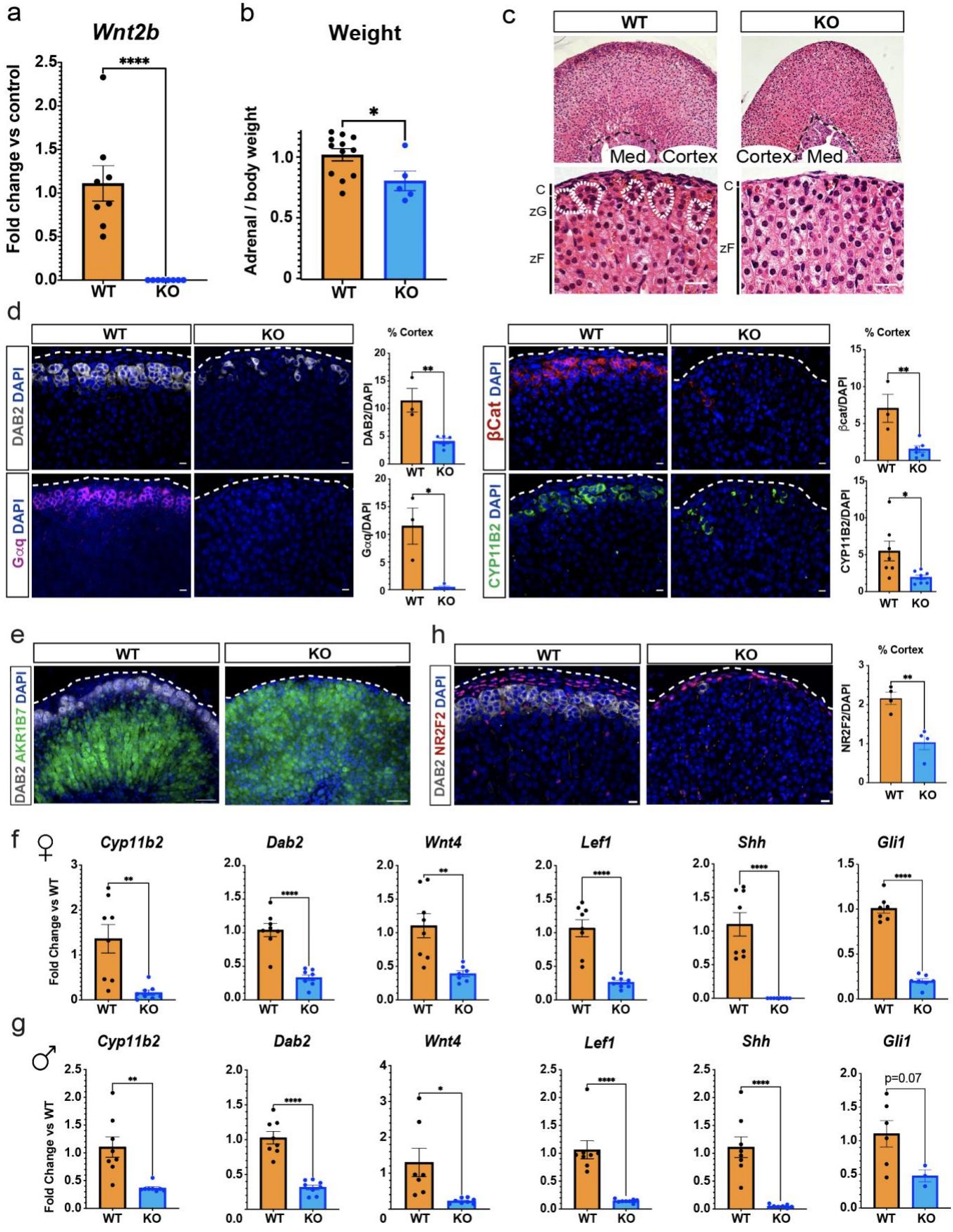
