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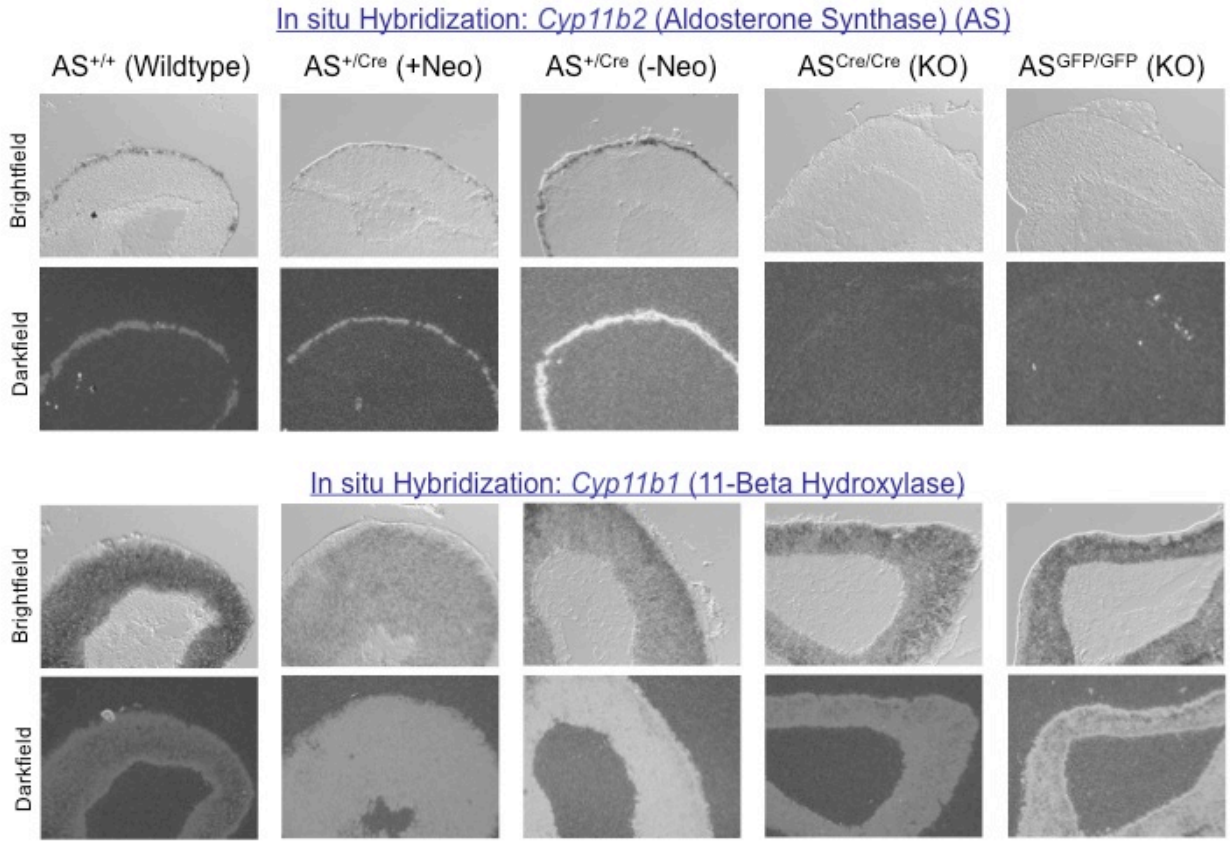
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AS+/+ (Wildtype)
AS+/Cre (+Neo)
AS+/Cre (-Neo)
ASCre/Cre (KO)
ASGFP/GFP (KO)
Brightfield
Darkfield
In situ Hybridization: Cyp11b1 (11-Beta Hydroxylase)
Brightfield
Darkfield

B

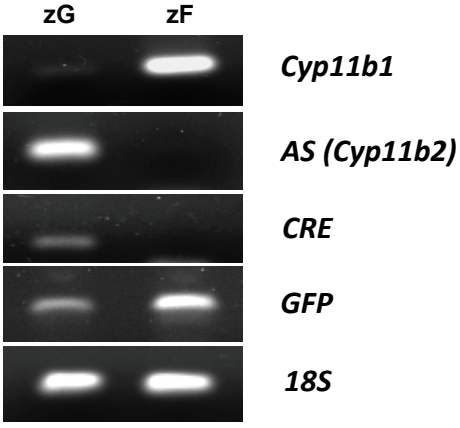
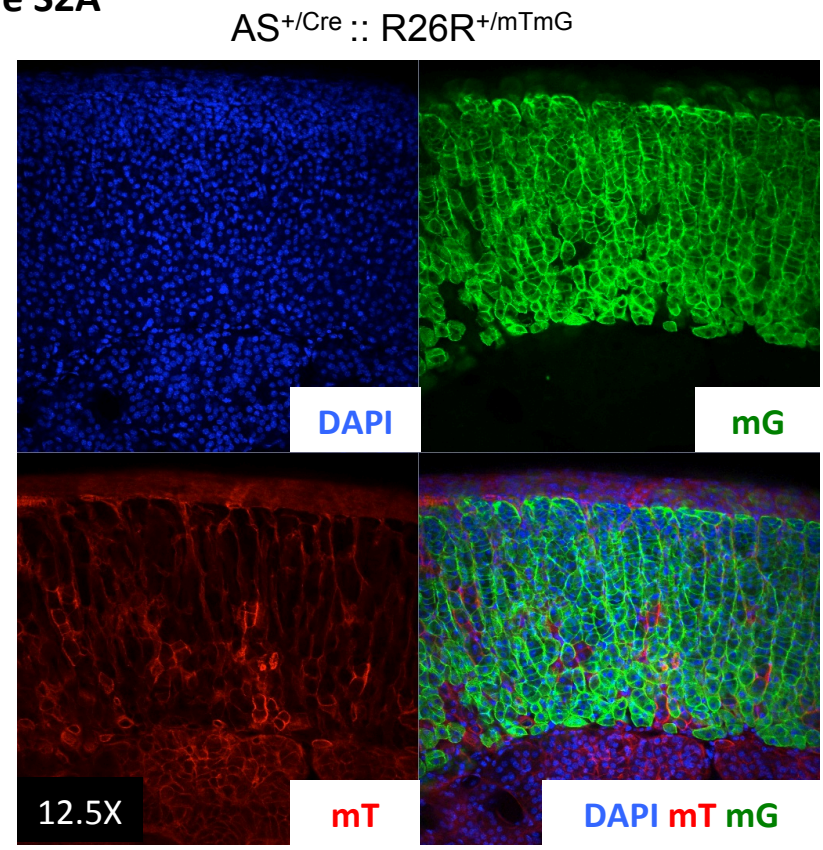

zG
zF
Cyp11b1
AS (Cyp11b2)
CRE
GFP
18S

Figure S2A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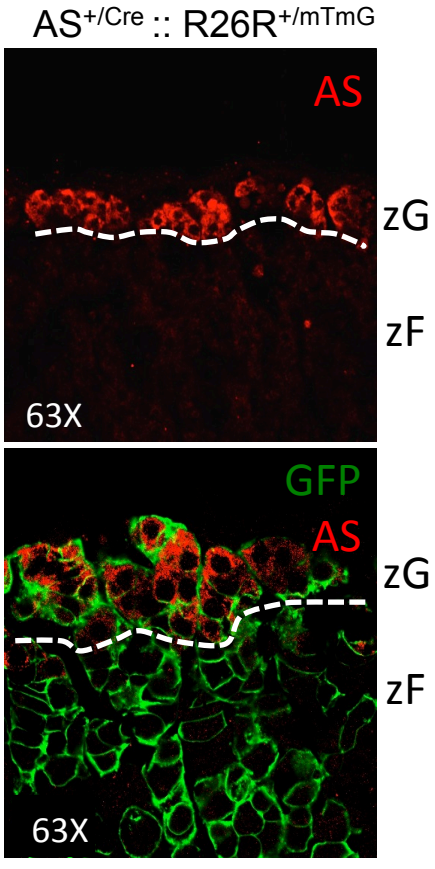
DAPI
mG
12.5X
mT
DAPI mT mG

