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Supplementary Information for

Steroidogenic differentiation and PKA signalling are programmed by histone methyltransferase EZH2 in the adrenal cortex

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Supplementary Materials and Methods

Other supplementary materials for this manuscript include the following:
Supplementary Dataset S1 to S2

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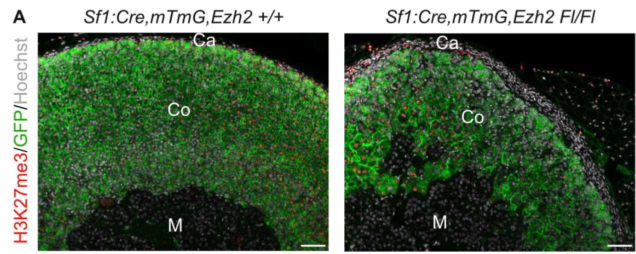
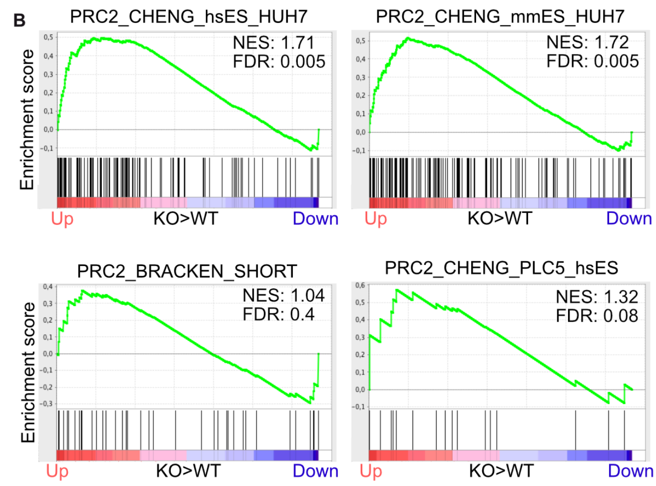
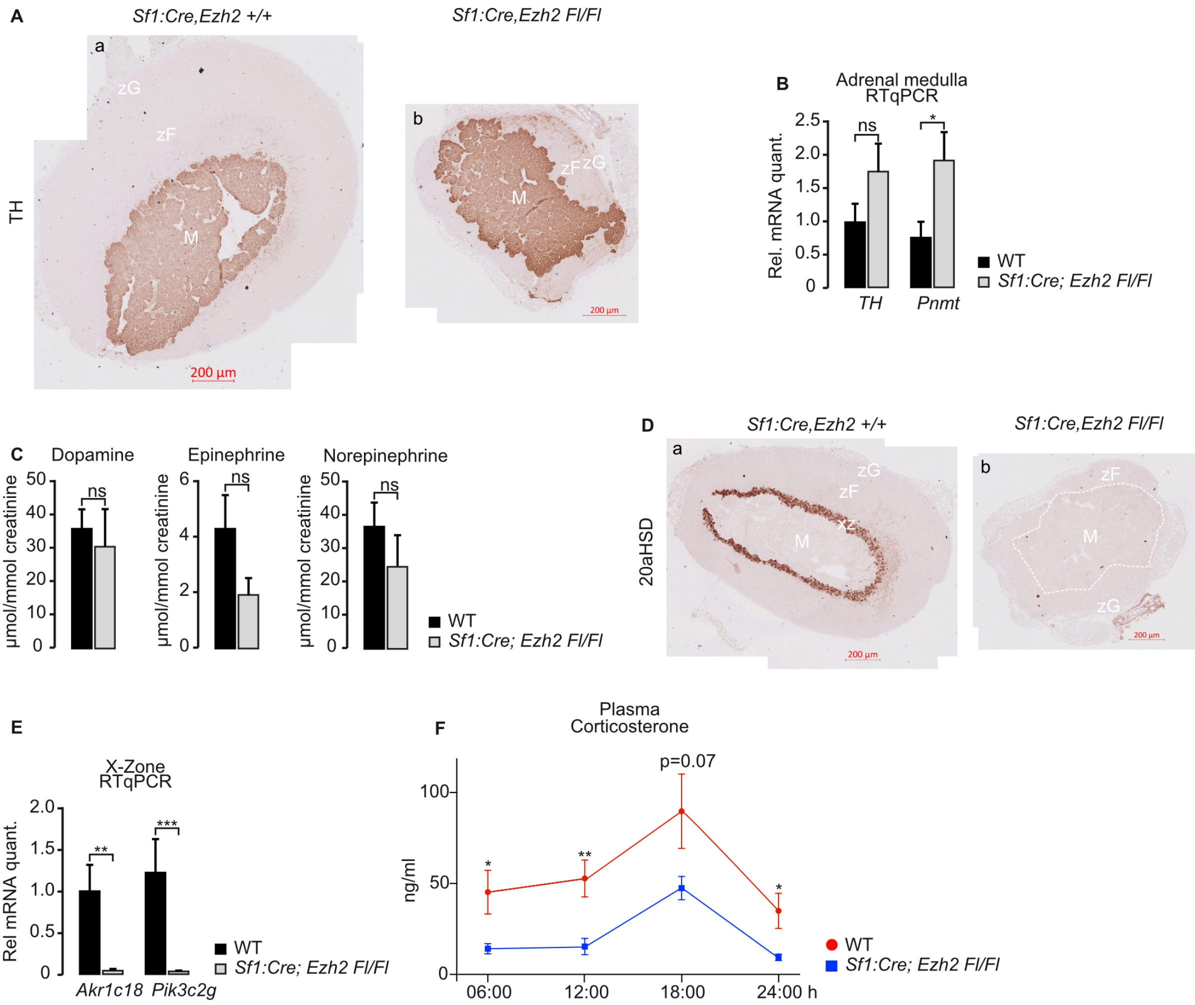
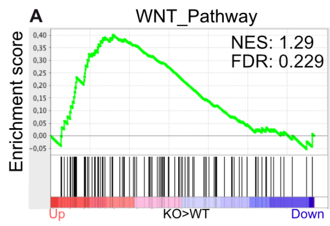

A
Sf1:Cre,mTmG,Ezh2 +/+
Sf1:Cre,mTmG,Ezh2 Fl/Fl
H3K27me3/GFP/Hoechst
Ca
Co
M
Ca
Co
M

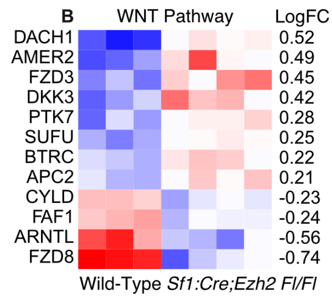

B
PRC2_CHENG_hsES_HUH7
NES: 1.71
FDR: 0.005
PRC2_CHENG_mmES_HUH7
NES: 1.72
FDR: 0.005
Enrichment score
Up
KO>WT
Down
PRC2_BRACKEN_SHORT
NES: 1.04
FDR: 0.4
PRC2_CHENG_PLC5_hsES
NES: 1.32
FDR: 0.08

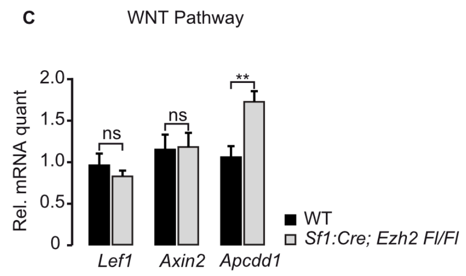
SI Appendix Figure S1 related to Figure 1. A- Immunohistochemical detection of H3K27me3 together with GFP expressed from the mTmG locus, following Sf1:Cre-mediated recombination, in 2 month-old wild-type (a, *Sf1:Cre,mTmG,Ezh2 +/+*) and Ezh2 KO female adrenals (b, *Sf1:Cre,mTmG,Ezh2 Fl/Fl*). Ca: capsule, Co: cortex, M: medulla. Scalebars: 50 µm **B-** Gene Set Enrichment Analysis (GSEA) of micro-array gene expression data (2 month-old knockout *versus* wild-type adrenals), using curated EZH2/PRC2 target gene lists.