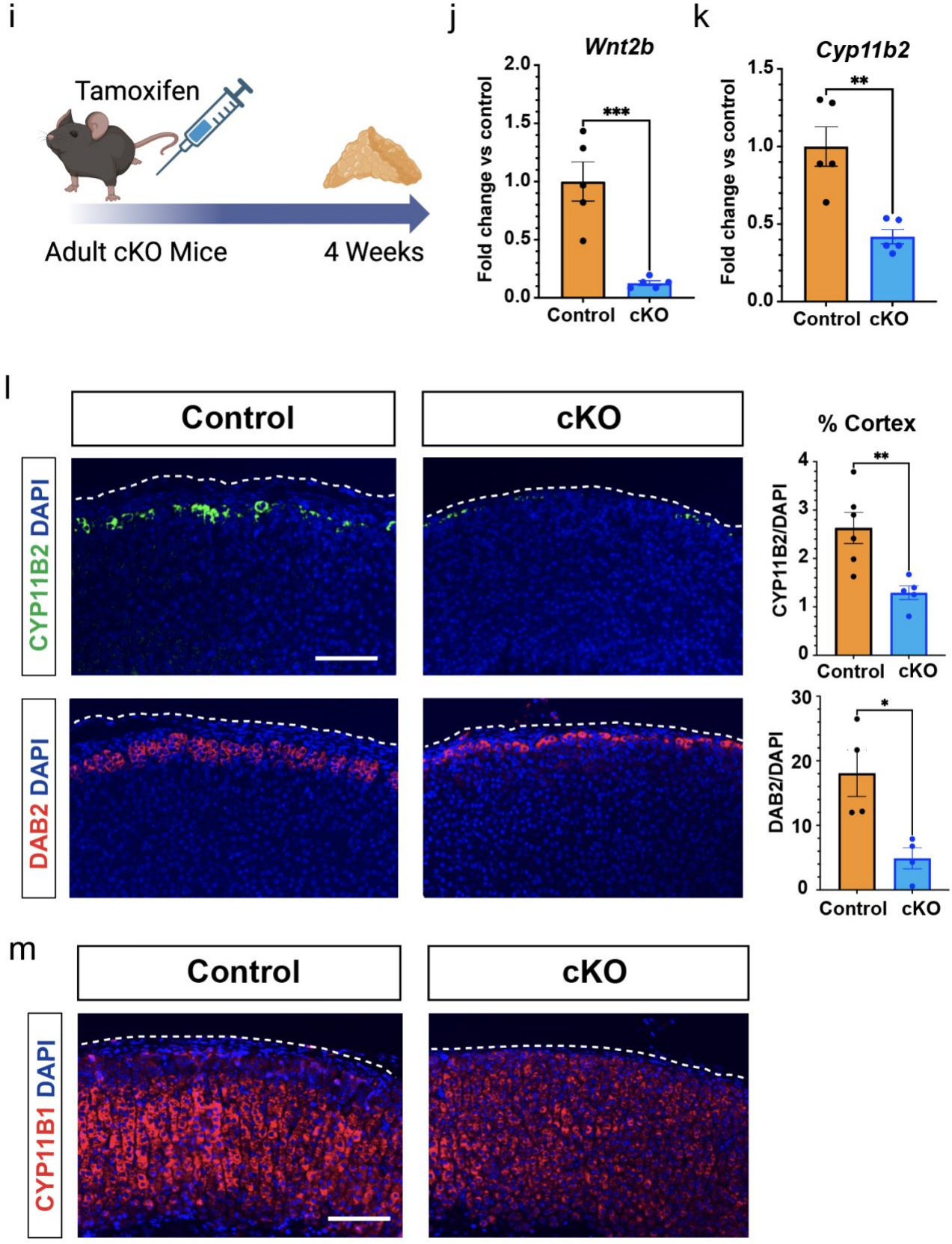
Supplemental Fig. 1