B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zF
63X
GFP
AS
zG
zF
63X

Figure S3

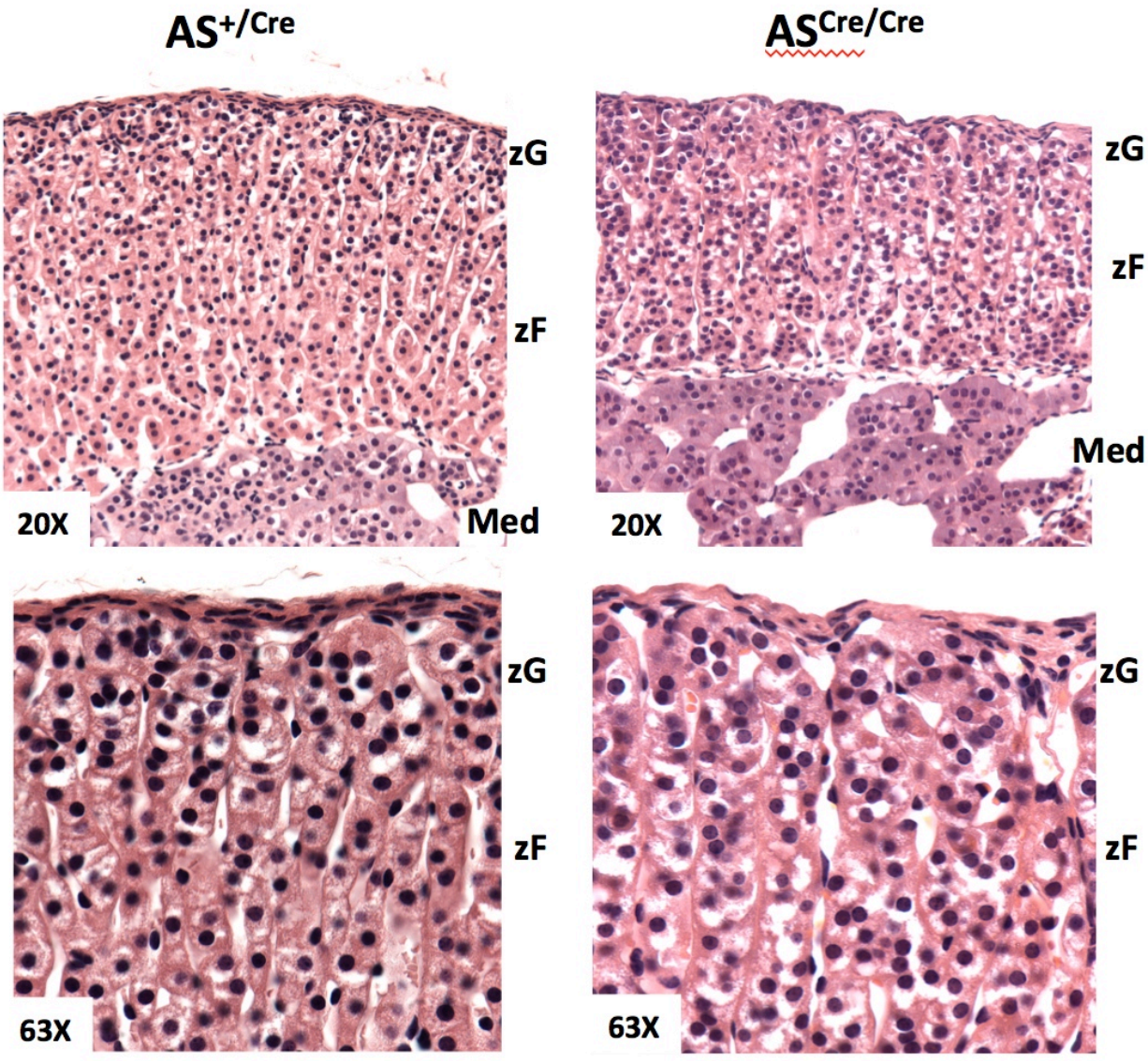
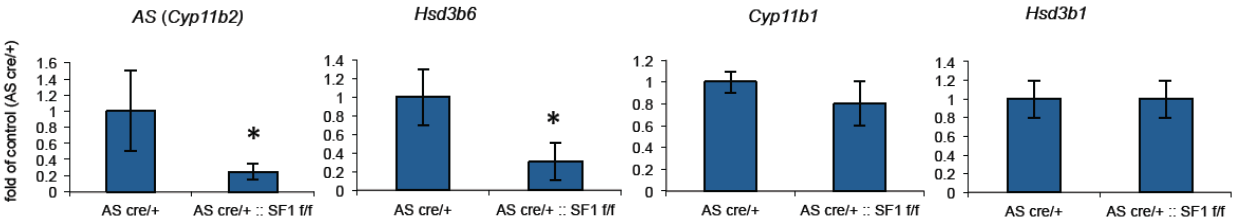


Figure S4A



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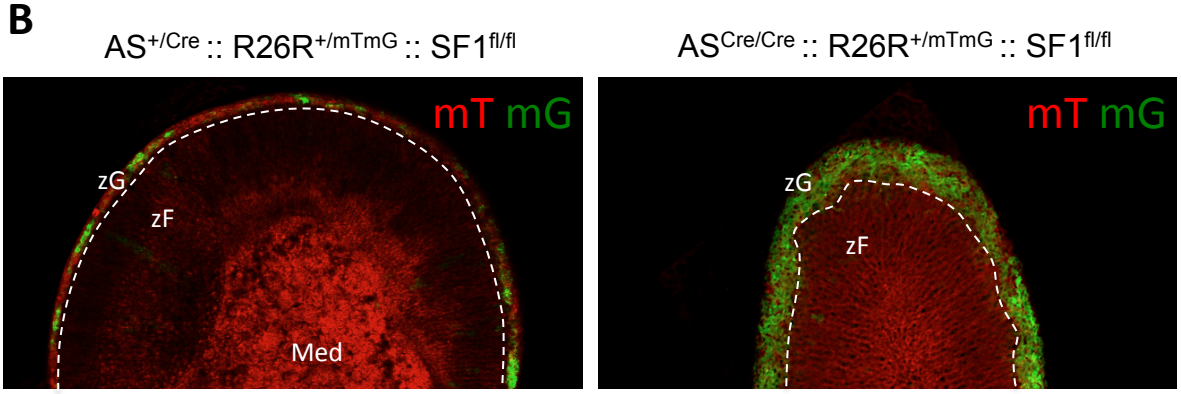