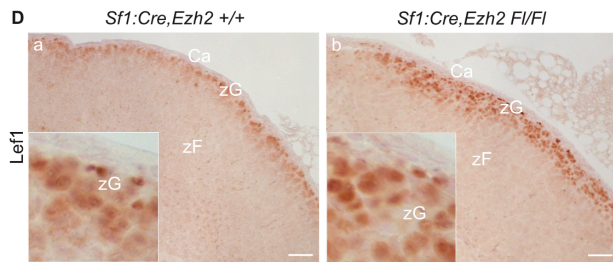

A
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
TH
a
zG
zF
M
200 µm
b
zF zG
M
200 µm
B
Adrenal medulla
RTqPCR
Rel. mRNA quant.
ns
*
TH
Pnmt
WT
Sf1:Cre; Ezh2 Fl/Fl
C
Dopamine
Epinephrine
Norepinephrine
µmol/mmol creatinine
ns
WT
Sf1:Cre; Ezh2 Fl/Fl
D
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
20aHSD
a
zG
zF
XZ
M
200 µm
b
zF
M
zG
200 µm
E
X-Zone
RTqPCR
Rel mRNA quant.
**
***
Akr1c18
Pik3c2g
WT
Sf1:Cre; Ezh2 Fl/Fl
F
Plasma
Corticosterone
p=0.07
ng/ml
06:00
12:00
18:00
24:00 h
WT
Sf1:Cre; Ezh2 Fl/Fl

SI Appendix Figure S2 related to Figure 2. A- Immunohistochemical detection of Tyrosine Hydroxylase (TH) in 2 month-old female wild-type and Ezh2 KO adrenals. **B-** RTqPCR analysis of accumulation of mRNAs encoding *TH and Pnmt* in 2 month-old female wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 7 animals per group ± SEM. **C-** Urinary concentrations of dopamine, epinephrine and norepinephrine in 2 month-old female wild-type and Ezh2 KO mice. Bars represent concentrations of each catecholamine standardised to creatinine in 6 animals per group ± SEM. **D-** Immunohistochemical detection of 20αHSD in 2 month-old nulliparous female wild-type and Ezh2 KO adrenals. E- RTqPCR analysis of accumulation of mRNAs encoding *Akr1c18* (20αHSD) and *Pik3c2g* in 2 month-old nulliparous female wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 7 animals per group ± SEM. **F-** Plasma corticosterone concentrations measured at 06:00, 12:00, 18:00 and 24:00 hours in 2 month-old female wild-type and Ezh2 KO mice. Graphs represent the mean concentration in 6 animals per group ± SEM. M: medulla, xZ: X-zone, zF: zona fasciculata, zG: zona glomerulosa. ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.


A
WNT_Pathway
Enrichment score
NES: 1.29
FDR: 0.229
Up
KO>WT
Down

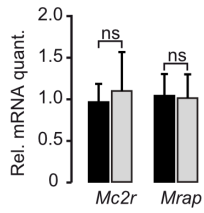

B
WNT Pathway
LogFC
DACH1 0.52
AMER2 0.49
FZD3 0.45
DKK3 0.42
PTK7 0.28
SUFU 0.25
BTRC 0.22
APC2 0.21
CYLD -0.23
FAF1 -0.24
ARNTL -0.56
FZD8 -0.74
Wild-Type Sf1:Cre;Ezh2 Fl/Fl


C
WNT Pathway
Rel. mRNA quant
2.0
1.5
1.0
0.5
0
ns
ns
**
Lef1
Axin2
Apcdd1
WT
Sf1:Cre; Ezh2 Fl/Fl

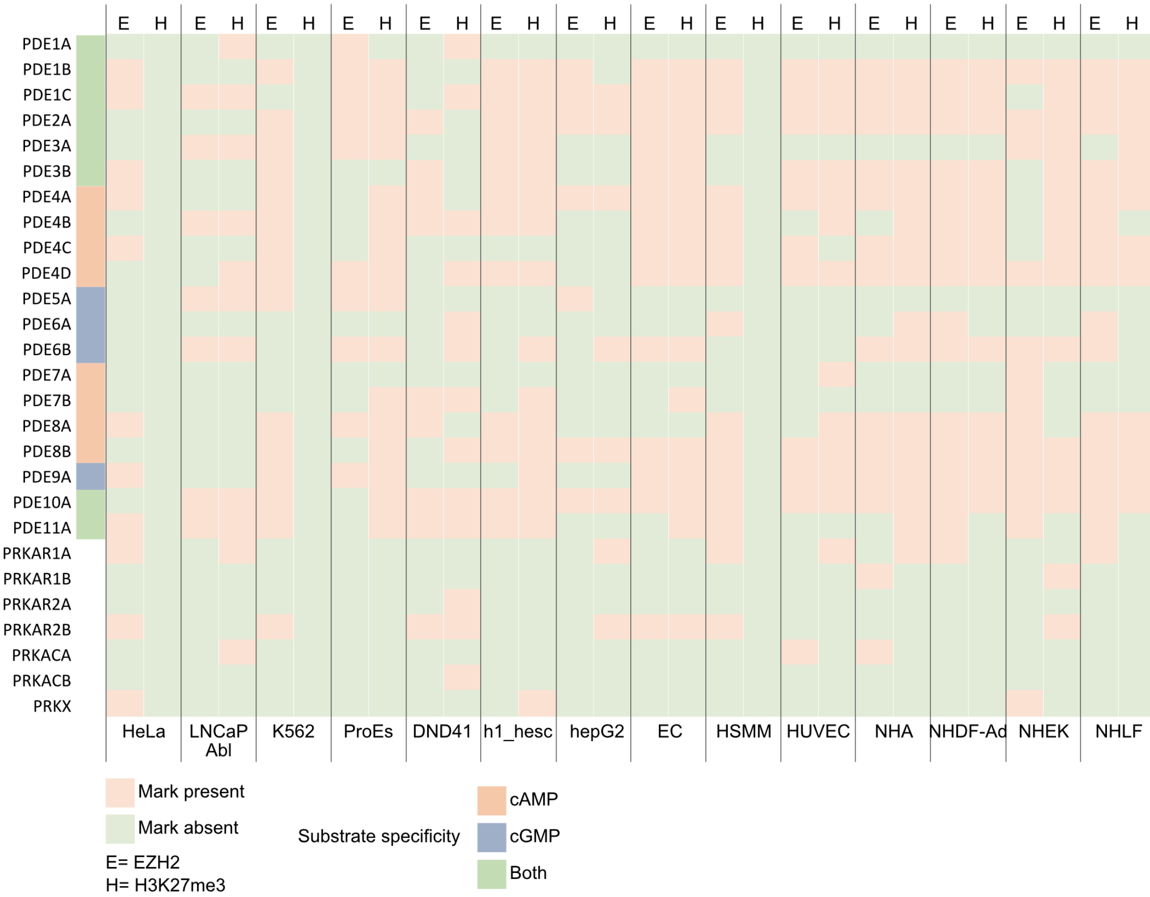

D
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
Lef1
a
b
Ca
zG
zF
zG
Ca
zG
zF
zG

SI Appendix Figure S3. ***Ezh2*** **ablation is associated with mild induction of canonical WNT/β-catenin signalling. A-** Gene Set Enrichment Analysis (GSEA) of micro-array gene expression data, using a curated WNT pathway regulators and targets gene list (1). **B-** Heatmap representing the median centred expression of significantly deregulated WNT regulators and target genes (FDR <0.1) in 2 month-old female Ezh2 KO compared with wild-type adrenals. **C-** RTqPCR analysis of accumulation of mRNAs encoding canonical WNT target genes in 2 month-old female wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 7 animals per group ± SEM. Ns, not significant, ** p <0.01. **D-** Immunohistochemical detection of the canonical WNT target gene LEF1 in 2 month-old female wild-type and Ezh2 KO adrenals. zF: zona fasciculata, zG: zona glomerulosa, Ca: capsule. Scalebars: 50µm.