Supplemental Fig. 1



Supplemental Figure 1. WNT2B deficiency results in a dysmorphic zG in mice.

a. QRT-PCR was performed on WT and KO male adrenals (n=8 WT, n=8 KO). Two-tailed Student's t-test. ****p < 0.0001. Data are represented as mean ± SEM.

b. Adrenal weight normalized to body weight from male mice (n=12 WT, n=5 KO). Two-tailed Student's t-test. *p < 0.05. Data are represented as mean fold change ± SEM.

c. Representative H&E images of WT and KO male adrenals. Scale bar: 10μm. C, capsule; zG, zona glomerulosa; zF, zona fasciculata; Med, medulla.

d. Representative images and quantification from male adrenals stained for DAB2 (gray, n=3 WT, n=5 KO), Gαq (magenta, n=3 WT, n=4 KO), β-catenin (β-cat, red, n=3 WT, n=6 KO) and CYP11B2 (green, n=7 WT, n=7 *Wnt2b* KO). Positive cells were quantified and normalized to nuclei (DAPI) in the cortex. Scale bars: 10μm. Two-tailed Student's t-test. *p < 0.05; **p < 0.01. Data are represented as mean ± SEM.

e. Representative images stained for DAB2 (gray), AKR1B7 (green) and DAPI (blue) from WT and KO adrenals. Scale bar: 50μm

f. QRT-PCR was performed on WT and KO female adrenals for *Cyp11b2* (n=8 WT, n=7 KO), *Dab2* (n=8 WT, n=7 KO), *Wnt4* (n=8 WT, n=7 KO), *Lef1* (n=8 WT, n=8 KO), *Shh* (n=8 WT, n=8 KO) and *Gli1* (n=7 WT, n=7 KO). Two-tailed Student's t-test. **p < 0.01; ****p < 0.0001. Data are represented as mean ± SEM.

g. QRT-PCR was performed on WT and KO male adrenals for *Cyp11b2* (n=8 WT, n=8 KO), *Dab2* (n=8 WT, n=8 KO), *Wnt4* (n=7 WT, n=8 KO), *Lef1* (n=8 WT, n=8 KO), *Shh* (n=8 WT, n=8 KO) and *Gli1* (n=6 WT, n=3 KO). Two-tailed Student's t-test. *p < 0.05; **p < 0.01; ****p < 0.0001. Data are represented as mean ± SEM.

h. Representative images and quantification of immunostaining for DAB2 (gray) and NR2F2 (red) from WT and KO adrenals (n=4 WT, n=4 KO). Positive cells were quantified and normalized to nuclei (DAPI, blue) in the cortex. Scale bars: 10μm. Two-tailed Student's t-test. **p < 0.01. Data are represented as mean ± SEM.

i. Treatment protocol of adult cKO mice at 6-7 weeks of age with tamoxifen and adrenal harvest after 4 weeks.

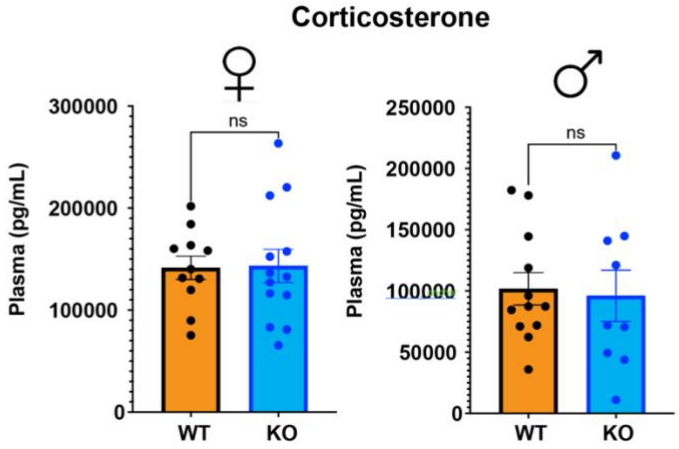
j. QRT-PCR was performed for *Wnt2b* in adrenals (n=5 Control and n=5 cKO) 4 weeks following tamoxifen injection. Two-tailed Student's t-test. ***p < 0.001. Data are represented as mean ± SEM.

k. QRT-PCR was performed for *Cyp11b2* in adrenals (n=5 Control and n=5 cKO) 4 weeks following tamoxifen injection. Two-tailed Student's t-test. **p < 0.01. Data are represented as mean ± SEM.

l. Representative images and quantification from adrenals stained for CYP11B2 (green, n=6 Control, n=5 cKO and DAB2 (red, n=4 Control, n=4 cKO) 4 weeks following tamoxifen injection. Positive cells were quantified and normalized to nuclei (DAPI) in the cortex. Scale bar: 100μm. Two-tailed Student's t-test. *p < 0.05; **p < 0.01. Data are represented as mean ± SEM.

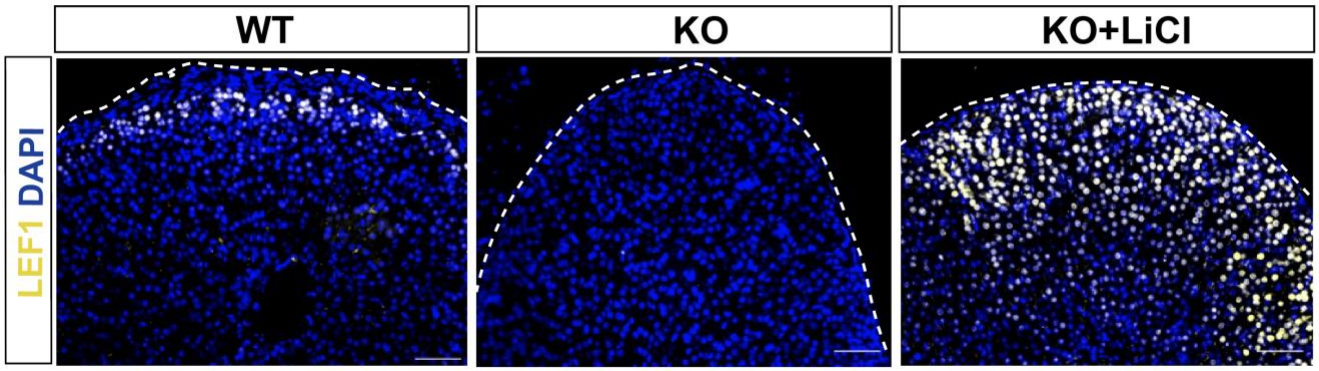
m. Representative images from Control and cKO adrenals stained for CYP11B1 (red) and DAPI (blue) 4 weeks following tamoxifen injection. Scale bar: 100μm.