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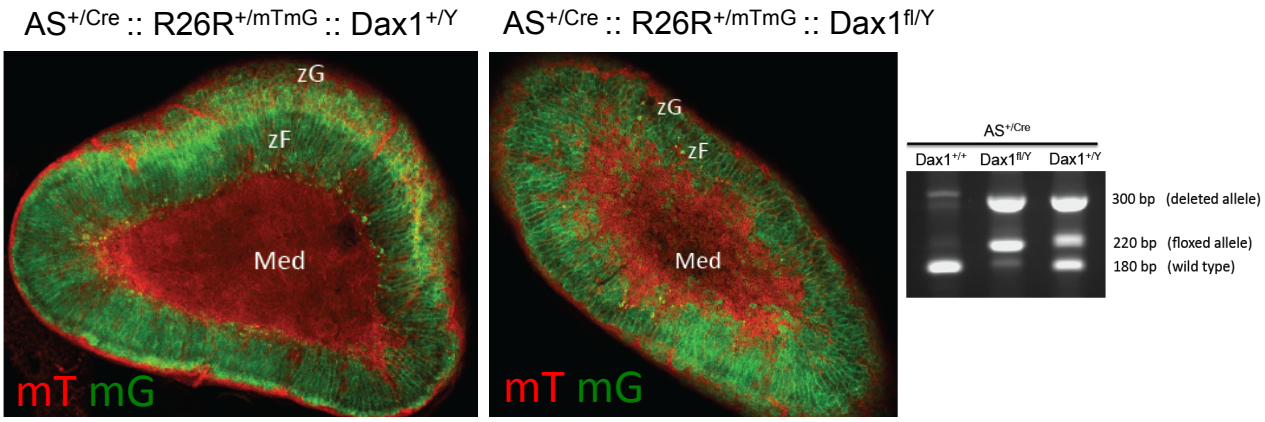

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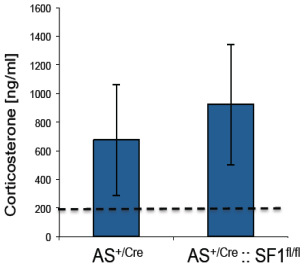

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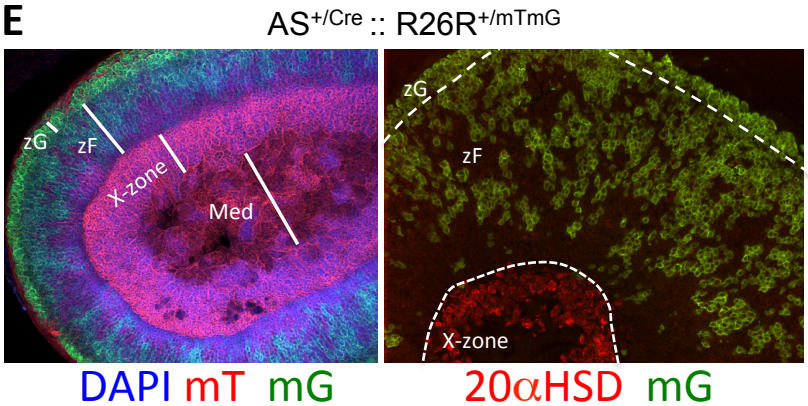

Table S1: Primer sequences and PCR conditions used for genotyping of mouse strains.

GENE /ALLELE	PRIMERS	PCR PRODUCTS
AS-CRE Tm = 65°C	Forward: GAGCTGGGGCCCATTTTCAGG	WT: 525 bp AS-CRE: 640 bp
	Reverse 1:GCTCCAGGTGCATCCGACGG	
	Reverse 2: AACTTGCACCATGCCGCCCA	
GFP Tm = 60°C	Forward: CACATGAAGCAGCACGACTT	300 bp
	Reverse: AGTTCACCTTGATGCCGTTC	
DAX-1 Tm = 56°C	Forward: CCTAGAAGTGTTGCTTCTG	WT: 180bp Floxed: 200 bp Deleted: 300 bp
	Reverse 1: GCACATTGTTCTGAGTGGCT	
	Reverse 2: ACAGCTCACCACAGGATCTT	
SF-1 Tm = 65°C	Forward: ACACCACACTAGCAATACATCTGC	WT: 160 bp Floxed: 200 bp
	Reverse: ATCCCTGTCTATCAGTATGGCCG	

Table S2: Primer sequences used for SYBR Green quantitative-PCR based gene expression analysis.

GENE	PRIMER SEQUENCE	REFERENCE
CYP11B1 11β hydroxylase	Fw: 5' CAGGAGCCTGACCCGATGGACA	This paper
	Rv: 5'GACTGTGGACGACCCTCTGCCA	
CYP11B2 / AS Aldosterone synthase	Fw: 5'CGTCGGATGCACCTGGAGCC	This paper
	Rv: 5'GCCGCAGTCGGTTGAGACGC	
HSD3B6 3β hydroxysteroid dehydrogenase typeVI	Fw: 5'CATCCTTCCACAGTTCTAGC	[Doi M, 2009, Nature Med]
	Rv: 5'TGGTGTGAGATTATTGTACA	
HSD3B1 3β hydroxysteroid dehydrogenase type I	Fw: 5'AGCATCCAGACACTCTCATC	[Doi M, 2009, Nature Med]
	Rv: 5'GGAGCTGGTATGATATAGGGTA	
18S	Fw: 5'CTCAACACGGGAAACCTCAC	This paper
	Rv: 5'AGACAAATCGCTCCACCAAC	

Supplemental Figure Legends

Figure S1, related to Figure 1. (A) *In situ* hybridization for *AS* (upper panel) and *Cyp11b1* (lower panel) expression in $AS^{+/+}$, $AS^{+/Cre}$ (+neo), $AS^{+/Cre}$ (-neo), $AS^{Cre/Cre}$ and $AS^{GFP/GFP}$ (Lee et al., 2005) mice. *AS* gene expression is detected in subcapsular region of $AS^{+/+}$ and mice heterozygous for the *Cre* allele. In contrast, AS expression is completely lost in $AS^{Cre/Cre}$ and $AS^{GFP/GFP}$ mice. Analysis of Cyp11b1 expression was comparable between all mice, indicating this allele was not disrupted in $AS^{+/Cre}$ mice.

(B) Confocal analysis of $AS^{+/Cre}::R26R^{+/mTmG}$ fetal adrenal at the day e16.5 showing occasional single (or rare doublet) GFP^+ cells in the subcapsular region.

(C) RNA was isolated from laser capture micro-dissections of zG and zF regions from $AS^{+/Cre}::R26R^{+/mTmG}$ mice, and expression of specific markers analyzed by qRT-PCR (as in Figure 1E) or semi-quantitative RT-PCR (shown here). PCR products were run on the gel and visualized by ethidium bromide staining. The zG-specific marker *AS* is expressed only in the zG along with *Cre*. The zF-specific marker *Cyp11b1* is expressed predominantly in the zF. *GFP* expression is detected in both zones as expected from the lineage tracing studies (Figure 2).

(D) FACS analysis of $AS^{+/Cre}\,R26R^{+/mTmG}$ mouse adrenals at different ages demonstrating increased GFP^+ cells. $AS^{+/+}\,R26R^{+/mTmG}$ mice containing only mT-labeled cells were used as a wild type controls. Quantification reveals an increase in GFP^+ cells. qRT-PCR analysis on sorted GFP^+ cells confirmed both *Cyp11b1* and *AS* gene expression in cells derived from 14 weeks old animals, Mean±SEM.