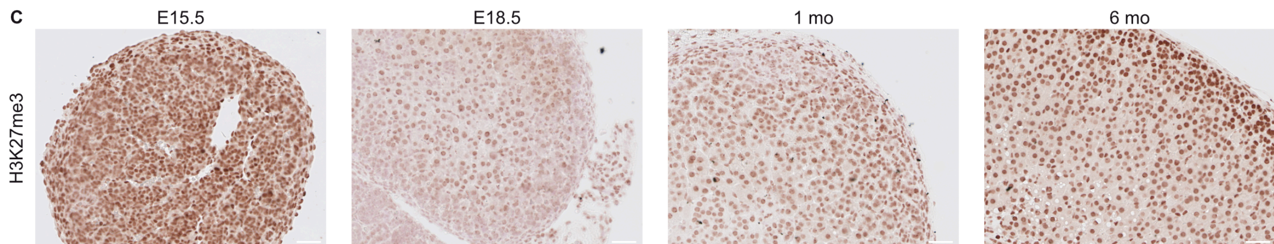
A ACTH signalling RTqPCR



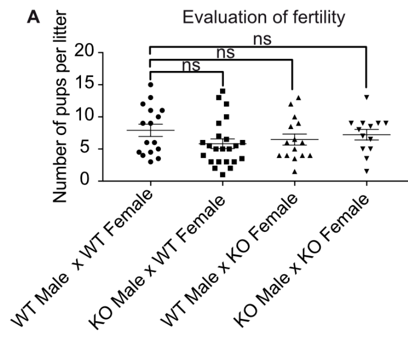
B EZH2 and H3K27me3 ChIP sequencing

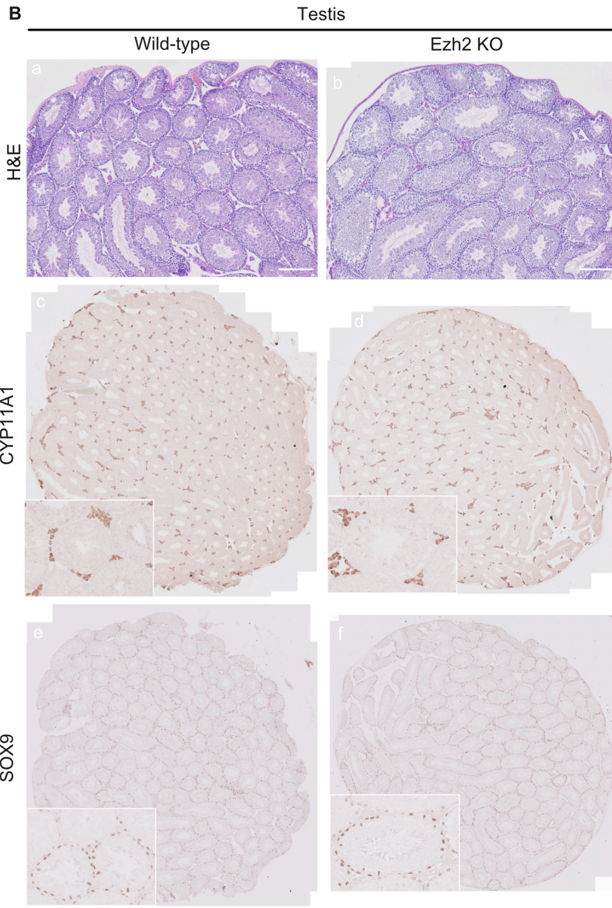


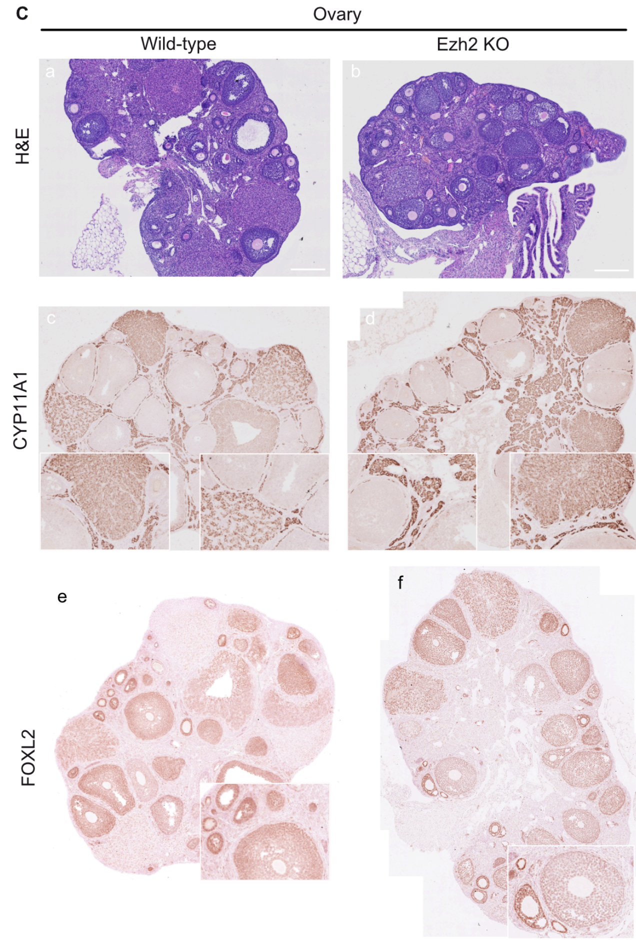
C

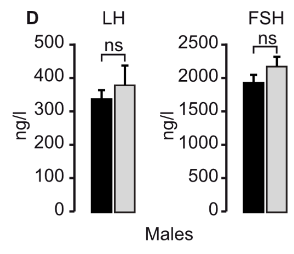


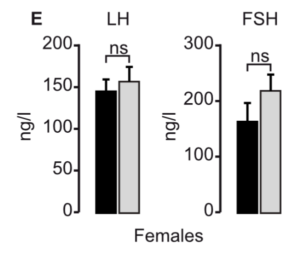
SI Appendix Figure S4 related to Figure 3. A- RTqPCR analysis of accumulation of mRNAs encoding the ACTH receptor *Mc2r* and the accessory protein *Mrap* in 2 month-old female wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 7 animals per group ± SEM. ns, not significant. **B-** Table recapitulating analysis of publically available ChIP sequencing data for EZH2 (E) and H3K27me3(H) in 14 cell lines and for genes encoding phosphodiesterases (*Pde1a-Pde11a*) and regulatory subunits of PKA. An orange box indicates the presence of a binding peak for either EZH2 and/or H3K27me3 within -2/+2kb from the transcription start site of each gene. A colour code in the leftmost lane indicates substrate specificity for cAMP (orange), cGMP (grey) or both (green). HeLa: Human cervix cancer, LNCaP Abl: Human castration resistant prostate cancer lymph node metastasis cells, K562: Human CML, ProES: Human primary fetal liver proerythroblasts, DND41: Human T cell leukaemia, h1_hesc: Human embryonic stem cells, hepG2: Human hepatocellular carcinoma, EC: Human primary epithelial cell, HSMM: Human skeletal muscle myoblast, HUVEC: Human umbilical vein endothelial cells, NHA: normal Human astrocyte cells, NHDF-Ad: adult Human dermal fibroblasts, NHEK: Human dermal keratinocyte, NHLF: Human lung fibroblast. **C-** Immunohistochemical detection of H3K27me3 in E15.5, E18.5, 1 month and 6 months adrenals from wild-type mice. Scalebars: 50 µm.


A
Evaluation of fertility
Number of pups per litter
ns
ns
ns
20
15
10
5
0
WT Male x WT Female
KO Male x WT Female
WT Male x KO Female
KO Male x KO Female