Supplemental Fig. 2



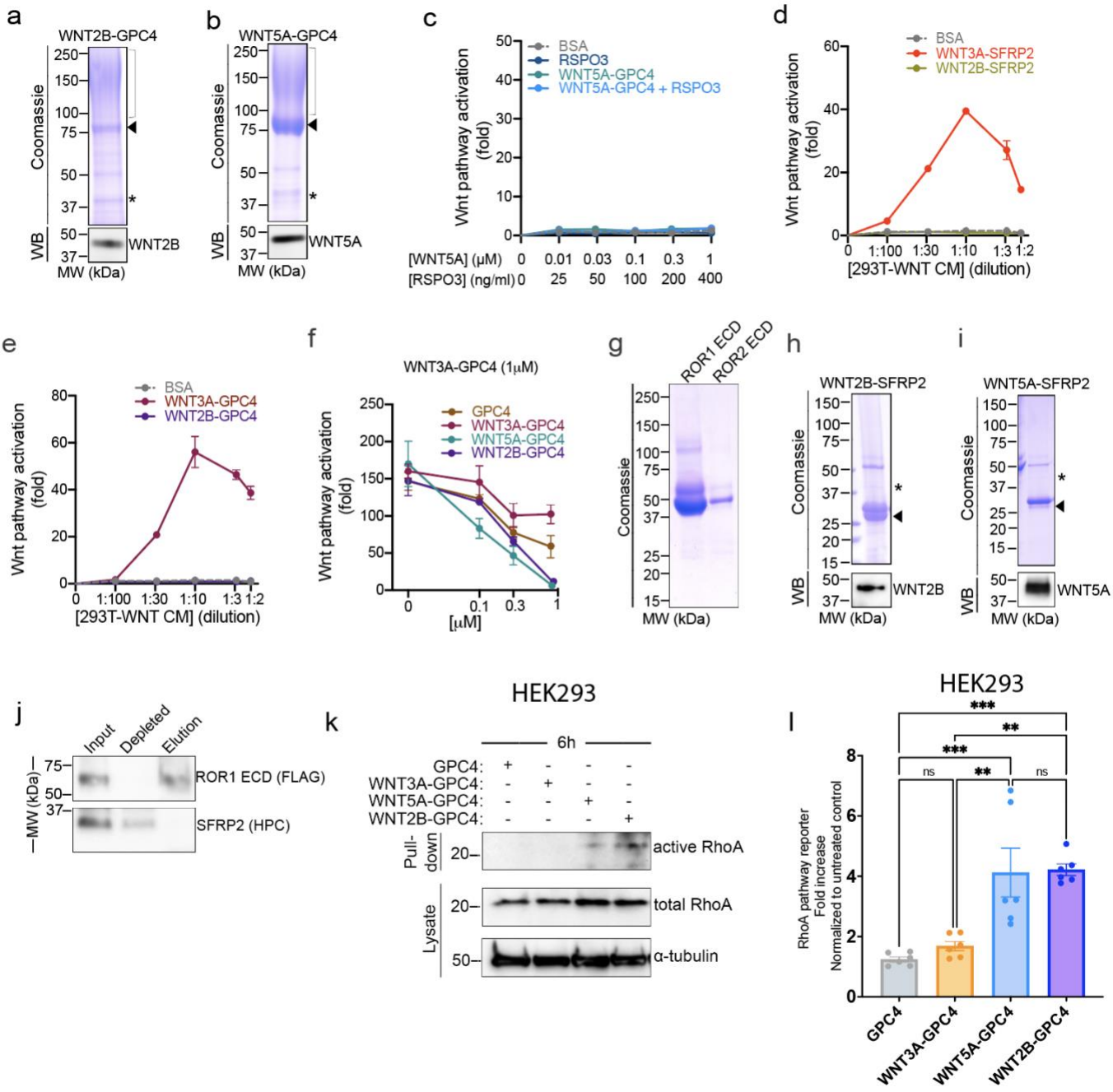
Supplemental Figure 2. WNT2B deficiency does not affect corticosterone levels in mice. Plasma corticosterone levels (female, n=11 WT, n=13 KO; male, n=12 WT, n=9 KO). Two-tailed Student's t-test. ns, not significant. Data are represented as mean ± SEM.