Figure S2, related to Figure 2. (A) Split view of confocal images from a 12 week old adrenal (see Figure 2F) from an $AS^{+/Cre}$ :: $R26R^{+/mTmG}$ mouse showing near complete replacement of the red fluorescent Tomato (mT) with GFP (mG) in the adrenal cortex, but not in capsule or adrenal medulla.

(B) Co-immunofluorescent analysis of endogenous AS and GFP expression in $AS^{+/Cre}::R26R^{+/mTmG}$ mice confirms co-expression in the zG region, but not in the zF region.

(C) Co-immunostaining for anti-CYP11B1 and GFP in the adrenal cortex of $AS^{+/Cre}::R26R^{+/mTmG}$ mouse. Arrows point to GFP cells co-expressing CYP11B1.

(D) Co-immunofluorescent analysis of $AS^{+/Cre}$ :: $Sf\text{-}1^{+/+}$ mice with anti-CYP11B1 and anti-AS (CYP11B2) antibodies in the adult adrenal cortex. Arrows indicate occasional cells present at the boundary of the two zones co-expressing both markers.

(E) Co-immunofluorescent analysis of $AS^{+/Cre}$ :: $Sf\text{-}1^{fl/fl}$ mice with anti-CYP11B1 and anti-AS (CYP11B2) in the adult adrenal cortex.

Figure S3, related to Figure 3. Histological analysis (H&E) of $AS^{+/Cre}$ and $AS^{Cre/Cre}$ mice shows a disorganized and thickened zG region in $AS^{Cre/Cre}$ knockout mice. Note the adrenal cortex in $AS^{Cre/Cre}$ mice is also generally smaller, as previously described (Lee et al., 2005).

Figure S4, related to Figure 4. (A) Expression of zone specific steroidogenic genes in 8 week old $AS^{+/Cre}$ and $AS^{+/Cre}$::$Sf\text{-}1^{fl/fl}$ whole adrenal glands. Expression levels of zG markers, *AS* (*Cyp11b2)* and *Hsd3b6* (3β-hydroxysteroid dehydrogenase type 6, shown to be specifically expressed in murine zG (Doi et al., 2010)) were significantly decreased in zG-specific *Sf-1* knock out mice, suggesting partial ablation of the zG whereas no change in expression of zF markers *(Cyp11b1* and *Hsd3b1*) was detected (Mean±SEM, * $p < 0.05$). (B) To further establish that conversion of zG cells into zF cells is SF-1 dependent, we generated $AS^{Cre/Cre}$::$R26R^{+/mTmG}$::$Sf\text{-}1^{fl/fl}$ mice (right panel) to accelerate lineage marking. Similar to $AS^{+/Cre}$::$R26R^{+/mTmG}$::$Sf\text{-}1^{fl/fl}$ mice (left panel), confocal analysis reveals *Sf-1* deficient zG cells are unable to contribute to zF cells.

(C) Measurement of random plasma corticosterone levels from $AS^{+/Cre}$ and $AS^{+/Cre}$::$Sf\text{-}1^{fl/fl}$ mice reveals no difference in glucocorticoid synthetic capacity. These results show no evidence for adrenocortical insufficiency after zG-specific ablation of *Sf-1*. Analysis of 10 animals per group. Adrenal sufficiency defined as ≥200 ng/ml, Mean±SEM.

(D) Quantification of GFP-positive cells co-expressing AS in the subcapsular region of $AS^{+/Cre}$::$R26R^{+/mTmG}$ and $AS^{+/Cre}$::$R26R^{+/mTmG}$::$Sf\text{-}1^{fl/fl}$ animals.

(E) Quantification of GFP-positive cells co-expressing SF-1 in the subcapsular region of $AS^{+/Cre}::R26R^{+/mTmG}$ and $AS^{+/Cre}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animals.

(F) Immunofluorescent analysis showing occasional GFP-positive cells expressing SF-1 in the adrenal cortex of a 13 week old $AS^{+/Cre}::R26R^{+/mTmG}$ and $AS^{+/Cr}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animal.

(G) Co-immunostaining of SF1 and GFP in $AS^{Cre/Cre}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animals showing a large number of accumulated GFP^+ cells devoid of SF-1 expression in the subcapsular region. Very few $SF\text{-}1^+$ cells are detected in the subcapsular region. Note that the cells of zF region still express SF-1. (H) Expression of Shh pathway genes and genes involved in the Wnt/β-catenin pathway in 8 week old $AS^{+/Cre}$ and $AS^{+/Cre}::Sf\text{-}1^{fl/fl}$ whole adrenal glands. Expression levels of Shh, a marker for adrenal progenitor cells, is significantly decreased, whereas expression of Gli1, a marker for capsular progenitor cells is increased zG-*specific Sf-1* knockout adrenals. Similarly, expression of the secreted WNT inhibitors (SFRP2 and SFRP3) are increased, whereas APCDD1 (a target of Wnt signaling) is decreased in zG-specific *Sf-1*-deficient adrenals (n= 4-5 samples per group, * $p< 0.05$); ** $p< 0.01$); *** $p< 0.001$).

(I) zG-specific ablation of nuclear receptor DAX-1 does not impact zG-dependent lineage development in the adult adrenal cortex. Confocal analysis of adrenal glands from $AS^{+/Cre}::R26R^{+/mTmG}::Dax\text{-}1^{+/Y}$ (left panel) and $AS^{+/Cre}::R26R^{+/mTmG}::Dax\text{-}1^{fl/Y}$ (middle panel) 28 week old mice. No difference in lineage conversion is evident. PCR analysis of genomic DNA from whole adrenals (right panel) (according to the method of Yu and Jameson (Yu et al., 1998)) confirms genetic deletion of the *Dax-1* allele in $AS^{+/Cre}::Dax\text{-}1^{fl/Y}$ males and $AS^{+/Cre}::Dax\text{-}1^{fl/+}$ *females* (heterozygotes). The smaller bands, 180 bp and 220 bp, correspond to the wild type and floxed alleles, respectively. Deletion of the floxed exon 2 is detected as a 300 bp PCR product.

(J) zG-dependent lineage conversion is restricted to the zF and does not contribute to the X-zone in adrenals of $AS^{+/Cre}::R26R^{+/mTmG}$ female mice. The X-zone is evident between the zF and the adrenal medulla and demonstrates bright pink endogenous fluorescence (upper panel). Co-immunofluorescent analysis of a paraffin embedded adrenal from $AS^{+/Cre}::R26R^{+/mTmG}$ female mouse stained for GFP and

20alpha-HSD, an X-zone specific marker (Hershkovitz et al., 2007), reveals no co-localization (lower panel).

Table S1, related to Figures S1B, S4D. Primer sequences and PCR conditions used for genotyping of mouse strains.

Table S2, related to Figures 1E, S4A. Primer sequences used for SYBR Green quantitative-PCR based gene expression analysis.

Supplemental Experimental Procedures

Mouse Experiments

All animal procedures were approved by the Children's Hospital Institutional Animal Care and Use Committee.

