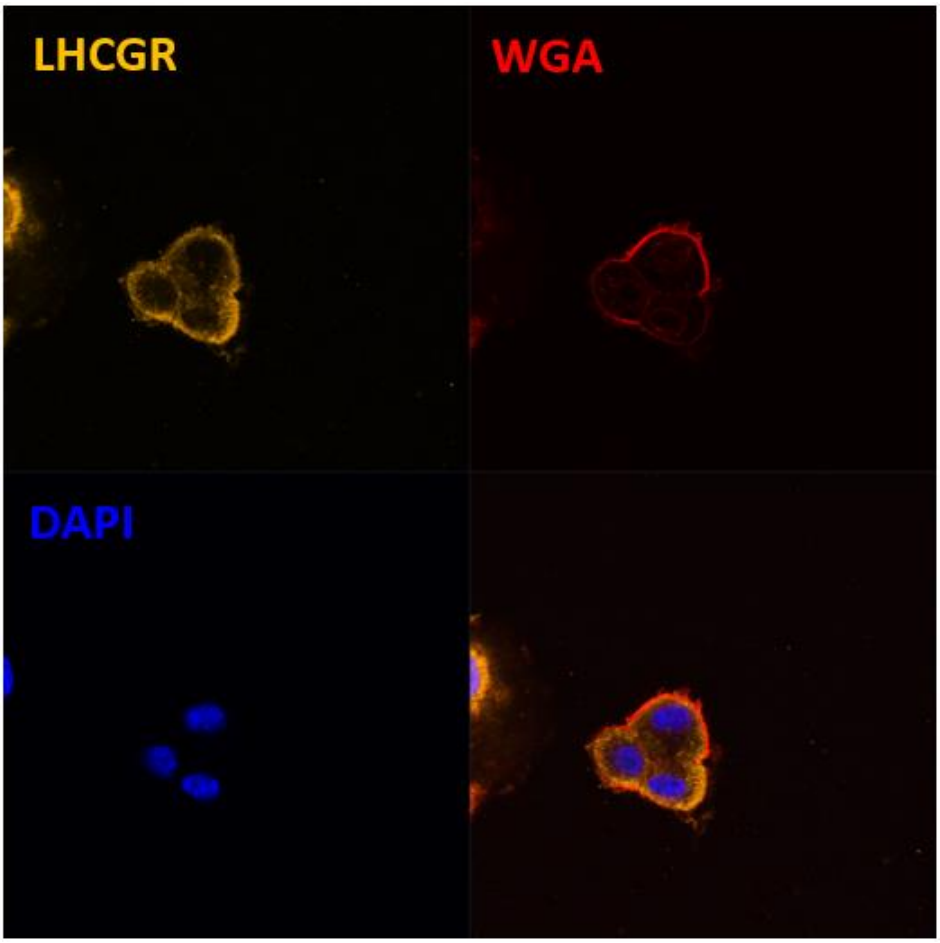


Figure S5. Positive control LHCGR staining in human ovarian carcinoma cell-line OVCAR-3. Orange, LHCGR; red, wheat germ agglutinin (WGA) staining of membrane; and blue, DAPI staining of nuclear DNA.