Supplemental Fig. 3



Supplemental Figure 3. Representative images from female adrenals immunostained for LEF1 (yellow) and DAPI (blue) from WT, KO and KO+LiCL mice. Scale bar: 50μm

Supplemental Fig. 4



Supplemental Figure 4. Characterization of WNT2B as a non-canonical ligand.

a. WNT2B-GPC4 ectodomain, C-terminally tagged with HaloTag7 (HT7) and HPC tag, was affinity purified from conditioned media on an anti-HPC antibody matrix, and analyzed by SDS-PAGE, followed by Coomassie staining or anti-WNT2B immunoblotting (WB). Arrowhead indicates unmodified GPC4, bracket indicates glycosaminoglycan (GAG)-modified species, and asterisks indicate WNT2B protein.

b. As in (a), but with WNT5A in complex with GPC4, and with anti-WNT5A immunoblotting.

c. R-Spondin 3 (RSPO3, 0, 25, 100, 200 and 400ng/ml) or purified WNT5A-GPC4 complex (0.01, 0.03, 0.1, 0.3 and 1μM with respect to WNT3A) with or without RSPO3 (400ng/ml) was added to Wnt reporter cells. After 24h, Wnt pathway activity was measured by luciferase assay. Incubation with BSA served as negative control. WNT5A-GPC4 does not activate canonical Wnt signaling, even when incubated with RSPO3. Points represent average activation for two biological replicates, normalized to untreated cells, and error bars represent SD.

d. SFRP2 (1μM) was added in serum-free media in WNT3A- or WNT2B-expressing HEK293 cells. Serial dilutions of the conditioned media were then added to Wnt reporter cells, and Wnt pathway activity was measured by Dual-Glo luciferase 24h later. BSA (1μM) served as negative control. WNT2B released by SFRP2 is unable to activate canonical Wnt signaling, in contrast to WNT3A-SFRP2 conditioned media. Points represent average activation for two biological replicates, normalized to the negative control, and error bars represent SD.

e. As in (d), but WNT-expressing cells were incubated with 1μM of GPC4.

f. As in (Fig. 4d), but purified WNT3A-GPC4 complex (1μM) was mixed with the indicated concentrations of GPC4 alone or in complex with WNT3A, WNT5A or WNT2B. WNT3A-SFRP2 activity is abolished by WNT5A-GPC4 and WNT2B-GPC4 complexes in a dose-dependent manner, which contrasts GPC4 alone or WNT3A-GPC4 complex.

g. Extracellular domains (ECD) of ROR1 and ROR2, N-terminally tagged with a FLAG tag, were affinity purified from conditioned media on an anti-FLAG antibody matrix. Purified proteins were analyzed by SDS-PAGE and Coomassie staining.

h. As in (a), but with WNT2B in complex with SFRP2, C-terminally tagged with 8x-His tag and HPC taI.

