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In vivo evidence for the crucial role of SF1 in steroid-producing cells of the testis, ovary and adrenal gland

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SUMMARY

Adrenal and gonadal steroids are essential for life and reproduction. The orphan nuclear receptor SF1 (NR5A1) has been shown to regulate the expression of enzymes involved in steroid production in vitro. However, the in vivo role of this transcription factor in steroidogenesis has not been elucidated. In this study, we have generated steroidogenic-specific Cre-expressing mice to lineage mark and delete *Sf1* in differentiated steroid-producing cells of the testis, the ovary and the adrenal gland. Our data show that SF1 is a regulator of the expression of steroidogenic genes in all three organs. In addition, *Sf1* deletion leads to a radical change in cell morphology and loss of identity. Surprisingly, sexual development and reproduction in mutant animals were not compromised owing, in part, to the presence of a small proportion of SF1-positive cells. In contrast to the testis and ovary, the mutant adult adrenal gland showed a lack of *Sf1*-deleted cells and our studies suggest that steroidogenic adrenal cells during foetal stages require *Sf1* to give rise to the adult adrenal population. This study is the first to show the in vivo requirements of SF1 in steroidogenesis and provides novel data on the cellular consequences of the loss of this protein specifically within steroid-producing cells.

KEY WORDS: Leydig cell differentiation, Adrenal development, Gonad development, Steroidogenic factor 1, Steroidogenic gene regulation, Mouse

INTRODUCTION

Endogenous steroid hormones are powerful small molecules produced in vertebrates through the metabolic process of steroidogenesis that impact a large number of normal homeostatic processes and disease development. In mammals, the sex steroids testosterone and oestrogen, principally produced in the testis and ovary, respectively, are crucial for reproduction, metabolism and behaviour. Adrenal glands produce corticosteroids that have numerous physiological impacts on, for example, stress and the immune response, metabolism, electrolyte levels and behaviour. Specific cells within each of these steroidogenic organs carry out the crucial function of converting cholesterol, the precursor to all steroids, into sex and corticosteroids by expression of a suite of cytochrome P450 and hydroxysteroid dehydrogenase enzymes, some of which are shared and others organ specific. Shared enzymes include CYP17, 3βHSD and P450 Side Chain Cleavage ($P450^{SCC}$). The latter protein, which is encoded by the *Cyp11a1* gene, catalyses the conversion of cholesterol to pregnenolone, the first rate-limiting enzymatic step in the biosynthesis of all steroid hormones.

The orphan nuclear hormone receptor steroidogenic factor 1 (SF1; also known as NR5A1 or Ad4BP) has been proposed to be a key regulator of steroidogenesis. It, and its family member LRH-1 (also known as NR5A2), bind specific DNA sequences found in the transcriptional regulatory promoters of many steroidogenic genes and can activate reporter constructs in vitro (reviewed by Omura and Morohashi, 1995; Parker and Schimmer, 1995; Hammer and Ingraham, 1999; Val et al., 2003). Consistent with this proposal, in vivo deletion of the 'P' site within the *Cyp11a1* gene, to which SF1 or LRH-1 can bind, leads to a dramatic decrease in *Cyp11a1* levels in the adult adrenal gland and testis (Shih et al., 2008). SF1 has been shown to have other functions, particularly during the development of the gonads and adrenal glands. SF1-deficient mice exhibit complete adrenal and gonadal agenesis owing to apoptosis in the developing genital ridge (Luo et al., 1994) and defects within the pituitary and ventral medial hypothalamus (Ingraham et al., 1994; Ikeda et al., 1995; Sadovsky et al., 1995; Shinoda et al., 1995). Although no humans bearing homozygous null mutations have been identified, a vast array of heterozygous alleles have been associated with adrenal failure, testis dysgenesis, androgen synthesis defects, hypospadias, anorchia and male factor infertility in 46,XY individuals (reviewed by Ferraz-de-Souza et al., 2011).