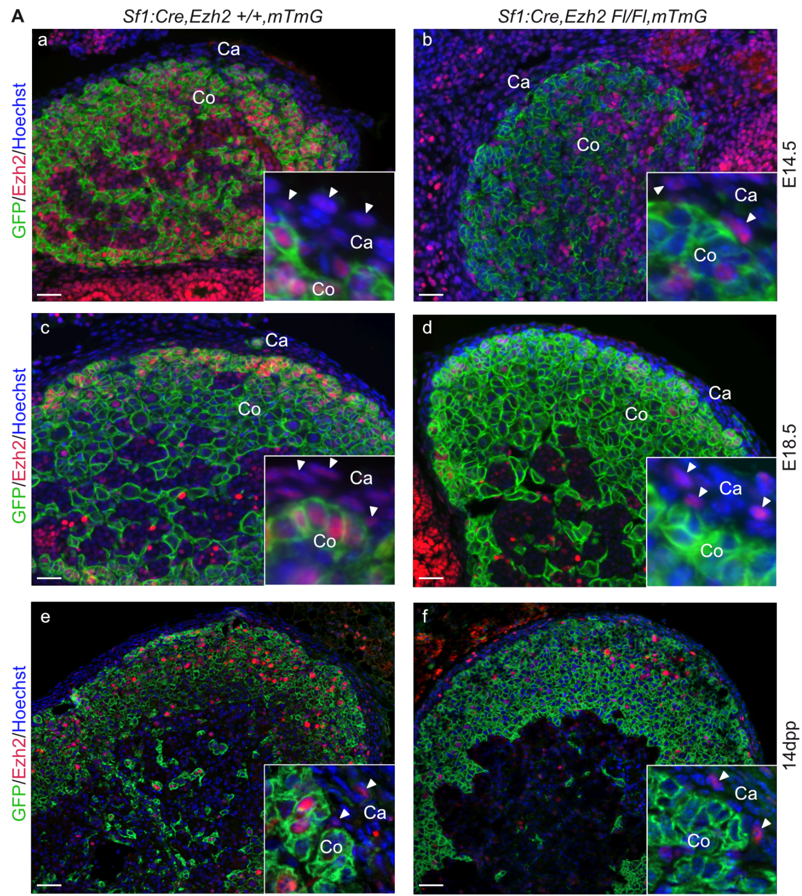

B
Testis
Wild-type
Ezh2 KO
H&E
CYP11A1
SOX9

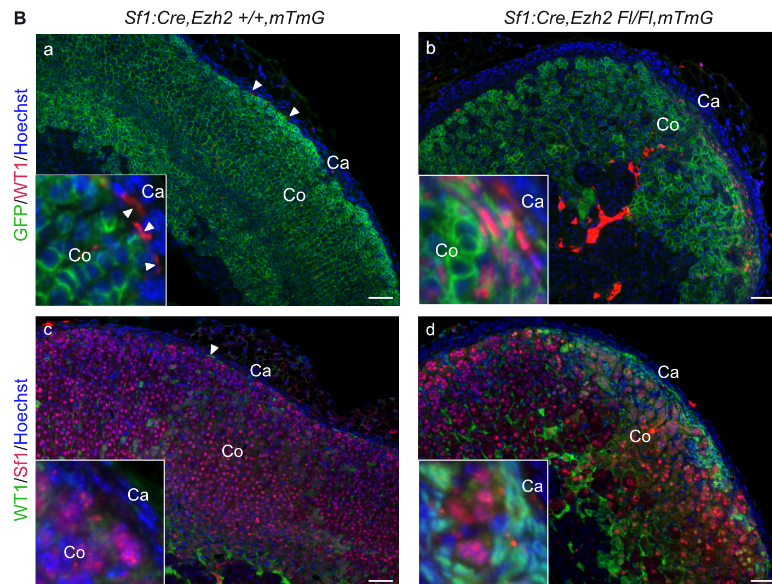

C
Ovary
Wild-type
Ezh2 KO
H&E
CYP11A1
FOXL2


D
LH
FSH
ns
ns
ng/l
500
400
300
200
100
0
2500
2000
1500
1000
500
0
Males

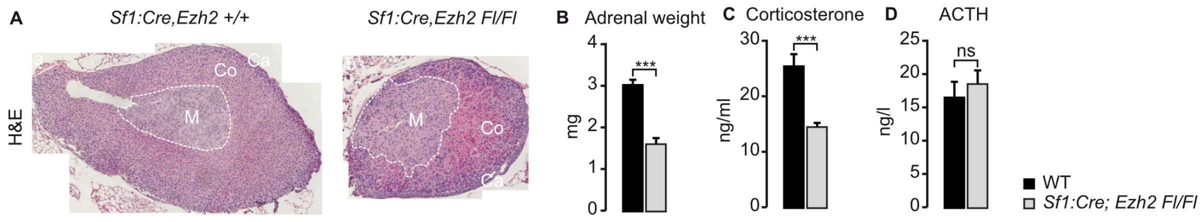

E
LH
FSH
ns
ns
ng/l
200
150
100
50
0
300
200
100
0
Females

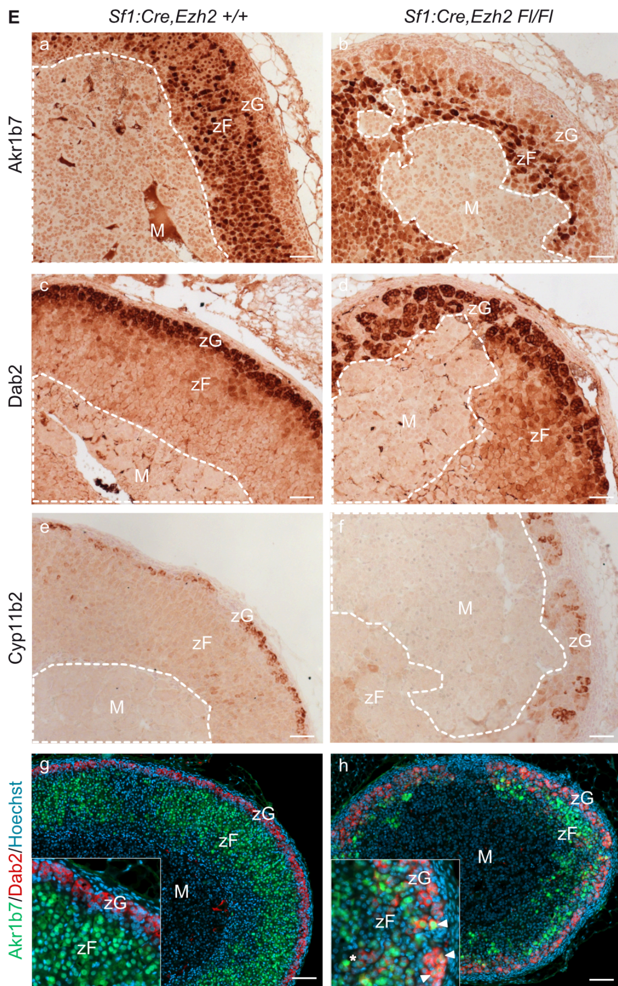
SI Appendix Figure S5 related to Figure 5. A- Number of pups per litter in different mating schemes. **B-** Hematoxylin-eosin staining (a,b) and immunohistochemical detection of CYP11A1 (c,d, Leydig cells) and SOX9 (e,f, Sertoli cells) in 2 month-old wild-type and Ezh2 KO testes. **C-** Hematoxylin-eosin staining (a,b) and immunohistochemical detection of CYP11A1 (c,d, theca cells and corpus luteum) and FOXL2 (e,f, granulosa cells) in 2 month-old wild-type and Ezh2 KO ovaries. **D-E-** Plasma LH and FSH concentrations in 2 month-old wild-type and Ezh2 KO male (D) and female (E) mice. Scalebars: 200 µm. ns, not significant.

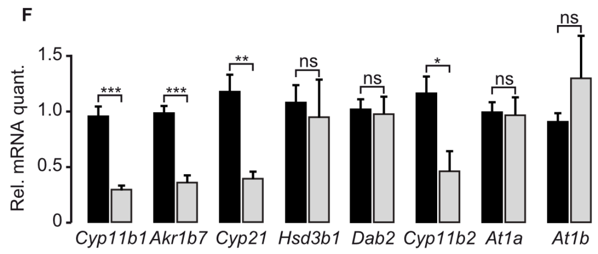

A
Sf1:Cre,Ezh2 +/+,mTmG
Sf1:Cre,Ezh2 Fl/Fl,mTmG
a
b
c
d
e
f
GFP/Ezh2/Hoechst
E14.5
E18.5
14dpp
Ca
Co

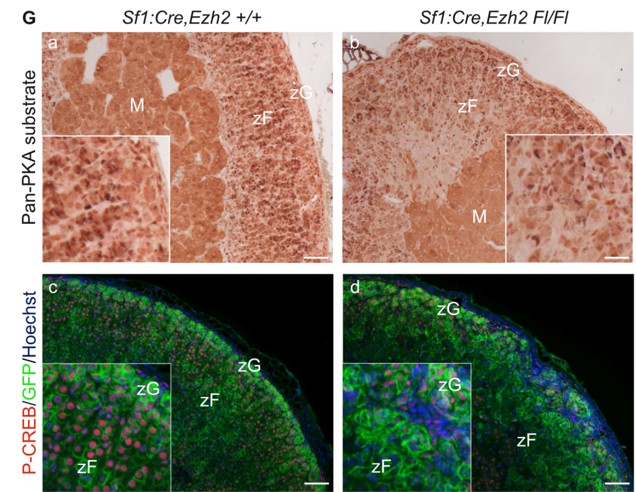

B
Sf1:Cre,Ezh2 +/+,mTmG
Sf1:Cre,Ezh2 Fl/Fl,mTmG
a
b
c
d
GFP/WT1/Hoechst
WT1/Sf1/Hoechst
Ca
Co

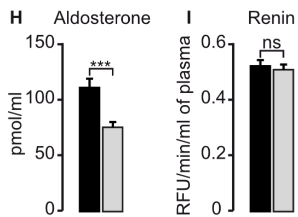
SI Appendix Figure S6 related to Figure 5. A- Co-immunostaining of EZH2 and GFP (cells of the steroidogenic lineage) in E14.5 (A-b), E18.5(c-d) and 14dpp (e-f) wild-type (a,c,e) and Ezh2 KO (b,d,f) adrenals. White arrowheads show EZH2-positive capsular cells. Ca: capsule, Co: cortex. **B-** Co-immunostaining of WT1 and GFP (a-b, cells of the steroidogenic lineage) and WT1 and SF1 (c-d) in 2 month-old female wild-type (a,c) and Ezh2 KO (b,d) female adrenals. White arrowheads show scarce WT1-positive cells in wild-type adrenals. Scalebars: 50 µm. Co: cortex, Ca: capsule.