Generation and Validation of $AS^{+/Cre}$ mice

Targeting arms for homologous recombination were obtained by screening a Lambda FIX II genomic library (Stratagene), generated from sv129 DNA, using a previously described AS-specific probe (Domalik et al., 1991). Two clones, covering the 5' and 3' portions of the *AS* gene, were subcloned into Bluescript-SK, restriction mapped and partially sequenced to confirm their identify. The knock-in targeting construct (Figure 1) includes the following additional key components: Cre recombinase (provided by Dr. K. Rajewsky, Harvard Medical School), the FTR-flanked neomycin resistance gene (provided by Dr. F. Alt, Harvard Medical School) and the gene for diphtheria toxin subunit A (DTA) (provided by F. Gertler, MIT). Modifications to the endogenous *AS* locus were designed to prevent translational initiation from the endogenous ATG. We chose to include as much endogenous regulatory sequence as possible within the final targeted allele to minimize the risk of creating a hypomorphic *AS* allele. The entire coding sequence of the gene was knocked out, retaining only exon 1, intron 1 and the

first portion of exon 2. All potential translation start sites (ATG) within exon 1 or exon 2 were excluded from the construct or mutated. The final targeting construct was validated using extensive restriction digests along with sequencing of all critical regions including the proximal promoter, exon 1, intron 1 and exon 2. J1 embryonic stem cells were transfected with the targeting construct by electroporation and plated on a feeder layer of embryonic fibroblasts. Three hundred and eighty four clones were screened by Southern blot analysis.

Southern blots were initially screened with a probe derived from sequences external to the 3' arm targeting. This probe was used to confirm site-specific integration of the targeting allele. Two additional probes were derived from sequences internal to the targeting region and used to further verify proper integration of the two targeting arms. In addition, inclusion of the mutated translation start sites within the recombined allele was verified to exclude the possibility that homologous recombination occurred within the first intron leading to exclusion of these mutated sequences. Southern blot analysis was also performed to confirm that *Cyp11b1*, which is highly homologous to the targeted locus, remained unaffected during gene targeting. *Aldosterone synthase* and *Cyp11b1* are organized in a head-to-tail orientation with approximately 6kb of intervening sequence. Finally, Southern blot screening to confirm proper recombination of the 3' targeting arm involved a Bcl1 restriction cut and use of a 3' probe (Figure 1B, C). The expected bands were 7.7 kb (wild-type) and 5.0 kb (recombined) (Figure 1C).

Two properly targeted ES cell clones were expanded for electroporation with Flp recombinase (pOG-Flpe6 was provided by Dr. S. Dymecki, Harvard Medical School) in order to delete the FRT-flanked positive selection marker. Individual colonies were selected and removal of the Neo^R gene (Figure 1B, C) was confirmed by Southern blot analysis. Two ES cell clones were expanded for karyotype analysis and then injected into blastocysts in the Boston Children's Hospital transgenic core facility. Mice were bred to germline heterozygosity and maintained on a mixed sv129-C57Bl/6 genetic background. To control for genetic background, experiments were performed using littermates when possible.

Generation of $AS^{+/Cre}$ :: $ROSA^{+/mTmG}$ mice

$AS^{+/Cre}$ mice were crossed with the *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (mT/mG) reporter line (Muzumdar et al., 2007) to generate $AS^{+/Cre}$*::R26R*$^{+/mTmG}$ mice. This reporter mouse expresses a floxed membrane bound modified red fluorescent protein (Tomato) (mT) upstream of a membrane bound enhanced green fluorescent protein (eGFP) (mG) at the endogenous *ROSA* locus ($R26R^{+/mTmG}$). These mice are useful as a Cre-reporter as they express red fluorescence prior to, and green fluorescence following Cre-mediated recombination (Figure 2A). The double-fluorescent system allows visualization of both recombined and non-recombined cells, and is ideal for lineage tracing experiments. These mice were intercrossed to generate $AS^{Cre/Cre}$*::R26R*$^{+/mTmG}$ mice.

Generation of $AS^{+/Cre}$ :: SF-$1^{fl/fl}$ mice

$AS^{+/Cre}$ mice were crossed with *Sf-*$1^{fl/fl}$ conditional mice (Zhao et al., 2001) generating $AS^{+/Cre}$*::Sf-*$1^{+/fl}$ and then intercrossed to generate $AS^{+/Cre}$*::Sf-*$1^{fl/fl}$ and $AS^{Cre/Cre}$*::Sf-*$1^{fl/fl}$ mice. These mice were also crossed with $R26R^{+/mTmG}$ mice to generate trigenic mice.

Generation of $AS^{+/Cre}$ :: DAX-$1^{fl/fl}$ mice

$AS^{+/Cre}$ mice were crossed with DAX-$1^{fl/fl}$ conditional mice (Yu et al., 1998) and then intercrossed to generate $AS^{+/Cre}$ :: $Dax1^{+/Y}$ and $AS^{+/Cre}$ :: $Dax1^{fl/Y}$. These mice were also crossed with $ROSA^{+/mTmG}$ mice to generate trigenic mice.

Histology

For immunohistochemical or immunofluorescent analysis adrenal were fixed in 4% paraformaldehyde for 1 hour at 4°C and either embedded in paraffin (cut as 6 μM sections) or embedded in OCT (cut as 10 μM sections). Paraffin sections were deparaffinized, antigen retrieval performed by boiling in 0.01M sodium citrate, endogenous peroxidase activity blocked by 0.3% hydrogen peroxide and endogenous biotin

background blocked by Biotin-Avidin Blocking Kit (Vector Laboratories). Nonspecific staining was blocked using 10% normal goat serum prior to immunostaining and primary antibodies used in following dilutions: chicken anti-GFP (Aves) 1:1000, rabbit anti-aldosterone synthase (C. Gomez-Sanchez, Lot 2066) 1:500, rabbit anti-SF-1 (a kind gift of R. Auchus and the late K. Parker, Lot 18433) 1:800, rabbit anti-P450scc (Millipore) 1:500, rabbit anti-CRE 1:400 (Novagen), 1:50, mouse anti-CYP11B1 and rabbit anti- 20α HSD 1:1000 (a kind gift of Y. Weinstein). Sections were incubated with primary antibodies overnight at 4°C with the exception of anti-SF1 staining where the sections were incubated overnight at room temperature. As a secondary antibody, goat anti-rabbit 633, goat anti-chicken 488 or goat anti-mouse 594 (all from Molecular Probes) were used at a dilution of 1:800. Sections were counterstained by short incubation with DAPI (1:1000) in PBS and visualized using a Nikon E800 Eclipse, a Zeiss LSM 700 or a Nikon 90i Eclipse. To facilitate the immunofluorescent detection of Cre, mice were water deprived for 48 hours prior to sacrifice.

In situ hybridization (ISH) was performed using 10 µm frozen sections from adult adrenals hybridized with ^{35}S-labeled anti-sense cRNA riboprobes for *AS, Cyp11b1* or sense controls. Anti-sense riboprobes were generated using T3 RNA polymerase and cDNA templates that were reverse transcribed from the 3'-untranslated region of either *Aldosterone Synthase* or *Cyp11b1*. After fixation with 4% paraformaldehyde, acetylation and dehydration, slides were hybridized with the probes at 65°C. The slides were then processed as described previously (Venihaki et al., 2000).