SF1 expression is first found in the adrenal and gonad anlagen, the adrenogonadal primordium (AGP), and continues in multiple differentiated cell types within the steroidogenic organs that are descendants of this embryological structure (Hatano et al., 1994; Ikeda et al., 1994; Hatano et al., 1996). Foetal and adult testes express high levels of SF1 in the testosterone-synthesizing Leydig cell and the non-steroidogenic, germline-supporting Sertoli cell. Analogous to the testes, ovaries express SF1 in non-steroidogenic (follicle or granulosa cells) and steroidogenic (stromal and theca cells) cell populations. Similar to the testis, the adrenal gland has a transient steroidogenic foetal population that is replaced by a definitive adult steroidogenic population and both express SF1 (reviewed by Kim and Hammer, 2007; Morohashi and Zubair, 2011).

The complete adrenal and gonadal agenesis and the perinatal lethality in $Sf1^{-/-}$ mutant mice have prevented in vivo analyses of this factor in the different differentiated steroidogeneic cell types in the foetus and adult. Previous studies with the $Amhr2^{tm3(cre)Bhr}$ targeted allele and the *Sf1* conditional allele attempted to circumvent the AGP apoptosis to study the role of SF1 in Leydig and follicle cells (Jeyasuria et al., 2004). Unfortunately, this genetic model deleted *Sf1*

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too early to allow normal testis development, as exemplified by the reduced cell proliferation one day prior to Leydig cell appearance. More importantly, recent data has shown that this Cre strain also induces abundant recombination in Sertoli cells, another crucial testis cell type (Tanwar et al., 2010). Furthermore, *Amhr2*$^{tm3(cre)Bhr}$ is not expressed in the adrenal gland or the steroidogenic stromal and theca cells in the ovary, precluding the in vivo characterization of SF1 in these steroidogenic cell types. Finally, these studies failed to genetically mark the *Sf1* mutant cells, which prevented the assessment of the fate of these cells in vivo. Therefore, no systematic study has addressed the in vivo function of SF1 in differentiated steroidogenic cells of the testis, ovary and adrenal gland.

We have overcome this by creating a novel mouse transgenic line, which, when combined with an *Sf1* conditional mutation, has allowed us to test the in vivo function of SF1 in the steroidogenic cell types residing in the three primary steroidogenic tissues in mammals. Importantly, we simultaneously lineage mark the mutant steroidogenic cells and provide the first report of the outcome of these cells in their native context. We find that SF1 acts as an in vivo major regulator of all steroidogenic cells of the testis whereas SF1 dependence in ovarian steroidogenic cells is cell type specific. In addition, in the testis, ovary and foetal adrenal gland, *Sf1* deletion leads to a radical change in cell morphology. In contrast to the testis and ovary, the mutant adult adrenal gland shows a lack of *Sf1*-deleted cells, suggesting that foetal *Cyp11a1*-expressing adrenal cells require SF1 to give rise to the adult adrenal population.

MATERIALS AND METHODS

Mouse strains and breeding

The *Sf1*^{Fl} conditional mutant mice were kindly provided by Dr Keith Parker (Zhao et al., 2001b). The *Gt(ROSA)26Sor*^{tm1Sor} (R26R^{lacZ}) and *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (R26R^{YFP}) R26 Cre reporter strains were developed by Drs Philippe Soriano and Frank Costantini, respectively (Soriano, 1999; Srinivas et al., 2001). *Sf1*$^{Fl/+}$;Tg(Cyp11a1-iCre) animals are shown as controls as initial experiments did not show any phenotypic difference between *Sf1*$^{Fl/+}$ and *Sf1*$^{+/+}$ mice carrying the Tg(Cyp11a1-iCre) transgene.

Bacterial artificial chromosome (BAC) construct

The Cyp11a1-iCre construct was generated using a previously characterized BAC containing the *Cyp11a1* transcriptional regulatory elements (Jeays-Ward et al., 2003). We used the previously described homologous recombination method in DY380 cells (Jeays-Ward et al., 2003). For the recombination fragment, the iCre coding region plus the rabbit β-globin polyadenylation signal sequences were introduced at 14 bp upstream of the *Cyp11a1* ATG codon, within the 5′ untranslated region of the mRNA. The resulting construct contained a 2.8 kb *Cyp11a1* fragment with the iCre + polyadenylation sequence insertion and a 100 bp deletion of *Cyp11a1* sequences, starting at the site of insertion and including the ATG codon. This fragment was introduced into DY380 cells containing the unmodified *Cyp11a1* BAC and homologous recombination was induced. For pronuclear injections, BAC DNA was prepared using the Qiagen Maxiprep Kit (Qiagen, UK), dialysed against microinjection buffer and injected as circular DNA at different concentrations.