A
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
H&E
Co
Ca
M
B Adrenal weight
mg
C Corticosterone
ng/ml
D ACTH
ng/l
ns
***
WT
Sf1:Cre; Ezh2 Fl/Fl

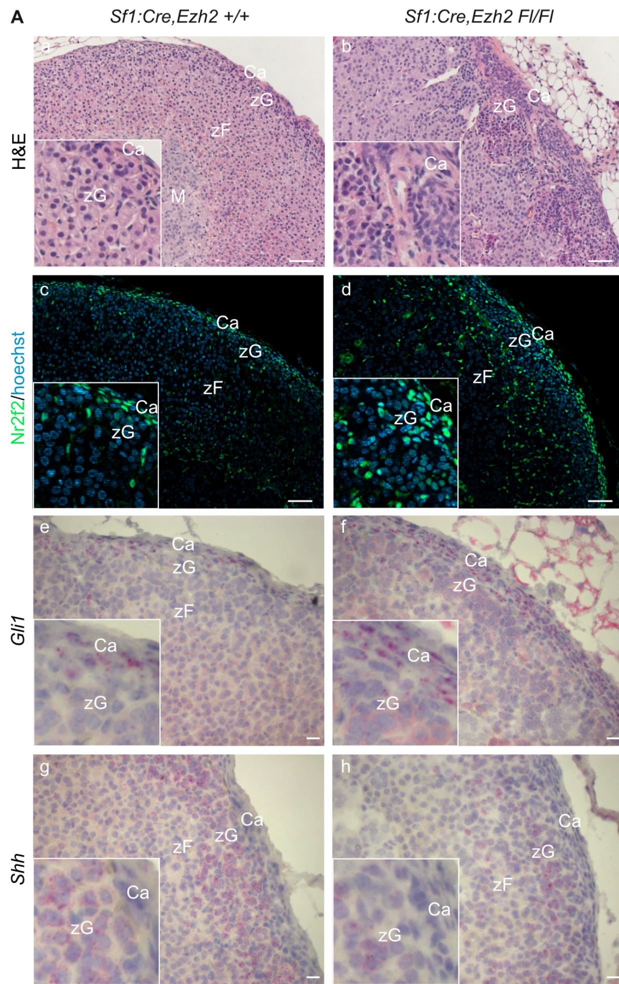

E
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
Akr1b7
Dab2
Cyp11b2
Akr1b7/Dab2/Hoechst
zG
zF
M

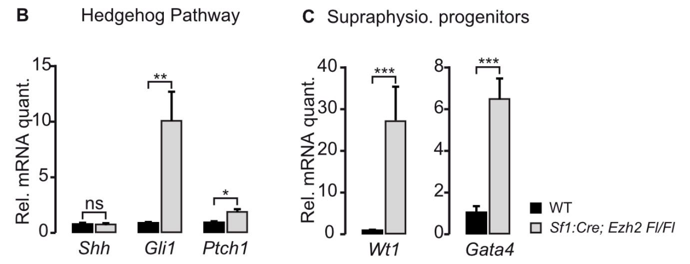

F
Rel. mRNA quant.
Cyp11b1
Akr1b7
Cyp21
Hsd3b1
Dab2
Cyp11b2
At1a
At1b
ns
***
**
*

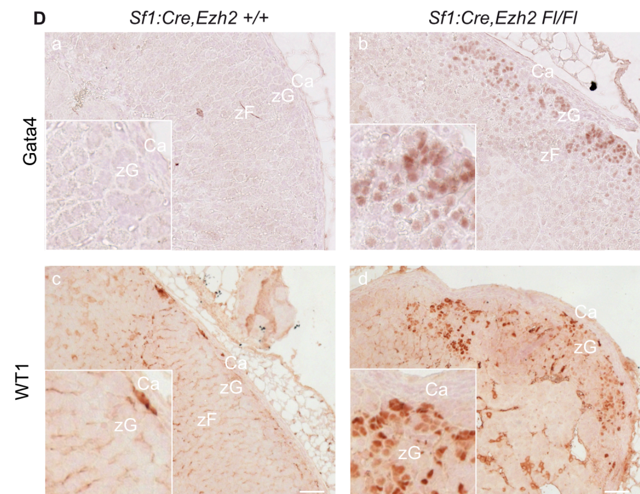

G
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
Pan-PKA substrate
P-CREB/GFP/Hoechst
zG
zF
M

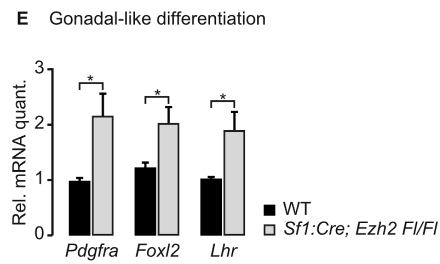

H Aldosterone
pmol/ml
I Renin
RFU/min/ml of plasma
***
ns

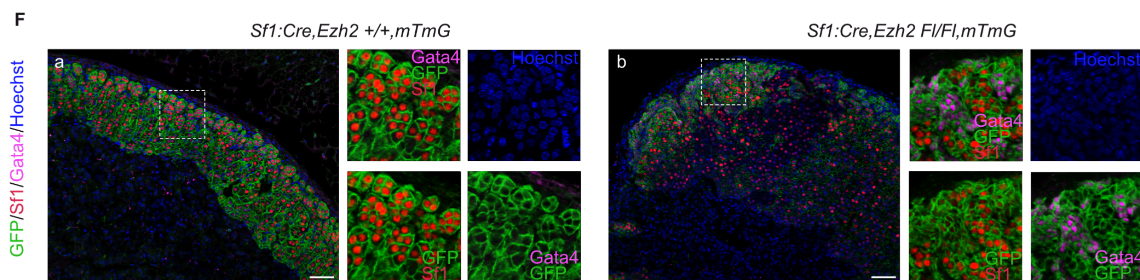
SI Appendix Figure S7. A- Haematoxylin & eosin staining of 2 month-old male wild-type and Ezh2 KO adrenals, showing a marked difference in size, cortical hypoplasia and dysplasia and misplaced adrenal medulla. Ca: capsule, Co: cortex, M: medulla. **B-** adrenal weight in 2 month-old male wild-type and Ezh2 KO mice. **C-** plasma corticosterone and **D-** ACTH in 2 month-old male wild-type and Ezh2 KO mice. **E-** Immunohistochemical detection of AKR1B7 (zF, a-b), DAB2 (zG, c,d), CYP11B2 (zG, e,f), and co-immunostaining for AKR1B7 (zF) and DAB2 (zG)(g,h) in 2 month-old male wild-type and Ezh2 KO adrenals. White arrowheads show cells with co-staining for DAB2 and AKR1B7, star shows misplaced DAB2-positive cells. **F-** RTqPCR analysis of accumulation of mRNAs encoding genes involved in steroidogenesis (*Cyp11b1*, *Cyp21*, *Hsd3b1*, *Cyp11b2*), zonal differentiation (*Akr1b7*, *Dab2*) and of angiotensin receptors (*At1a*, *At1b*) in 2 month-old male wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 11 animals per group ± SEM. **G-** Immunohistochemical detection of Pan-PKA substrates (a,b) and phosphorylated CREB protein together with GFP (cells of the steroidogenic lineage, c,d) in 2 month-old male wild-type and Ezh2 KO adrenals. **H-** plasma aldosterone and **I-** Renin in 2 month-old male wild-type and Ezh2 KO mice. Bars represent mean concentrations in 13 mice per group ± SEM. ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Scalebars : 50µm. M: medulla, zF: zona fasciculata, zG: zona glomerulosa.

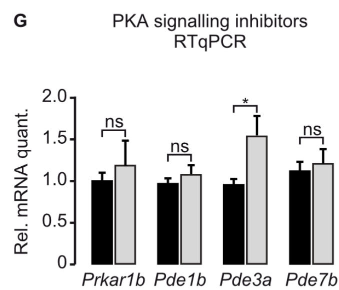

A
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
H&E
Nr2f2/hoechst
Gli1
Shh
Ca
zG
zF
M

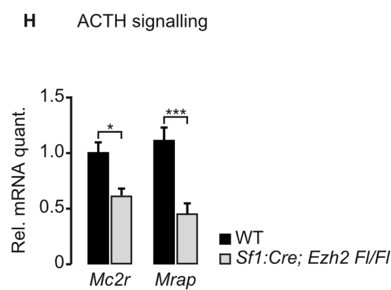

B
Hedgehog Pathway
Rel. mRNA quant.
Shh
Gli1
Ptch1
ns
**
*
C
Supraphysio. progenitors
Wt1
Gata4
***
WT
Sf1:Cre; Ezh2 Fl/Fl