Measurement of Hormonal Levels

Blood samples of animals for hormonal measurements were collected by retro-orbital bleeding in the morning hours using heparin-coated capillaries for corticosterone and aldosterone measurements. For measurement of plasma renin activities blood was collected in non-heparinized capillaries and treated with EDTA. Samples were centrifuged for 15 min at 3000 rpm, plasma fraction collected and kept frozen at -80°C until further processing. Corticosterone levels were analyzed by Cortisterone ^{125}I RIA kit (MP

Biomedicals, LLC, Orangenburg, NY, USA), aldosterone levels were measured by aldosterone RIA (Diagnostics Products Corporation, LA, CA) and plasma renin activity by RIA assay (Diasorin, Stillwater, MN). Aldosterone was also measured in 24-hr urine samples from adult male mice housed in metabolic cages as previously described (Davies et al., 2008; Guagliardo et al., 2012). Briefly, mice were fed a normal Na (NS, 0.3% Na) for one week. Urine was collected daily and analyzed on the last 4 days of each diet. Urinary aldosterone concentration was measured by RIA and standardized to urinary creatinine (colorimetric assay kit, Cayman Chemical Company, Ann Arbor, MI) to control for differences in urine volume and glomerular filtration rate. On the last day, tail vein blood was sampled and plasma renin concentration measured by RIA.

Genotyping

Genomic DNA was isolated by alkaline lysis (0.2 M NaOH) at 95 C for 45 min, followed by neutralization in 20 mM EDTA (final concentration). Sequences of primers and PCR conditions used for genotyping are listed in Table 1. Briefly, aldosterone synthase (*AS*) alleles were genotyped using three primers PCR reactions yielding either WT allele PCR product (500 bp) or mutant allele PCR product (650 bp). Floxed *Sf-1* or *Dax-1* allele detection resulted in 220 bp mutant allele whereas wild type allele gives shorter 180 bp product. For detection of deleted *Dax-1* allele at the genomic level, DNA from $AS^{+/Cre}$*::Dax-1*$^{fl/fl}$ or $AS^{+/Cre}$*::Dax-1*$^{fl/Y}$ adrenal gland was isolated and amplified using the same primers as for genotyping. Deleted allele, missing exon 2, will result in amplification of specific 300 bp PCR product (Table 1). The $R26R^{mTmG}$ allele was detected by PCR amplification of the *GFP* coding region (Table 1).

FACS sorting

Adrenals were dissected, cleaned of fat and digested in Hank's balanced salt solution with 1mM $MgCl_2$ and 5 mg/ml BSA containing 1 mg/ml collagenase 1 (Worthington) and 0.5 mg/ml DNAse I (Sigma) for 1 hour at 37°C. The resulting cell suspension was filtered through a 40 μM cell strainer, centrifuged and re-suspended in DMEM:F12 medium containing 2%BSA and 1mM EDTA. FACS analysis was

performed using $AS^{+/+}::R26R^{+/mTmG}$ and $AS^{+/Cre}::R26R^{+/mTmG}$ mice with an Avalon Cell Sorter using v.1.0.0 software (Propel Labs). Red/mTomato and green/mGFP cells were sorted directly into Trizol (Sigma). RNA was isolated using RNeasy Micro Kit (Qiagen). cDNA synthesis and quantitative PCR was performed as described above.

Supplemental References

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Supplemental Figure Legends

Figure S1, related to Figure 1. (A) *In situ* hybridization for *AS* (upper panel) and *Cyp11b1* (lower panel) expression in $AS^{+/+}$, $AS^{+/Cre}$ (+neo), $AS^{+/Cre}$ (-neo), $AS^{Cre/Cre}$ and $AS^{GFP/GFP}$ (Lee et al., 2005) mice. *AS* gene expression is detected in subcapsular region of $AS^{+/+}$ and mice heterozygous for the *Cre* allele. In contrast, AS expression is completely lost in $AS^{Cre/Cre}$ and $AS^{GFP/GFP}$ mice. Analysis of Cyp11b1 expression was comparable between all mice, indicating this allele was not disrupted in $AS^{+/Cre}$ mice.

(B) Confocal analysis of $AS^{+/Cre}::R26R^{+/mTmG}$ fetal adrenal at the day e16.5 showing occasional single (or rare doublet) GFP^+ cells in the subcapsular region.

(C) RNA was isolated from laser capture micro-dissections of zG and zF regions from $AS^{+/Cre}::R26R^{+/mTmG}$ mice, and expression of specific markers analyzed by qRT-PCR (as in Figure 1E) or semi-quantitative RT-PCR (shown here). PCR products were run on the gel and visualized by ethidium bromide staining. The zG-specific marker *AS* is expressed only in the zG along with *Cre*. The zF-specific marker *Cyp11b1* is expressed predominantly in the zF. *GFP* expression is detected in both zones as expected from the lineage tracing studies (Figure 2).

(D) FACS analysis of $AS^{+/Cre}\,R26R^{+/mTmG}$ mouse adrenals at different ages demonstrating increased GFP^+ cells. $AS^{+/+}\,R26R^{+/mTmG}$ mice containing only mT-labeled cells were used as a wild type controls. Quantification reveals an increase in GFP^+ cells. qRT-PCR analysis on sorted GFP^+ cells confirmed both *Cyp11b1* and *AS* gene expression in cells derived from 14 weeks old animals, Mean±SEM.

Figure S2, related to Figure 2. (A) Split view of confocal images from a 12 week old adrenal (see Figure 2F) from an $AS^{+/Cre}$ :: $R26R^{+/mTmG}$ mouse showing near complete replacement of the red fluorescent Tomato (mT) with GFP (mG) in the adrenal cortex, but not in capsule or adrenal medulla.

(B) Co-immunofluorescent analysis of endogenous AS and GFP expression in $AS^{+/Cre}::R26R^{+/mTmG}$ mice confirms co-expression in the zG region, but not in the zF region.

(C) Co-immunostaining for anti-CYP11B1 and GFP in the adrenal cortex of $AS^{+/Cre}$::$R26R^{+/mTmG}$ mouse. Arrows point to GFP cells co-expressing CYP11B1.

(D) Co-immunofluorescent analysis of $AS^{+/Cre}$ :: $Sf\text{-}1^{+/+}$ mice with anti-CYP11B1 and anti-AS (CYP11B2) antibodies in the adult adrenal cortex. Arrows indicate occasional cells present at the boundary of the two zones co-expressing both markers.

(E) Co-immunofluorescent analysis of $AS^{+/Cre}$ :: $Sf\text{-}1^{fl/fl}$ mice with anti-CYP11B1 and anti-AS (CYP11B2) in the adult adrenal cortex.

Figure S3, related to Figure 3. Histological analysis (H&E) of $AS^{+/Cre}$ and $AS^{Cre/Cre}$ mice shows a disorganized and thickened zG region in $AS^{Cre/Cre}$ knockout mice. Note the adrenal cortex in $AS^{Cre/Cre}$ mice is also generally smaller, as previously described (Lee et al., 2005).

Figure S4, related to Figure 4. (A) Expression of zone specific steroidogenic genes in 8 week old $AS^{+/Cre}$ and $AS^{+/Cre}$::$Sf\text{-}1^{fl/fl}$ whole adrenal glands. Expression levels of zG markers, *AS* (*Cyp11b2)* and *Hsd3b6* (3β-hydroxysteroid dehydrogenase type 6, shown to be specifically expressed in murine zG (Doi et al., 2010)) were significantly decreased in zG-specific *Sf-1* knock out mice, suggesting partial ablation of the zG whereas no change in expression of zF markers *(Cyp11b1* and *Hsd3b1*) was detected (Mean±SEM, * $p < 0.05$). (B) To further establish that conversion of zG cells into zF cells is SF-1 dependent, we generated $AS^{Cre/Cre}$::$R26R^{+/mTmG}$::$Sf\text{-}1^{fl/fl}$ mice (right panel) to accelerate lineage marking. Similar to $AS^{+/Cre}$::$R26R^{+/mTmG}$::$Sf\text{-}1^{fl/fl}$ mice (left panel), confocal analysis reveals *Sf-1* deficient zG cells are unable to contribute to zF cells.