Whole-mount in situ hybridization

Whole-mount RNA in situ hybridization was performed as previously described (Buaas et al., 2009). Digoxigenin (DIG)-labelled probes were synthesized using a DIG RNA Labeling Mix (Roche) from previously described templates (Jeays-Ward et al., 2003; Val et al., 2006; Val et al., 2007).

Quantitative RT-PCR

For each genotype, three or four microdissected gonad pairs were processed separately. Total RNA was extracted from frozen tissue using the RNAeasy Micro Kit (Qiagen). Oligo(dT) primed single-stranded cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). The SYBR Green system (Applied Biosystems) was used for real-time PCR quantification. Primer sequences are shown in supplementary material Table S1. The relative mRNA accumulation was determined using the ΔΔCt method with *Rpl2* for normalization (Livak and Schmittgen, 2001). For statistical analysis, the unpaired *t*-test was used and $P<0.05$ was considered to be statistically significant.

Histology, immunofluorescence and β-galactosidase staining

For histological staining, tissues were immersed in Bouin's fixative, wax embedded and subjected to periodic acid-Schiff (PAS) staining. Immunofluorescence staining was performed on foetal and adult frozen tissue as described (Val et al., 2007). For double immunostaining with primary antibodies raised in the same species, a serial staining protocol was carried out using the Tyramide Signal Amplification (TSA) Kit (PerkinElmer) (see supplementary material Table S2 for antibody dilutions).

Various modifications were performed for studies on the adult adrenal gland. For double YFP/P450^{SCC} immunostaining, P450^{SCC} was always the primary antibody amplified with the TSA Kit, as steroidogenic P450^{SCC}-expressing cells in the adrenal cortex harbour endogenous biotin activity (Paul and Laufer, 2011). We found that SF1 immunofluorescence on adult adrenal glands was incompatible with formaldehyde-based fixation. Therefore, adult adrenal glands for SF1/P450^{SCC} staining on adjacent sections were fresh frozen in OCT on dry ice and stored at –80°C. Fixation was achieved in pre-cooled acetone for 10 minutes at –20°C immediately prior to immunostaining. β-Galactosidase staining on frozen sections was performed as described (Jeays-Ward et al., 2003).

Testis YFP cell number quantification

YFP cells and the number of testis cord cross sections were counted in control (n=4) and mutant (n=4) samples with a range of 51-201 YFP cells per image section (taken at 20×) with two sections per biological replicate.

Lysotracker labelling of cultured foetal adrenal glands and testes

Dissected embryonic day (E) 14.5 adrenal glands and gonads were processed for apoptosis detection according to the Lysotracker protocol developed by Schmahl and Capel (Schmahl and Capel, 2003).

Testosterone measurements

Intratesticular testosterone levels (n=3 for control and mutants) were measured by ELISA, using a Total Testosterone Kit from Diagnostics Biochem according to the manufacturer's instructions, and expressed relative to testicular weight. Data are expressed as mean ± standard error.

RESULTS

Cyp11a1-Cre transgene characterization and conditional *Sf1* ablation

We used a previously characterized BAC construct to generate transgenic mouse strains expressing Cre recombinase under the control of the *Cyp11a1* transcriptional regulatory elements (referred to as *Cyp11a1*-Cre). One *Cyp11a1*-Cre transgenic founder line was characterized by mating it to reporter mouse strains that express YFP (R26R^{YFP}) or β-galactosidase (R26R^{lacZ}) in cells that have been exposed to a functional Cre recombinase protein and their descendants. Foetal testes and adrenal glands, but not ovaries, are steroidogenic and express *Cyp11a1*. We observed robust Cre-mediated reporter expression in E14.5 testes and adrenal glands derived from foetuses carrying the *Cyp11a1*-Cre transgene and R26R^{YFP} (Fig. 1A) or R26R^{lacZ} reporter strains. YFP or β-galactosidase expression could initially be detected in a few cells of E13.5 testes whereas foetal ovaries failed to show reporter expression (data not shown). Adult testis, ovary and adrenal gland all express *Cyp11a1* and revealed high level of Cre-mediated recombination (Fig. 1B-D). Sections through foetal and adult tissues revealed reporter expression restricted to *Cyp11a1*-