D
Sf1:Cre,Ezh2 +/+
Sf1:Cre,Ezh2 Fl/Fl
Gata4
WT1
Ca
zG
zF

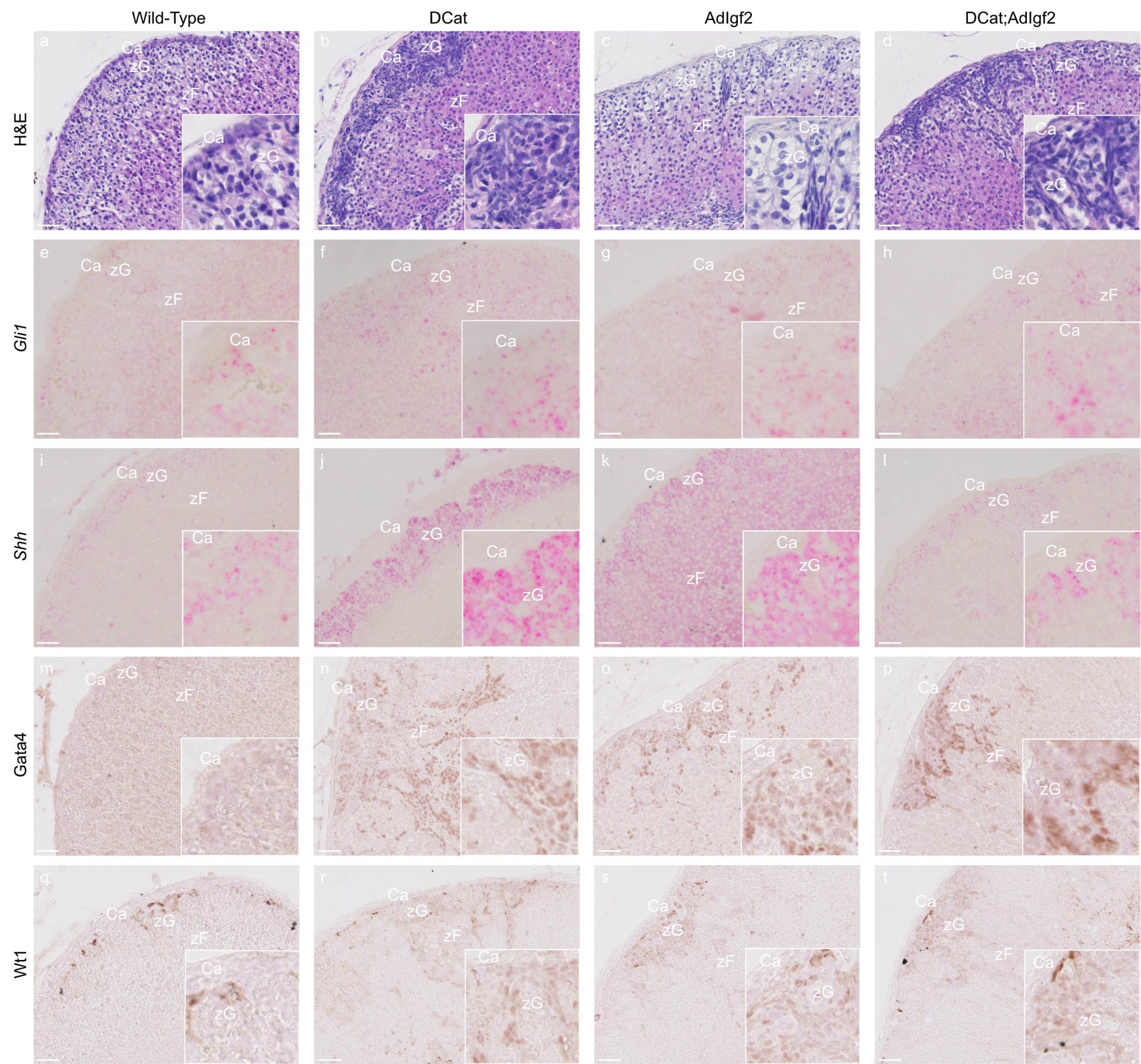

E
Gonadal-like differentiation
Rel. mRNA quant.
Pdgfra
Foxl2
Lhr
WT
Sf1:Cre; Ezh2 Fl/Fl


F
GFP/Sf1/Gata4/Hoechst
Sf1:Cre,Ezh2 +/+,mTmG
Sf1:Cre,Ezh2 Fl/Fl,mTmG
Gata4
GFP
Sf1
Hoechst


G
PKA signalling inhibitors
RTqPCR
Rel. mRNA quant.
Prkar1b
Pde1b
Pde3a
Pde7b
ns
*


H
ACTH signalling
Rel. mRNA quant.
Mc2r
Mrap
*
***
WT
Sf1:Cre; Ezh2 Fl/Fl

SI Appendix Figure S8. A- haematoxylin-eosin staining (a-b), immunohistochemical detection of NR2F2 (c,d) and RNAScope analysis of *Gli1* (e,f) and *Shh* (g,h) expression in 2 month-old male wild-type and Ezh2 KO adrenals. **B-** RTqPCR analysis of accumulation of mRNAs encoding key actors in Hedgehog pathway and **C-** markers of supraphysiological progenitors in 2 month-old male wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 11 animals per group ± SEM. **D-** Immunohistochemical analysis of GATA4 (a,b) and WT1 (c,d) expression in 2 month-old male wild-type and Ezh2 KO adrenals. **E-** RTqPCR analysis of accumulation of mRNAs encoding markers of gonadal-like differentiation progenitors in 2 month-old male wild-type and Ezh2 KO adrenals. Bars represent the mean expression in 11 animals per group ± SEM. **F-** Co-immunostaining of SF1 (actual steroidogenic cells), GATA4 and GFP (cells of the steroidogenic lineage) in 2 month-old male wild-type (a, bearing Sf1:Cre and the mTmG reporter) and Ezh2 KO (b) adrenals. Right panels show different overlays of the region delineated by the dashed rectangle to the left. **G-** RTqPCR analysis of accumulation of mRNAs encoding inhibitors of PKA signalling pathway and **H-** of ACTH signalling pathway in 2 month-old male wild-type and Ezh2 KO adrenals. ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Scalebars in D, E, F, I, J : 50µm. M: medulla, zF: zona fasciculata, zG: zona glomerulosa, Ca: capsule.



Wild-Type
DCat
AdIgf2
DCat;AdIgf2
H&E
Gli1
Shh
Gata4
Wt1

SI Appendix Figure S9. Haematoxylin & eosin staining (a-d), RNAScope analysis for *Gli1* (e-h) and *Shh* (i-l) and immunohistochemical detection of GATA4 (m-p) and WT1 (q-t) in 10-12 month-old ΔCat (constitutive WNT pathway), AdIgf2 (overexpression of Igf2) and ΔCat;AdIgf2 (combination of both) transgenic mouse models. Scalebars : 50 µm. zF: zona fasciculata, zG: zona glomerulosa, Ca : capsule.

Antibody	Supplier	Reference	Host	Dilution	Unmasking	Amplification?
Ezh2	Abcam	Ab191080	Rabbit	1/250	Tris-EDTA	Y
GFP	Abcam	Ab5450	Goat	1/1000	Tris-EDTA	Y
GFP	Invitrogen	A11122	Rabbit	1/1000	Tris-EDTA	Y
H3K27me3	Millipore	07-449	Rabbit	1/8000	Citrate-Tween	Y
Akr1b7	Santa Cruz	SC-27763	Goat	1/200	Citrate-Tween	Y
Dab2	BD Biosciences	610464	Mouse	1/500	Citrate-Tween	Y
CYP11B2	C. Gomez Sanchez	Homemade	Rabbit	1/500	Tris-EDTA-SDS 10%	Y
Lef1	Abcam	Ab137872	Rabbit	1/400	Tris-EDTA	Y
Pan-PKA Substrate	Cell Signaling	9621	Rabbit	1/200	Citrate-Tween	Y
Phospho-CREB Ser 133	Cell Signaling	9198	Rabbit	1/50	Citrate-Tween	Y
NR2F2	Perseus Proteomics	PP-H7147-00	Mouse	1/200	Vector	Y
WT1	Leica Novocastra	NCL-L-WT1-562	Mouse	1/200	Citrate-Tween	Y
GATA4	Santa Cruz	SC-1237	Goat	1/500	Vector	Y
Ki67	Leica Novocastra	NCL-Ki67p	Rabbit	1/100	Tris-EDTA	N
Ki67	Spring Biosciences	M3062	Rabbit	1/200	Tris-EDTA	Y
CYP17A1	Corbin Laboratory	Homemade	Rabbit	1/1500	Citrate-Tween	Y
CYP11A1	Santa Cruz	SC-18043	Goat	1/200	Tris-EDTA	Y
FOXL2	Abcam	Ab5096	Goat	1/500	Citrate-Tween	Y
TH	Chemicon	AB152	Rabbit	1/2000	Citrate-Tween	Y
20αHSD	Beuschlein lab.	Homemade	Rabbit	1/20000	Citrate-Tween	Y
SOX9	Millipore	AB5535	Rabbit	1/500	Citrate-Tween	Y
SF1	Cosmo Bio	KAL-KO611	Rat	1/100	Tris-EDTA	Y

SI Appendix Table S1. Antibodies and conditions for immunohistochemistry.