(C) Measurement of random plasma corticosterone levels from $AS^{+/Cre}$ and $AS^{+/Cre}$::$Sf\text{-}1^{fl/fl}$ mice reveals no difference in glucocorticoid synthetic capacity. These results show no evidence for adrenocortical insufficiency after zG-specific ablation of *Sf-1*. Analysis of 10 animals per group. Adrenal sufficiency defined as ≥200 ng/ml, Mean±SEM.

(D) Quantification of GFP-positive cells co-expressing AS in the subcapsular region of $AS^{+/Cre}::R26R^{+/mTmG}$ and $AS^{+/Cre}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animals.

(E) Quantification of GFP-positive cells co-expressing SF-1 in the subcapsular region of $AS^{+/Cre}::R26R^{+/mTmG}$ and $AS^{+/Cre}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animals.

(F) Immunofluorescent analysis showing occasional GFP-positive cells expressing SF-1 in the adrenal cortex of a 13 week old $AS^{+/Cre}::R26R^{+/mTmG}$ and $AS^{+/Cr}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animal.

(G) Co-immunostaining of SF1 and GFP in $AS^{Cre/Cre}::R26R^{+/mTmG}::Sf\text{-}1^{fl/fl}$ animals showing a large number of accumulated GFP^+ cells devoid of SF-1 expression in the subcapsular region. Very few $SF\text{-}1^+$ cells are detected in the subcapsular region. Note that the cells of zF region still express SF-1. (H) Expression of Shh pathway genes and genes involved in the Wnt/β-catenin pathway in 8 week old $AS^{+/Cre}$ and $AS^{+/Cre}::Sf\text{-}1^{fl/fl}$ whole adrenal glands. Expression levels of Shh, a marker for adrenal progenitor cells, is significantly decreased, whereas expression of Gli1, a marker for capsular progenitor cells is increased zG-*specific Sf-1* knockout adrenals. Similarly, expression of the secreted WNT inhibitors (SFRP2 and SFRP3) are increased, whereas APCDD1 (a target of Wnt signaling) is decreased in zG-specific *Sf-1*-deficient adrenals (n= 4-5 samples per group, * $p< 0.05$); ** $p< 0.01$); *** $p< 0.001$).

(I) zG-specific ablation of nuclear receptor DAX-1 does not impact zG-dependent lineage development in the adult adrenal cortex. Confocal analysis of adrenal glands from $AS^{+/Cre}::R26R^{+/mTmG}::Dax\text{-}1^{+/Y}$ (left panel) and $AS^{+/Cre}::R26R^{+/mTmG}::Dax\text{-}1^{fl/Y}$ (middle panel) 28 week old mice. No difference in lineage conversion is evident. PCR analysis of genomic DNA from whole adrenals (right panel) (according to the method of Yu and Jameson (Yu et al., 1998)) confirms genetic deletion of the *Dax-1* allele in $AS^{+/Cre}::Dax\text{-}1^{fl/Y}$ males and $AS^{+/Cre}::Dax\text{-}1^{fl/+}$ *females* (heterozygotes). The smaller bands, 180 bp and 220 bp, correspond to the wild type and floxed alleles, respectively. Deletion of the floxed exon 2 is detected as a 300 bp PCR product.

(J) zG-dependent lineage conversion is restricted to the zF and does not contribute to the X-zone in adrenals of $AS^{+/Cre}::R26R^{+/mTmG}$ female mice. The X-zone is evident between the zF and the adrenal medulla and demonstrates bright pink endogenous fluorescence (upper panel). Co-immunofluorescent

analysis of a paraffin embedded adrenal from $AS^{+/Cre}::R26R^{+/mTmG}$ female mouse stained for GFP and 20alpha-HSD, an X-zone specific marker (Hershkovitz et al., 2007), reveals no co-localization (lower panel).

Table S1, related to Figures S1B, S4D. Primer sequences and PCR conditions used for genotyping of mouse strains.

Table S2, related to Figures 1E, S4A. Primer sequences used for SYBR Green quantitative-PCR based gene expression analysis.

Supplemental Experimental Procedures

Mouse Experiments

All animal procedures were approved by the Children's Hospital Institutional Animal Care and Use Committee.

Generation and Validation of $AS^{+/Cre}$ mice

Targeting arms for homologous recombination were obtained by screening a Lambda FIX II genomic library (Stratagene), generated from sv129 DNA, using a previously described AS-specific probe (Domalik et al., 1991). Two clones, covering the 5' and 3' portions of the *AS* gene, were subcloned into Bluescript-SK, restriction mapped and partially sequenced to confirm their identify. The knock-in targeting construct (Figure 1) includes the following additional key components: Cre recombinase (provided by Dr. K. Rajewsky, Harvard Medical School), the FTR-flanked neomycin resistance gene (provided by Dr. F. Alt, Harvard Medical School) and the gene for diphtheria toxin subunit A (DTA) (provided by F. Gertler, MIT). Modifications to the endogenous *AS* locus were designed to prevent translational initiation from the endogenous ATG. We chose to include as much endogenous regulatory sequence as possible within the final targeted allele to minimize the risk of creating a hypomorphic *AS*

allele. The entire coding sequence of the gene was knocked out, retaining only exon 1, intron 1 and the first portion of exon 2. All potential translation start sites (ATG) within exon 1 or exon 2 were excluded from the construct or mutated. The final targeting construct was validated using extensive restriction digests along with sequencing of all critical regions including the proximal promoter, exon 1, intron 1 and exon 2. J1 embryonic stem cells were transfected with the targeting construct by electroporation and plated on a feeder layer of embryonic fibroblasts. Three hundred and eighty four clones were screened by Southern blot analysis.

Southern blots were initially screened with a probe derived from sequences external to the 3' arm targeting. This probe was used to confirm site-specific integration of the targeting allele. Two additional probes were derived from sequences internal to the targeting region and used to further verify proper integration of the two targeting arms. In addition, inclusion of the mutated translation start sites within the recombined allele was verified to exclude the possibility that homologous recombination occurred within the first intron leading to exclusion of these mutated sequences. Southern blot analysis was also performed to confirm that *Cyp11b1*, which is highly homologous to the targeted locus, remained unaffected during gene targeting. *Aldosterone synthase* and *Cyp11b1* are organized in a head-to-tail orientation with approximately 6kb of intervening sequence. Finally, Southern blot screening to confirm proper recombination of the 3' targeting arm involved a Bcl1 restriction cut and use of a 3' probe (Figure 1B, C). The expected bands were 7.7 kb (wild-type) and 5.0 kb (recombined) (Figure 1C).

Two properly targeted ES cell clones were expanded for electroporation with Flp recombinase (pOG-Flpe6 was provided by Dr. S. Dymecki, Harvard Medical School) in order to delete the FRT-flanked positive selection marker. Individual colonies were selected and removal of the Neo^R gene (Figure 1B, C) was confirmed by Southern blot analysis. Two ES cell clones were expanded for karyotype analysis and then injected into blastocysts in the Boston Children's Hospital transgenic core facility. Mice were bred to germline heterozygosity and maintained on a mixed sv129-C57Bl/6 genetic background. To control for

genetic background, experiments were performed using littermates when possible.