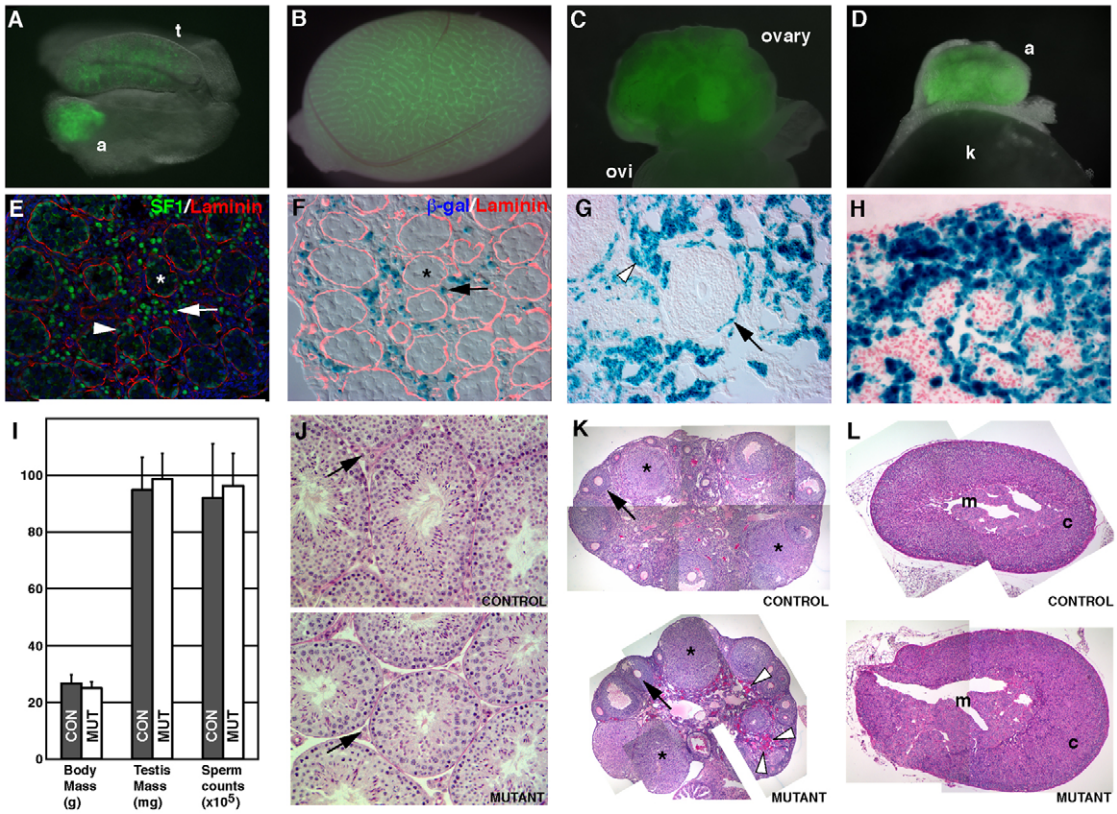

Fig. 1. Reporter gene expression and deletion of *Sf1* in mouse foetal and adult steroidogenic cells. (**A-D**) YFP fluorescence from Cyp11a1-Cre;*R26R*^{YFP} E14.5 testis (t) and adrenal (a) (A), adult testis (B), adult ovary and adjacent oviduct (ovi) (C), and adult adrenal (a) juxtaposed to the kidney (k) (D). (**E**) Double immunofluorescence staining on E14.5 wild-type testis of laminin surrounding the testicular cords, and SF1 in the interstitial region (arrow) and in Sertoli cells (arrowhead), within cords (asterisk). (**F-H**) β-Gal staining of sections from Cyp11a1-Cre;*R26R*^{lacZ} tissues in E14.5 testicular interstitial cells (F, arrow), presumptive theca (black arrow) and stroma (white arrowhead) in the adult ovary (G) and E16.5 adrenal gland (H). (**I**) Body mass, testis mass and epididymal sperm counts from mutant (MUT) and control (CON) adult (2- to 3-month-old) male mice. Error bars represent s.e.m. (**J-L**) PAS-stained sections of adult control and mutant testes (J), ovaries (K) and adrenal glands (L). (J) Presumptive Leydig cells (arrows) based on position and morphology. (K) Follicles (black arrows) and corpora lutea (asterisks). Increased PAS-positive cells in mutant stroma (white arrowheads). (L) Medulla (m) and cortical (c) regions of adrenal glands.

expressing steroidogenic cell types. Foetal testes showed reporter expression in interstitial cells with morphological characteristics of Leydig cells (Fig. 1E,F). Adult ovaries showed reporter expression in thin cells surrounding the growing follicles, where theca cells reside, and in the stromal region (Fig. 1G). Reporter expression was prevalent in the foetal adrenal gland, prior to the formation of different cortical layers and was found in the steroidogenic cortical layer at later stages (Fig. 1H). Finally, reporter-expressing cells (YFP) co-expressed steroidogenic markers in all of these organs as assayed by co-labelling methods (shown in later figures).