	Forward	Reverse
Pde1b-ChIP	CGTGGAATCTTGGGACCACAT	AGGACTTCGTGTGGATGGT
Pde3a-ChIP	ATCCTGCTAGCTGCCAACTTGA	GATCTACGCGAAGTTTCAGCC
Pde7b-ChIP	AGGGAAAACCGGAGACCAATC	CCCTTTGACGTCACTCTCGAA
Prkar1b-ChIP	TGCCCACATTCCCTATCGTTC	CTGGTGAATGTGGCAAGAGGT
Wt1-ChIP	TAGGGCTGCTGTGTGAATGGA	CCAGCTTCCCAAAGCTCAAA
Gata4-ChIP	TTTGCTGCTCTGCCAAGGAT	AGGTCACCTTCTCCTCTACCA
GAPDH-ChIP	TCAGGGCGCGAAAGTAAAGA	GTATTAGGAACAACCCCACGC
Cyp11b1	GCAGAGATGATGCTCCTGCTT	GAGAGGGCAATGTGTCATCAGAA
Akr1b7	ATGGGGAGTAAGAAACAGCTCC	GCAAGTGGACCTCAGTATTCCTCG
Cyp21	GCTGTGGCTTTCCTGCTTCAC	GGCCCAGCTTGAGGTCTAACT
Hsd3b1	CCTACATTCTGAACTGAGCGGCTGC	GGTCTGTCCTTCCCAGTGATTGATAAAC
Dab2	CCTGCATCTTCTGATCCCCAC	CATGTTTCTGGCTGTCTGCTT
Cyp11b2	GTCATCTCTGAGTATGCCAACAGA	GTGAGCTCCATAGAGTT
At1a	TCACCTGCATCATCATCTGG	AGCTGGTAAGAATGATTAGG
At1b	TCACCTGCATCATCATCTGG	AGCTGGTGAGAATAATAACG
Lef1	GACGAGCACTTTTCTCCGGG	TGGGGTGATCTGTCCAACGC
Axin2	GCCGATTGCTGAGAGGAACTG	AAAGTTTTGGTATCCTTCAGGTTCAT
Apcdd1	TGGCACGGAGTTTGTGTTCA	CTCAGCCCCACACTCATTCC
Prkar1b	GAAGTCTACACTGAAGAAGATGCTGTCTC	GATGTGAGTGACAGGAAACATGGCGT
Pde1b	CCAGAGATGCTGGAGTCGGATTGC	CATTCTCCAACTGCTTCACCATGTAGCG
Pde3a	GAACTATACCTGCTCGGACTCTGAGG	GTTGTCCAAGTTGACGAGACTCTTCTC
Pde7b	CCAGAGATGATCAGGATGGTAAAGCTG	GTAGACGGAAGTCAATGAATGGGTAGG
Cyp11b2	GTCATCTCTGAGTATGCCAACAGA	GTGAGCTCCATAGAGTT
Shh	GCGGCAGATATGAAGGGAAGATC	GTTCATCACAGAGATGGCCAAGGC
Gli1	CCTGGTGGCTTTCATCAACTCTCG	CACAGGGCTGGACTCCATAGG
Ptch1	TCAGGCAATACGAAGCACAGC	GACAAGGAGCCAGAGTCCAG
Wt1	CGGCGCAGTTCCCCAACCATTC	GCACGGAGTACTGCTGCTCGC
Gata4	AACCAGAAAACGGAAGCCCA	TTGATGGGGCGCATCTCTTC
Pdgfra	TGGCATGATGGTCGATTCTA	CTCGCTGAGGTGGTAGAAGG
Foxl2	TCCGGCATCTACCAGTACATCA	TATTCTGCCAGCCCTTCTTGTT
Lhr	GCCATGCATTCAATGGGACG	GGCCTGCAATTTGGTGGAAG
Ki67	TAGAGGATCTGCCTGGCTTC	TGTCCTTGGTTGGTTCCTCC
Mc2r	CAAACACCACCCCCGTCTTA	TCTTGCGGTGTCATTGGTGT
Mrap	CAGAAGCCCTACAGGGGAAC	AGAATCACCCGGCTTGTCTG
Th	CCGTCTCAGAGCAGGATACC	CAATGGGTTCCCAGGTTCCG
Pnmt	TAGCTGTCGCCTTGGCTTAC	ATCAATGAGAACCCGTCCCG
Akr1c18	GGCTTTGGCACCTATGCAAC	TGGGTCTGACCAACTCTGGA
Pik3c2g	CCATTTGTGGACCCAGGTGA	GGGTCAGTGCATTTTGGAACA

SI Appendix Table S2. Primers used for RTqPCR and ChIP qPCR.

SI Materials and Methods

Mice

All experiments with mice were approved by Auvergne Ethics committee (CEMEAA). Ezh2 Fl/Fl mice were obtained from the MMRRC repository (strain #015499_UNC) and were initially generated by Dr Tarakhovsky (2). Sf1:Cre mice were generated by Bingham et al. (3). mTmG mice were generated by Muzumdar et al. (4). Data on adult mice from Figure 1 to Figure 5 were generated on tissues from 2 month-old females. Data for Figure S7 and S8 and Supplementary Figure 6 were generated on tissues from 2 month-old males. For embryonic analyses, embryos were used irrespective of sex. The morning of the vaginal plug was considered as E0.5. Control animals were littermates of *Sf1:Cre,Ezh2 Fl/Fl* mice. All mice were maintained on a mixed background mostly composed of C57BL/6J and 129. There was no randomization of samples nor blinding of the investigator. At the end of experimental procedures, mice were euthanized by decapitation and blood was collected in vacuum blood collection tubes (VF-053STK, Terumo). Adrenals were either frozen in liquid nitrogen or fixed in 4% PFA. Total mRNAs were extracted using RNAII nucleotide extraction kit (Macherey Nagel) according to manufacturer's instructions. ΔCat (5), AdIgf2 and ΔCat;AdIgf2 (6, 7) mice were previously described. Ten to twelve month-old adrenals from these models were used in our experiments.

Immunohistochemistry

Immunohistochemistry was performed on tissues embedded in paraffin, after unmasking by boiling for 20 min in sodium citrate 10 mM, Tween 0.05%; Tris 10mM, EDTA 1mM, pH 9.0; or Vector Unmasking Solution (H3300, Vector Laboratories), depending on the primary antibody and/or combinations of antibodies to be used. For CYP11B2, unmasking with Tris-EDTA was followed by 5 min incubation in 10% SDS. After blocking for 1h, slides were incubated overnight at room temperature, with primary antibodies at the indicated concentrations (Supplementary table 1). Primary antibodies were detected with appropriate polymers (ImmPress Polymer Detection Kit, Vector Laboratories). Polymer-coupled HRP activity was then detected with either Vectastain ABC (PK-4000, Vector Laboratories) for brightfield images or TSA-Alexa-coupled fluorochromes for fluorescence (Invitrogen). All immunohistochemical analyses were conducted on an automated processor (Intavis InSitu Pro VSi) to ensure homogeneity and reproducibility of detections. Images were acquired with a Zeiss Axioplan 2, Zeiss AxioImager with Apotome2 or Zeiss Axioscan Z1 slide scanner. They were minimally processed for global levels and white balance using Affinity Photo®. Image settings and processing were identical across genotypes.

RNAScope™ analysis

RNA in situ hybridisation to detect *Shh* and *Gli1* expression was conducted on 5 µm paraffin sections using RNAScope probes for *Shh* (#314361) and *Gli1* (#311001) with the RNAScope 2.5HD detection reagent-Red system (#322360, Adanced Cell Diagnostics), following manufacturer's instructions.

ChIP sequencing analysis

Publically available ChIP sequencing data were retrieved by searching for a combination of "EZH2", "H3K27me3" and "ChIP" on the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.gov/geo). We selected datasets for which .fastq, peaks.bed and input data were available for download. Mapping of ChIP seq reads was performed with Bowtie2 (version 2.3.0) on the hg38 Human reference genome. Output files were converted to .bam format using

SAMtools (version 1.3.1). ChIP enrichment for each of the marks was determined using MACS2 (version 2.1.1), using a qvalue (minimal FDR) of 0.05. Peaks annotation was performed using the ChIPseeker package (version 1.12.1) under R (version 3.4.3). To detect potential target genes, peaks were annotated using the "TxDb.Hsapiens.UCSC.hg38.knownGene" package, using a -2/+2kb window surrounding the transcription start site.

Micro-array analyses

Adrenal gene expression profiles for three 2 month-old female wild-type and four Ezh2 KO littermates were analyzed using Affymetrix Mouse Gene 2.0 ST Arrays (Raw and processed data are deposited on NCBI GEO platform). Gene expression was normalised by RMA (Affy R package) and genotype comparisons were performed using the Limma package. All p-values were adjusted by the Benjamini-Hochberg correction method (Supplementary Dataset 1). Heatmaps were generated with R and represent colour-coded individual median centered gene expression levels (in the Log2 space) in 3 WT and 4 knockout adrenals. Genes were ordered according to the mean Fold change (Log2) in knockout versus WT. Genes with adjusted p value (FDR) <0.1 and Abs(Log 2 fold change) > 1.0 were considered significantly deregulated.