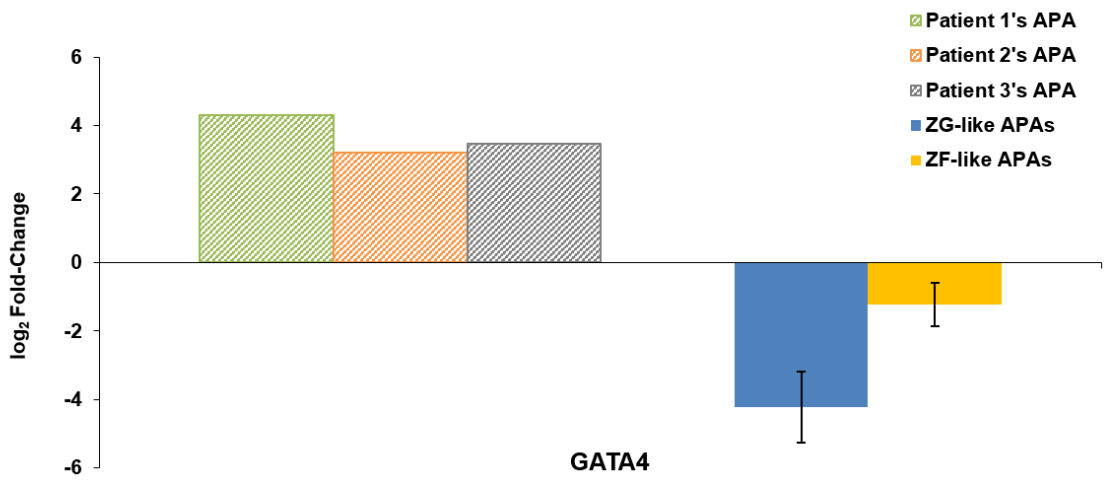


Figure S6. *GATA4* mRNA levels in all three mutant APAs vs their adjacent adrenals, compared to 6 ZG-like APAs and 5 ZF-like APAs, as measured by qPCR.

In a previous microarray [2], the expression of gonadal differentiation marker *GATA4* in patient 3 was 3.6-fold higher in the APA than the adjacent adrenal. On qPCR, *GATA4* was similarly upregulated in all three *CTNNB1*-mutant APAs than in their adjacent adrenals. As a marker of gonadal differentiation, the transcription factor *GATA4* should be absent in adrenal cells [9], and its presence in these tumours suggest a de-differentiation process.

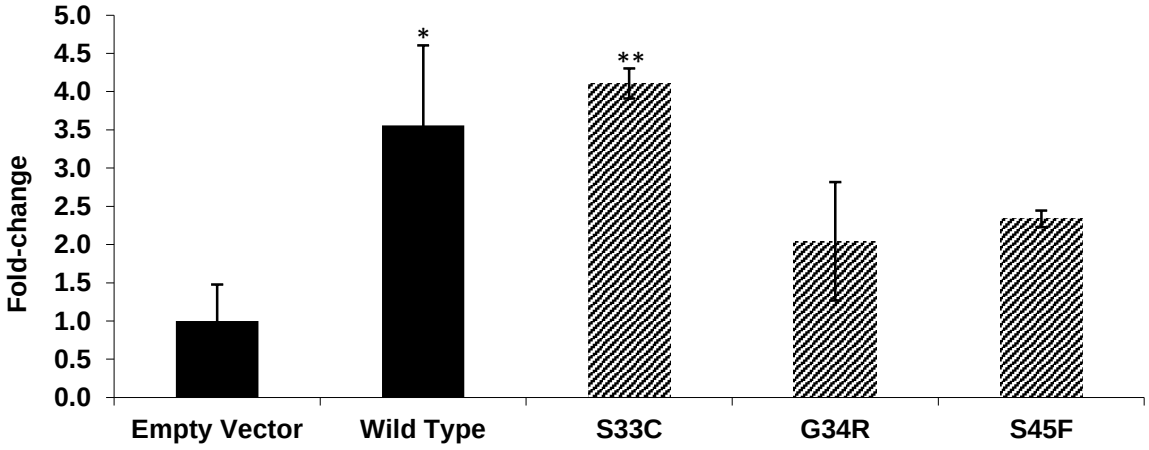


Figure S7. *GATA4* mRNA levels in primary ZG-like APA cells transfected with empty vector, wild-type or mutant-CTNNB1 constructs, as measured by qPCR.

Transfection of primary ZG-like APA cells with either wild-type or mutant-*CTNNB1* also led to between 2- and 4-fold increases in *GATA4* expression compared to cells transfected with the empty vector.

Results are from 72h post-transfection (n=2-3, * represents $p \leq 0.05$, ** represents $p \leq 0.01$)