Generation of $AS^{+/Cre}$ :: $ROSA^{+/mTmG}$ mice

$AS^{+/Cre}$ mice were crossed with the *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (mT/mG) reporter line (Muzumdar et al., 2007) to generate $AS^{+/Cre}$*::R26R*$^{+/mTmG}$ mice. This reporter mouse expresses a floxed membrane bound modified red fluorescent protein (Tomato) (mT) upstream of a membrane bound enhanced green fluorescent protein (eGFP) (mG) at the endogenous *ROSA* locus ($R26R^{+/mTmG}$). These mice are useful as a Cre-reporter as they express red fluorescence prior to, and green fluorescence following Cre-mediated recombination (Figure 2A). The double-fluorescent system allows visualization of both recombined and non-recombined cells, and is ideal for lineage tracing experiments. These mice were intercrossed to generate $AS^{Cre/Cre}$*::R26R*$^{+/mTmG}$ mice.

Generation of $AS^{+/Cre}$ :: SF-$1^{fl/fl}$ mice

$AS^{+/Cre}$ mice were crossed with *Sf-*$1^{fl/fl}$ conditional mice (Zhao et al., 2001) generating $AS^{+/Cre}$*::Sf-*$1^{+/fl}$ and then intercrossed to generate $AS^{+/Cre}$*::Sf-*$1^{fl/fl}$ and $AS^{Cre/Cre}$*::Sf-*$1^{fl/fl}$ mice. These mice were also crossed with $R26R^{+/mTmG}$ mice to generate trigenic mice.

Generation of $AS^{+/Cre}$ :: DAX-$1^{fl/fl}$ mice

$AS^{+/Cre}$ mice were crossed with DAX-$1^{fl/fl}$ conditional mice (Yu et al., 1998) and then intercrossed to generate $AS^{+/Cre}$ :: $Dax1^{+/Y}$ and $AS^{+/Cre}$ :: $Dax1^{fl/Y}$. These mice were also crossed with $ROSA^{+/mTmG}$ mice to generate trigenic mice.

Histology

For immunohistochemical or immunofluorescent analysis adrenal were fixed in 4% paraformaldehyde for 1 hour at 4°C and either embedded in paraffin (cut as 6 µM sections) or embedded in OCT (cut as 10 µM sections). Paraffin sections were deparaffinized, antigen retrieval performed by boiling in 0.01M sodium

citrate, endogenous peroxidase activity blocked by 0.3% hydrogen peroxide and endogenous biotin background blocked by Biotin-Avidin Blocking Kit (Vector Laboratories). Nonspecific staining was blocked using 10% normal goat serum prior to immunostaining and primary antibodies used in following dilutions: chicken anti-GFP (Aves) 1:1000, rabbit anti-aldosterone synthase (C. Gomez-Sanchez, Lot 2066) 1:500, rabbit anti-SF-1 (a kind gift of R. Auchus and the late K. Parker, Lot 18433) 1:800, rabbit anti-P450scc (Millipore) 1:500, rabbit anti-CRE 1:400 (Novagen), 1:50, mouse anti-CYP11B1 and rabbit anti- 20α HSD 1:1000 (a kind gift of Y. Weinstein). Sections were incubated with primary antibodies overnight at 4°C with the exception of anti-SF1 staining where the sections were incubated overnight at room temperature. As a secondary antibody, goat anti-rabbit 633, goat anti-chicken 488 or goat anti-mouse 594 (all from Molecular Probes) were used at a dilution of 1:800. Sections were counterstained by short incubation with DAPI (1:1000) in PBS and visualized using a Nikon E800 Eclipse, a Zeiss LSM 700 or a Nikon 90i Eclipse. To facilitate the immunofluorescent detection of Cre, mice were water deprived for 48 hours prior to sacrifice.

In situ hybridization (ISH) was performed using 10 μm frozen sections from adult adrenals hybridized with ^{35}S-labeled anti-sense cRNA riboprobes for *AS, Cyp11b1* or sense controls. Anti-sense riboprobes were generated using T3 RNA polymerase and cDNA templates that were reverse transcribed from the 3'-untranslated region of either *Aldosterone Synthase* or *Cyp11b1*. After fixation with 4% paraformaldehyde, acetylation and dehydration, slides were hybridized with the probes at 65°C. The slides were then processed as described previously (Venihaki et al., 2000).

Measurement of Hormonal Levels

Blood samples of animals for hormonal measurements were collected by retro-orbital bleeding in the morning hours using heparin-coated capillaries for corticosterone and aldosterone measurements. For measurement of plasma renin activity blood was collected in non-heparinized capillary tubes treated with EDTA. Samples were centrifuged for 15 min at 3000 rpm, plasma fraction collected and kept frozen at -

80°C until further processing. Corticosterone levels were analyzed by Cortisterone ^{125}I RIA kit (MP Biomedicals, LLC, Orangenburg, NY, USA), aldosterone levels were measured by aldosterone RIA (Diagnostics Products Corporation, LA, CA) and plasma renin activity was measured using the saturating substrate concentration method (Diasorin, Stillwater, MN). Aldosterone was also measured in 24-hr urine samples from adult male mice housed in metabolic cages as previously described (Davies et al., 2008; Guagliardo et al., 2012). Briefly, mice were fed a normal Na (NS, 0.3% Na) for one week. Urine was collected daily and analyzed on the last 4 days of each diet. Urinary aldosterone concentration was measured by RIA and standardized to urinary creatinine (colorimetric assay kit, Cayman Chemical Company, Ann Arbor, MI) to control for differences in urine volume and glomerular filtration rate. On the last day, tail vein blood was sampled and plasma renin concentration measured by RIA.

Genotyping

Genomic DNA was isolated by alkaline lysis (0.2 M NaOH) at 95 C for 45 min, followed by neutralization in 20 mM EDTA (final concentration). Sequences of primers and PCR conditions used for genotyping are listed in Table 1. Briefly, aldosterone synthase (*AS*) alleles were genotyped using three primers PCR reactions yielding either WT allele PCR product (500 bp) or mutant allele PCR product (650 bp). Floxed *Sf-1* or *Dax-1* allele detection resulted in 220 bp mutant allele whereas wild type allele gives shorter 180 bp product. For detection of deleted *Dax-1* allele at the genomic level, DNA from $AS^{+/Cre}$*::Dax-1*$^{fl/fl}$ or $AS^{+/Cre}$*::Dax-1*$^{fl/Y}$ adrenal gland was isolated and amplified using the same primers as for genotyping. Deleted allele, missing exon 2, will result in amplification of specific 300 bp PCR product (Table 1). The *R26R*^{mTmG} allele was detected by PCR amplification of the *GFP* coding region (Table 1).

FACS sorting

Adrenals were dissected, cleaned of fat and digested in Hank's balanced salt solution with 1mM $MgCl_2$ and 5 mg/ml BSA containing 1 mg/ml collagenase 1 (Worthington) and 0.5 mg/ml DNAse I (Sigma) for 1 hour at 37°C. The resulting cell suspension was filtered through a 40 μM cell strainer, centrifuged and

re-suspended in DMEM:F12 medium containing 2%BSA and 1mM EDTA. FACS analysis was performed using $AS^{+/+}::R26R^{+/mTmG}$ and $AS^{+/Cre}::R26R^{+/mTmG}$ mice with an Avalon Cell Sorter using v.1.0.0 software (Propel Labs). Red/mTomato and green/mGFP cells were sorted directly into Trizol (Sigma). RNA was isolated using RNeasy Micro Kit (Qiagen). cDNA synthesis and quantitative PCR was performed as described above.

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