Gene set enrichment analyses

Gene set enrichment analysis were conducted using GSEA 2.2.1 (8) with custom curated gene sets (Supplementary Datdaset S1). Permutations were set to 1000 and were performed on gene sets. PRC2_CHENG_PLC5_mmES, PRC2_CHENG_PLC5_hsES, PRC2_CHENG_hsES_HUH7, and PRC2_CHENG_HUH7_mmES were extracted from Cheng et al. (9). They represent genes with binding of EZH2, SUZ12 and H3K27me3 in both PLC5 and mouse ES cells (PRC2_CHENG_PLC5_mmES), in both PLC5 and human ES cells (PRC2_CHENG_PLC5_hsES), in both HUH7 and human ES cells (PRC2_CHENG_hsES_HUH7) and in both HUH7 and mouse ES cells (PRC2_CHENG_HUH7_mmES). PRC2_LEE represents genes with binding of SUZ12, EED and H3K27me3 in human ES cells and was extracted from Lee et al. (10). PRC2_VELICHTUNA_hsES_CB_K27_EZH2 represents genes with binding of EZH2 and H3K27me3 in both human ES cells and germinal center B cells and was extracted from Velichtuna et al. (11). PRC2_BRACKEN_SHORT represents genes presenting PRC2 binding and showing increased expression after Polycomb depletion. It was extracted from Bracken et al. (12). Adrenal_progenitors gene set was curated from Pubmed searches on regulators of adrenal progenitors homeostasis. Zf_Nishimoto and Zg_Nishimoto correspond to genes that are expressed in laser-captured rat zona fasciculata and zona glomerulosa respectively (13). WNT_pathway corresponds to WNT pathway regulators and targets extracted from Drelon et al. (1). Steroidogenis represents key players in adrenal steroidogenesis. Cholesterol_synthesis represents key players in de novo cholesterol synthesis through the mevalonate pathway.

Hormonal measurements

Plasma corticosterone (AR E-8100, LDN) and aldosterone (CAN-ALD-450, Diagnostics Biochem Canada) concentrations were determined using commercially available ELISA kits, following manufacturer's instructions. Renin activity rate in plasma was determined using a Fluorimetric Sensolyte 520 Mouse Renin Assay Kit (AnaSpec Inc). ACTH, LH and FSH concentrations were determined using a multiplex assay (MPTMAG-49K, Merck Millipore). For 9 AM sampling, blood was taken by decapitation. For kinetic evaluation of corticosterone concentrations, blood was drawn from a small incision of the tip of the tail at 06:00, 12:00, 18:00 and 24:00 hours. Animals were maintained in a 12h light (07:00-19:00) / 12h dark cycle. Catecholamines concentrations

were evaluated in urines collected for 24h using metabolic cages. Urines were stabilised by addition of 5 µl 6N HCl to collection tubes. The extraction procedure was based on the previously validated method of Cai et al (14). Six calibration curve points were constructed in the concentration ranges and four different quality controls. The analytical samples were prepared by adding 20µL of IS to 100µL of biological sample. Sample solutions (standards, controls and samples) were derived by 50µL of dansyl chloride at pH 11 with carbonate buffer. After 15 min at 50°C, samples were centrifuged and the upper phase was transferred to an analytical vial. Liquid chromatography and high-resolution mass spectrometric detection methods were used to simultaneously quantify catecholamines. 10µL of standard, control or sample preparation were injected into liquid chromatography systems (transcends TLX2, Thermo Fisher Scientific). On-line chromatography purification was carried out using a turbulent flow column (C2XL cyclone 0.5 x 50 mm) at 1.5 ml/min between 30 seconds. Elution step used the mobile phase of chromatographic elution: acetonitrile/water (0.1% formic acid). Chromatographic separation was performed using a Hypersil GOLD C18 column (50 x 2.1 mm, 1.9 µm) (Thermo Fisher Scientific) in reverse phase at 30°C. A gradient system usesd a mobile phase with a solvent A (0.1%; v/v; formic acid in water) and a solvent B (0.1%; v/v; formic acid in acetonitrile) at a rate of 500µL/min. Run time was set to 13.50min. The autosampler was maintained at 4°C. On-line LC/HRMS analyses were performed on a QExactive Plus mass spectrometer equipped with an HESI II atmospheric ionization source operated in electrospray mode, and an orbitrap® technology detection (Thermo Fisher Scientific). MS experiments were performed with a full scan mass acquisition (m/z 400 to 900) at 70,000FWH resolution. A parallel reaction monitoring was performed similarly, to generate specific m/z fragments for analyte confirmation (resolution at 17,500FWH). Dopamine, epinephrine and norepinephrine were monitored at m/z 853.23940, 883.24996 and 869.23431 respectively. Lower limits of quantification (LLOQ) for each compound were 30 nmol/L for dopamine and 5 nmol/L for epinephrine and norepinephrine. Upper limit of quantification (ULOQ) were 1500 nmol/L, 600 nmol/L and 800nmol/L in urine for dopamine, epinephrine and norepinephrine respectively. The concentration of catecholamine in urine samples, were determined by their area ratios to that of the IS using a weight quadratic fit. All absolute urinary concentrations were adjusted to creatinine measurement to limit fluctuations between samples from different animals.

RTqPCR

One microgram of total mRNAs (from tissues or cell culture) was reverse transcribed for 1 hour at 37°C with 5 pmoles of random hexamers primers, 200 units reverse transcriptase (M-MLV RT, M1701, Promega), 2 mM dNTPs and 20 units RNAsin (N2615, Promega). One microliter of a one-tenth dilution of cDNA was used in each quantitative PCR. PCR reactions were conducted with SYBR qPCR Premix Ex Taq II Tli RNase H+ (TAKRR820W, Takara). Primer pairs are listed in Supplementary table 2. For each experiment and primer pairs, efficiency of PCR reactions was evaluated by amplification of serial dilutions of a mix of cDNAs. Relative gene expression was obtained by the $\Delta\Delta Ct$ method with normalization to average expression of three housekeeping genes, *36b4, Ppib and Actin*.

Western Blot

Twenty micrograms of total proteins were loaded on 10% SDS-PAGE gel, transferred onto nitrocellulose and detected with either total CREB (1/1000, #9197, Cell Signaling) or Phospho-Serine133-CREB (1/1000, #9198, Cell Signaling) antibodies in 5% BSA. Signals were quantified with

a DNR MF ChemiBis 3.2 camera system and Multi Gauge software suite (Fujifilm). Expression of the phospho-protein was normalized to expression of the corresponding total protein.

PKA Activity

PKA activity was measured using PKA Kinase Activity Assay Kit (ab139435, Abcam), following manufacturer's instructions. It was measured on 10µg of protein extracts from pairs of adrenals from four 2 month-old female wild-type and four Ezh2 KO adrenals.

Chromatin Immunoprecipitation qPCR

H3K27me3 and EZH2 chromatin immunoprecipitation (ChIP) were performed using the iDeal ChIP-seq kit for histones (Diagenode) according to manufacturer's instructions, using the following antibodies: C15410195 (rabbit polyclonal, Diagenode) for H3K27me3 and C15410039 (rabbit polyclonal, Diagenode) for EZH2. Briefly, 20 to 35 mg of tissue from 2 month-old female WT and Ezh2 KO mice (pools of 6 adrenals from 3 mice, in triplicate) were first grinded using a dounce homogenizer and then cross-linked with 1% formaldehyde for 10 min. Cross-linking was stopped by adding glycine (125mM final) for 5 min at room temperature. Lysis buffer was then added and chromatin was sheared using a BioruptorPico (Diagenode) to obtain fragments of ~300 bp in average. Two percent of chromatin used for IPs was used as input control. IPs were conducted overnight at 4 °C on a rotating wheel. Two micrograms of antibody were used for H3K27me3 and 3µg for EZH2. After 4 successive washing, elution of chromatin was performed for 30 min at room temperature. The reversion of the cross-linking was performed by incubating chromatin 4 h at 65 °C with proteinase K. The Ipure kit (Diagenode) was used to precipitate and purify the DNA. qPCR were performed to validate ChIP for each antibody on specific genomic regions using SensiMix™ SYBR® (Bioline). 5µl of input or precipitated chromatin were used for the qPCR in the presence of 250pM primers (Supplementary table 2). The results were normalised as percentage of input (%(meIP/Input) = 2*((Ct(2%input) - 5.643856) - Ct(IP))*100) and are expressed as relative enrichment of the same marks on the regulatory regions of the housekeeping gene GAPDH.

Statistical analyses

Minimal sample size was set at n=5 allowing for detection of 40% increases/decreases with α=0.05, 1-β=80%, σ=0.3 and mean of control group=1.0. Statistical analyses were conducted with R and GraphPad Prism 7. Normality of data was assessed using D'Agostino & Pearson normality test. Statistical analysis of normally distributed data was performed by two-tailed Student's t test (two groups) with or without Welch's correction (as a function of variance) or one-way ANOVA (multiple groups), followed by Tukey's multiple comparisons test. Analysis of non-normally distributed data was performed by two-tailed Mann & Whitney test (two groups) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (multiple groups). All bars represent the mean ± SEM.

Data Availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. Microarray data are deposited on Gene Expression Omnibus repository with reference GSE109578.

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