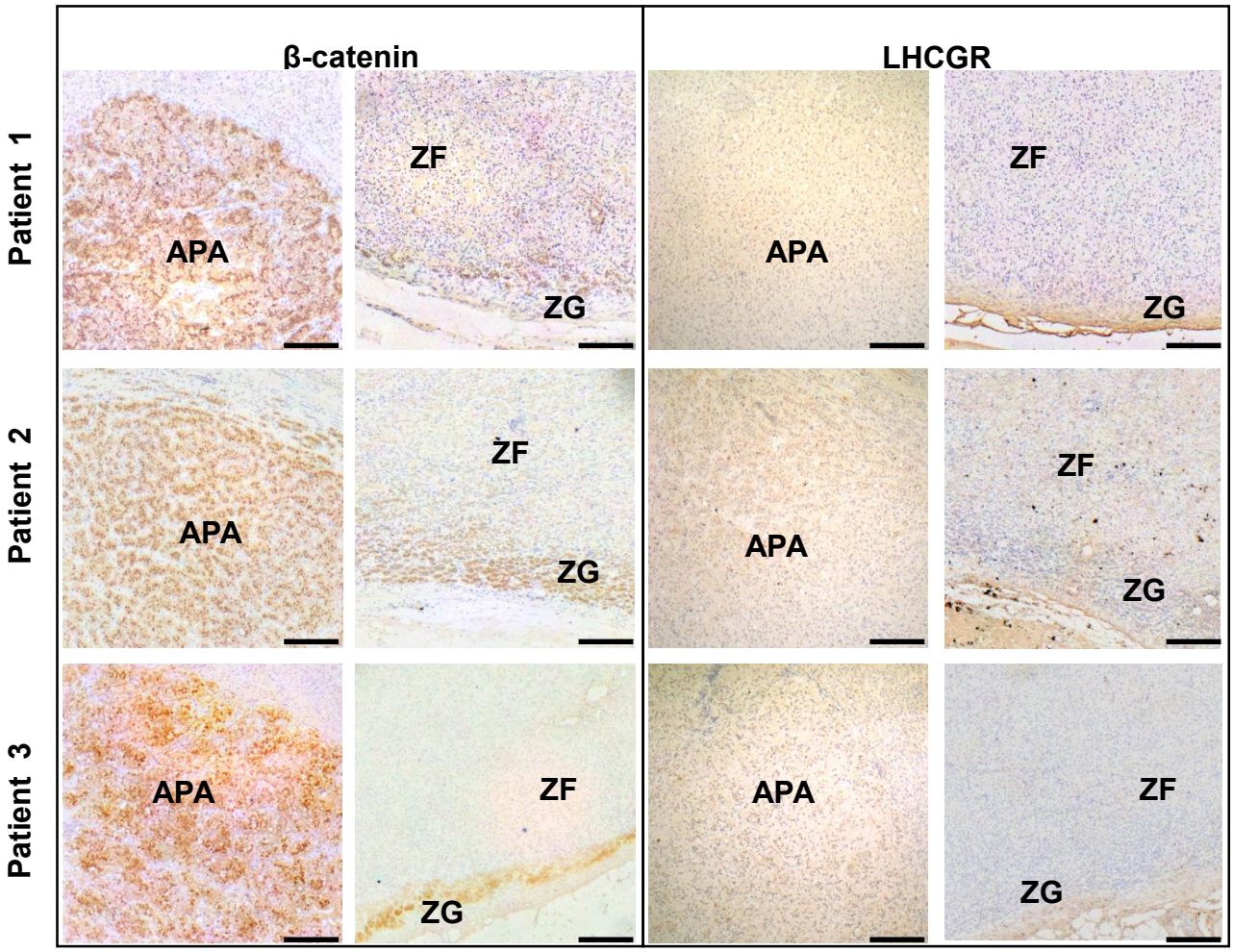


Figure S8. Low magnification immunohistochemical staining for β-catenin and LHCGR on APA of all three patients compared to their adjacent adrenal.

4x magnification compared to 20x in Figure 3C. Scale bar, 250µm. APA and adjacent adrenal mounted on the same slide.

In line with a previous study by Boulkroun *et al.* [10], β-catenin staining was stronger in the APAs than in the adjacent adrenal. The localisation of β-catenin staining has been shown to vary between normal adrenal and tumour, and the staining of these three tumours are within the previously-reported spectrum. However, unlike the three representative tumours in the study by Boulkroun *et al.*, all three of our tumours show nuclear staining of β-catenin.

Some APAs have previously been shown to stain positively for LHCGR and GnRHR [11]. For normal adrenal, no human adrenal has shown positive staining for LHCGR and GnRHR, but staining for GnRH has been shown locally in mouse adrenals [12].

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