To define the functional role of SF1 in *Cyp11a1*-expressing cells, we produced mice that contained the *Cyp11a1*-Cre transgene, the *Sf1* conditional loss-of-function mutation (hereafter referred to as *Sf1*^{Fl}) (Zhao et al., 2001b) and the R26R^{YFP} reporter allele. This genetic design allows cells to first become steroidogenic before SF1 is deleted. Furthermore, our aim was to identify, through YFP expression, cells in which *Sf1* deletion was likely to have taken place and analyse their phenotype. Mice with the *Cyp11a1*-Cre transgene and homozygous for *Sf1*^{Fl} (mutant) were viable and showed no decrease in body mass (Fig. 1I). Furthermore, male mutants showed no fertility phenotypes as assayed by testis mass, epididymal sperm counts, impregnation rate or litter size (Fig. 1I; data not shown). Consistent with this observation, intratesticular testosterone levels were similar between mutant and control animals (9509±863 versus 9262±1585 ng testosterone/g tissue). Histological analysis of sections from the adult testis, ovary and adrenal gland showed no major organ abnormalities, although subtle effects were found, which will be discussed below (Fig. 1J-L).

Testes with *Sf1*-deleted Leydig cells show defects in steroidogenic gene expression

Mammalian testes have a remarkable capability to compensate for developmental defects, prompting us to investigate steroidogenic development during the foetal phase when Leydig cells are first arising. Morphologically, E14.5 mutant testes are indistinguishable from controls. However, whole-mount RNA in situ hybridization (WISH) revealed numerous gene expression changes associated with the steroid-producing foetal Leydig cell population. *Sf1* is expressed at high levels in foetal Leydig cells but is observed at lower levels in Sertoli cells, which reside in the testis cords (Fig. 1E; Fig. 2A). As expected, mutant testes showed a notable *Sf1* reduction in the high-expressing interstitial Leydig cells but no change in the Sertoli cell *Sf1* expression (Fig. 2A). *Cyp11a1*,