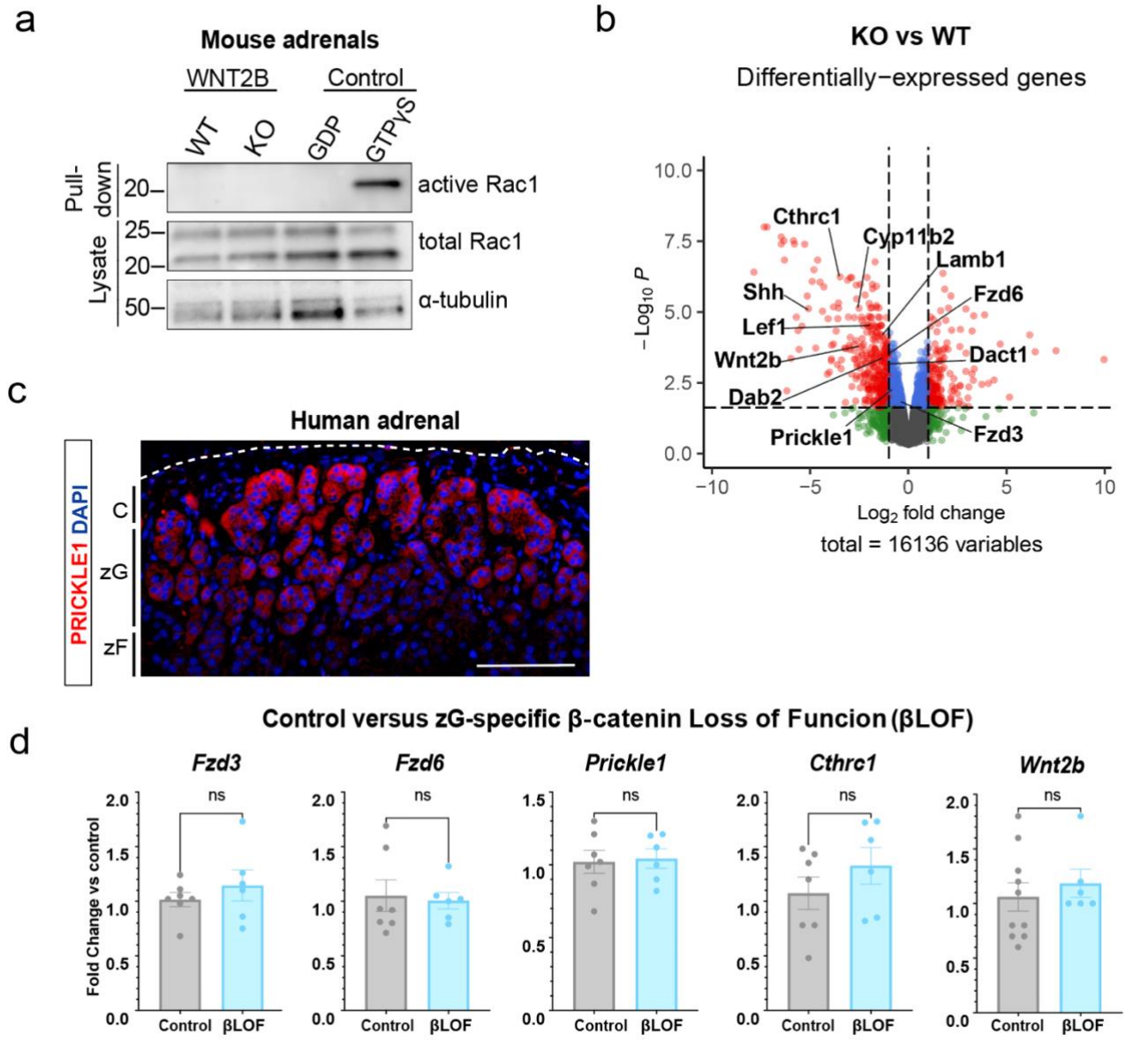
i. As in (b), but with WNT5A in complex with SFRP2.

j. Purified SFRP2 (5μM) was incubated with FLAG-tagged ROR1-ECD (2.5μM), followed by immunoprecipitation with antibodies against the FLAG tag. Samples were analyzed by SDS-PAGE and immunoblotting. SFRP2 does not interact with ROR1-EcD.

k. Activity of RhoA in cell lysates of HEK293 cells treated for 6h with GPC4 alone or in complex with WNT3A, WNT5A or WNT2B (2μM) was assessed by Rhotekin-RBD pull-down assay. RhoA endogenous levels are shown in the lysates. Both WNT5A-GPC4 and WNT2B-GPC4 complexes induce activity of RhoA, in contrast to GPC4 alone or in complex with WNT3A. Blotting for α-tubulin served as loading control.

l. HEK293 cells were co-transfected with the firefly luciferase reporter (pGL4.34) and the renilla luciferase thymidine kinase reporter (pRL-TK). They were then used to assay RhoA activation by purified GPC4 alone or in complex with WNT3A, WNT5A, and WNT2B (1 μM). We found that the activity of RhoA is induced by WNT2B-GPC4 or WNT5A-GPC complexes, but not by WNT3A-GPC4 or GPC4 alone. The bars represent the average from three independent experiments performed in duplicate, normalized to untreated cells. Statistical significance was determined using one-way ANOVA with Tukey's post-test (ns, not significant; **$p < 0.01$; ***$p < 0.001$). Data are represented as mean ± SEM.

Supplemental Fig. 5



Supplemental Figure 5. WNT2B deficiency disrupts Wnt/PCP signaling in the adrenal.