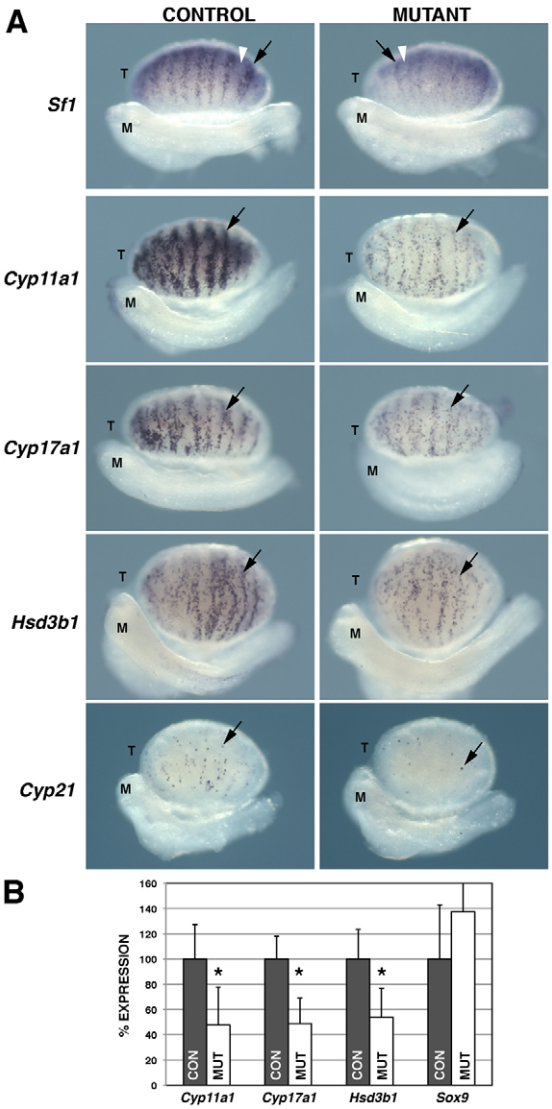

Fig. 2. The steroidogenic gene expression pathway is reduced in Cyp11a1-Cre;*Sf1*$^{Fl/Fl}$ foetal testes. (**A**) Whole mount in situ hybridization of representative E14.5-15 testes from control and mutant mouse embryos were analysed for the expression of steroidogenic genes as indicated. Anterior is to the left with the testis (T) positioned above the mesonephros (M). Steroidogenic Leydig cells (black arrows) reside between seminiferous tubules, which contain *Sf1*-expressing Sertoli cells (white arrowhead). (**B**) Quantitative RT-PCR on RNA from E14.5 control (CON) and mutant (MUT) testes for steroidogenic genes as indicated. *P<0.05. Error bars represent s.e.m.

Cyp17a1 and *Hsd3b1* are steroidogenic genes that are crucial for testosterone production and have been shown to be direct transcriptional targets of SF1 in vitro. Mutant testes showed dramatic reductions in all three genes compared with controls (Fig. 2A). Furthermore, the more recently recognized adrenal-like steroidogenic cells that reside in the foetal testis (Val et al., 2006) were also compromised in mutant testes, as indicated by the reduction in *Cyp21* gene expression (Fig. 2A). Quantitative (q)RT-PCR analysis confirmed our WISH observations and showed *Cyp11a1*, *Cyp17a1* and *Hsd3b1* to be reduced 48%, 49% and 53% relative to controls, respectively (Fig. 2B). Importantly, the direct transcriptional target of SF1 in Sertoli cells, *Sox9*, was unaffected in mutants, as expected, as the *Cyp11a1*-Cre transgene is not expressed in Sertoli cells (Fig. 2B). These data suggest that loss of SF1 in foetal Leydig cells reduces their steroidogenic capacity in the foetal testis.

Foetal Leydig cells require SF1 cell-autonomously for steroidogenesis

In order to investigate the mutant foetal testis phenotype further, we performed co-marker immunohistochemical analyses on sections to study individual *Sf1*-deleted cells. Two steroidogenic enzymes were analysed, P450^{SCC} and 3βHSD, together with YFP. Double immunostaining of control E14.5 testes, which express a nuclear and cytoplasmic localized YFP after exposure to Cre, showed that 100% of the interstitial YFP cells were positive for the mitochondrial and endoplasmic reticulum localized markers P450^{SCC} and 3βHSD, respectively (Fig. 3A; supplementary material Fig. S1). These data confirm that the *Cyp11a1*-driven Cre activity targets foetal Leydig cells. In contrast to control foetal Leydig cells, mutants showed a marked reduction in P450^{SCC} (68% of YFP+ cells were P450^{SCC}+) whereas 3βHSD levels were more subtly reduced (78% of YFP+ cells were 3βHSD+) (Fig. 3C; supplementary material Fig. S1). The continued expression of steroidogenic markers in E14.5 mutant Leydig cells raised the possibility that the Cre activity was not efficiently deleting both *Sf1* conditional alleles. Therefore, we analysed SF1 expression in mutant testes. All YFP-positive Leydig cells in control testes express SF1 (Fig. 3B). By contrast, 88% of mutant Leydig cells (YFP positive) were deficient in SF1, whereas the SF1 expression in the non-Cre expressing Sertoli cells remained unchanged (Fig. 3B,C). These data are consistent with our previous RNA expression studies.

The lack of concordance between the number of SF1-deficient YFP cells (88%) in comparison with the number of cells lacking the steroidogenic proteins (32% of mutant cells were P450^{SCC} negative) suggested that the SF1 protein turnover might be more rapid than that of steroidogenic enzymes, which reside in the mitochondria and endoplasmic reticulum. To investigate this hypothesis, we looked at testes from later stages. Antibody staining at postnatal day (P) 1 revealed notably more mutant YFP-positive cells lacking P450^{SCC} (82%) and 3βHSD (80%), compared with observations in E14.5 testis, confirming a slower decay of these proteins (Fig. 3C). In addition, SF1/P450^{SCC} co-staining at this stage showed that all mutant cells that had lost SF1 had also lost P450^{SCC} (data not shown). Our data therefore show that Leydig cells require cell-autonomous SF1 function for steroidogenic gene expression. The delayed decay of steroidogenic enzymes in foetal Leydig cells is likely to account for the lack of an overt feminization phenotype during foetal development.

SF1 loss leads to steroidogenic cell shape changes without cell death

Sf1 deletion has been shown to induce apoptosis in the early adrenal gland and gonad (Luo et al., 1994). The loss of Leydig cells through apoptosis could account for the reduction in Leydig cell-specific gene expression. However, we failed to detect increased apoptosis in E14.5 mutant testes using Lysotracker and TUNEL assays (supplementary material Fig. S1C; data not shown). Although the YFP intensity in the mutant cells was not as strong as in control Leydig cells, we did not observe a reduced number of mutant YFP cells, supporting the conclusion that cell death is not induced in the SF1-deficient foetal Leydig cells (Fig. 3D). However, we did observe that mutant Leydig cells exhibited a