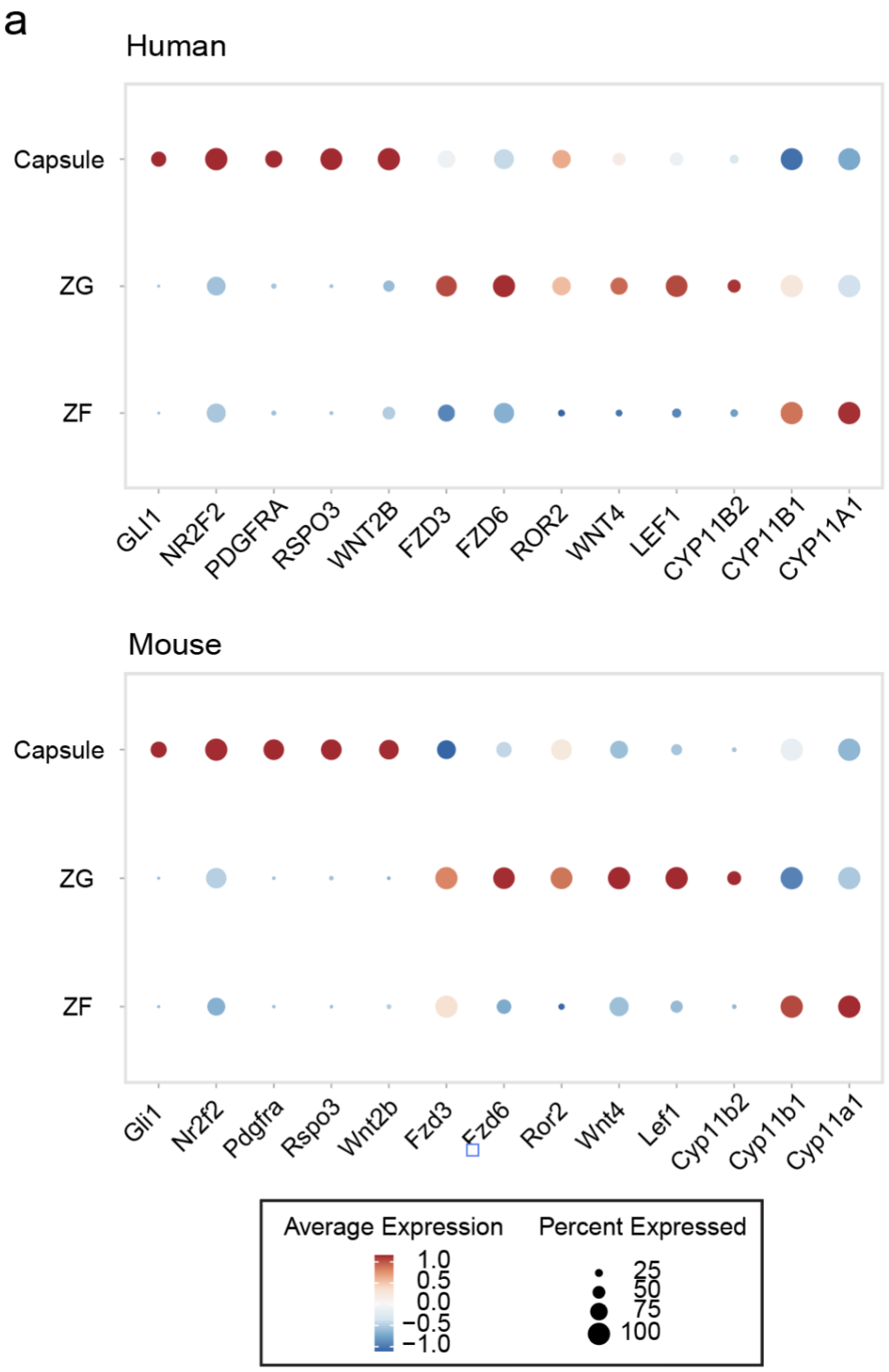
a. Activity of Rac1 in WT and KO adrenals assessed by Rhotekin-RBD pull-down assay using adrenal lysates. GTPγS and GDP treated adrenal lysates served as positive and negative controls, respectively. Total Rac1 and α-tubulin served as loading controls.

b. Volcano plot showing differentially-expressed genes between WT and KO adrenals. Dots representing genes down- and up-regulated in KO are displayed on the left and right sides of the plot, respectively. Red dots represent genes that exhibit a fold-change > 2-fold with a FDR-adjusted p-value < 0.05. Selected zonal markers, including zG genes, are indicated.

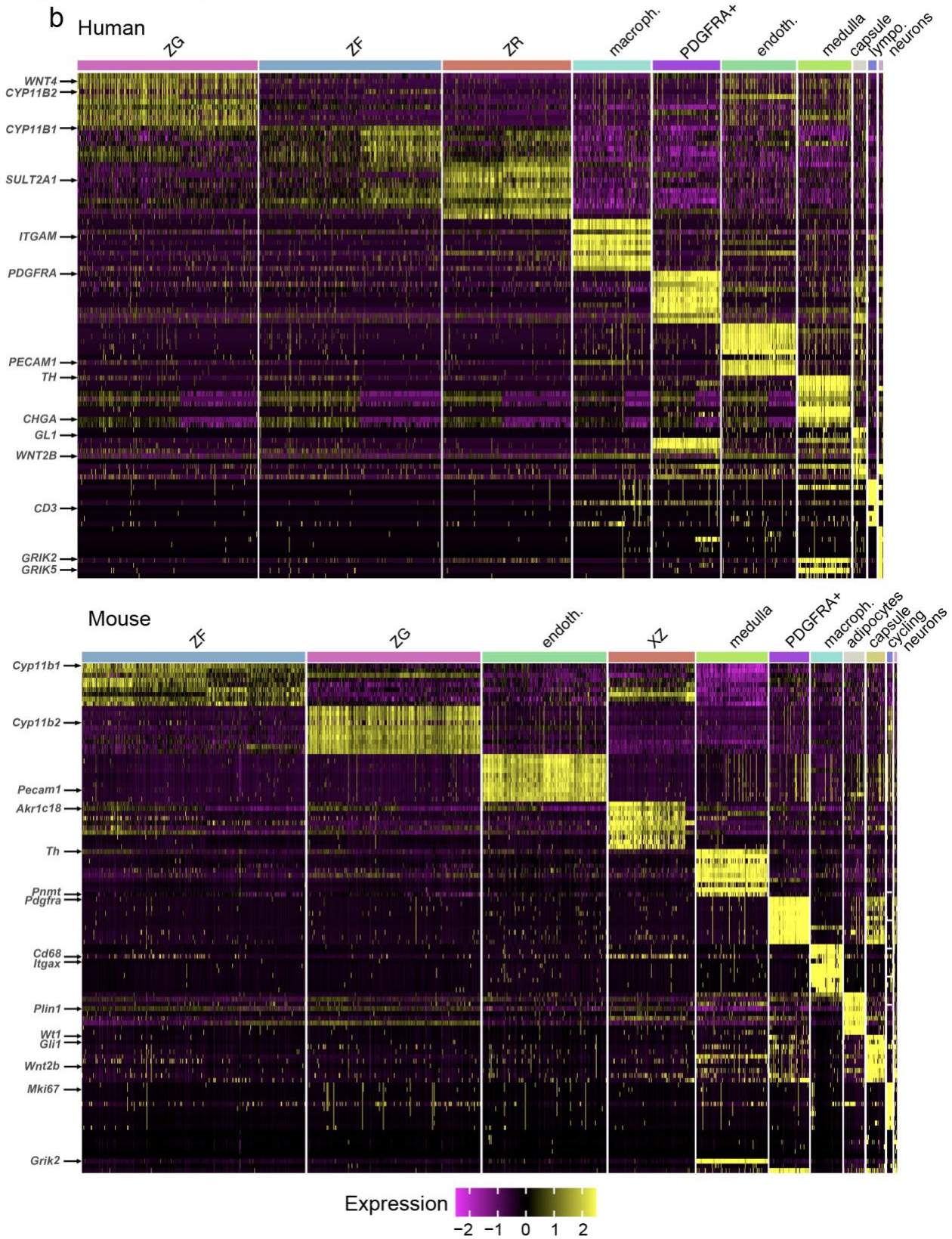
c. Representative image stained for PRICKLE1 (red) and DAPI (blue) from human adrenals. Scale bar: 100μm. C, capsule; zG, zona glomerulosa; zF, zona fasciculata.

d. QRT-PCR was performed in WT and zG-specific β−catenin LOF adrenals for Fzd3 (n=7 Control, n=6 βLOF), Fzd6 (n=7 Control, n=6 βLOF), Prickel1 (n=7 Control, n=6 βLOF), Cthrc1 (n=7 Control, n=6 βLOF), and Wnt2b (n=10 Control, n=6 βLOF). Two-tailed Student's t-test. ns, not significant. Data are represented as mean ± SEM.

Supplemental Fig. 6



Supplemental Fig. 6



Supplemental Figure 6.
a. Dot plot showing average expression of genes in the capsule, zG or zF from human or mouse adrenals.
b. Heatmap visualization showing gene expression patterns of cellular clusters identified in human and mouse adrenals.