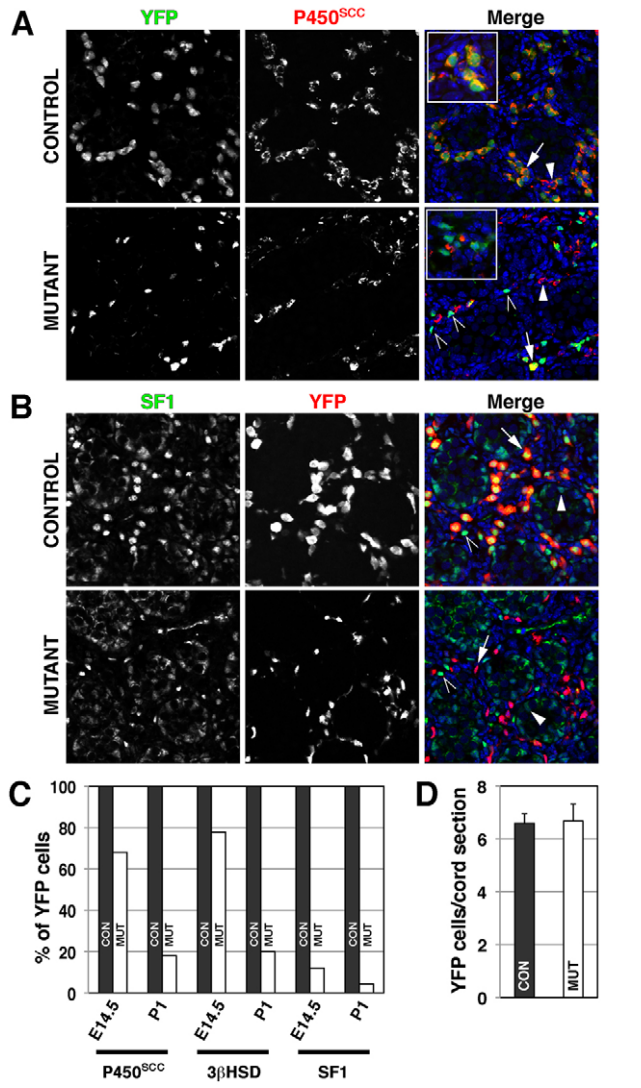

Fig. 3. Foetal Leydig cells require SF1 for steroidogenesis. (**A**) Protein expression of the nuclear and cytoplasmic YFP (green) and mitochondrial P450^{SCC} (red) in control and mutant E14.5 testes (blue, DAPI). YFP-positive cells in controls are always positive for P450^{SCC} (arrow and magnified inset). P450^{SCC} single-positive cells were also present (filled arrowhead). Mutant testes have fewer double-positive cells (arrow) with the continued presence of P450^{SCC} single positives (filled arrowhead). YFP single positives (unfilled arrowheads) are present throughout the interstitium. Mutant YFP cells show reduced P450^{SCC} and a flattened morphology (inset). (**B**) Protein expression of nuclear SF1 (green) and nuclear and cytoplasmic YFP (red) in control and mutant E14.5 testes. Control YFP cells show SF1 expression (arrow) whereas Sertoli cells in the testicular cords express SF1 but no YFP (filled arrowhead). SF1-positive interstitial cells, not expressing YFP, are infrequent in control and mutants (unfilled arrowheads). Mutant interstitial YFP cells (arrow) do not express SF1, exhibit a reduced size and flattened morphology. SF1 expression in Sertoli cells is unaffected (filled arrowhead). (**C**) Frequency of YFP cells expressing P450^{SCC}, 3βHSD and SF1 in E14.5 and P1 control (CON) and mutant (MUT) testes (n=2 control and mutant, two sections per sample and >65 YFP cells counted per section). (**D**) Quantification of E14.5 testis sections for the number of YFP cells per testis cord. Error bars represent s.e.m.

major cell shape change. Control Leydig cells express YFP in the nuclear and cytoplasmic compartments and typically appear as large round or ovoid cells (Fig. 3A,B). In striking contrast, mutant Leydig cells are not only smaller, but exhibit a dramatically different cell morphology and appear flat or crescent-shaped, as seen most clearly in the YFP-stained samples (Fig. 3A,B).

Adult Leydig cells require SF1 cell-autonomously for steroidogenesis

Foetal Leydig cells are removed from the prepubertal testis by an ill-defined mechanism referred to as regression, with the de novo development of adult Leydig cells occurring during the prepubertal phase (reviewed by Habert et al., 2001). We therefore examined the adult Leydig cell phenotype of the *Cyp11a1*-Cre;*Sf1*$^{Fl/Fl}$ animals. Adult Leydig cells, are large pyramidal shaped P450^{SCC}-positive cells positioned in the interstitial space between several seminiferous tubule cross sections. Similar to the foetal Leydig cell findings, we observed that all YFP-positive cells from control animals expressed P450^{SCC}, confirming the specificity of this transgene (Fig. 4A). However, YFP–/P450^{SCC}+ cells were observed in control testes, suggesting that a small percentage of adult Leydig cells did not express the *Cyp11a1*-Cre transgene (Fig. 4A). Mutant testes showed an obvious reduction in P450^{SCC} staining with numerous YFP-positive cells lacking P450^{SCC} expression (Fig. 4A). Notably, their shape and position were dramatically impacted (Fig. 4B). As in control animals, a low number of P450^{SCC}-expressing cells in the mutant testes did not express YFP, probably representing fully functioning Leydig cells that were not targeted by the Cre transgene (Fig. 4A). To confirm that *Sf1* had been deleted in the mutant testis, we carried out double immunostaining for SF1 and YFP. All YFP cells in control testes, which expressed P450^{SCC}, were positive for SF1 (Fig. 4B). By contrast, mutant testes contained an abundance of YFP cells that were negative for SF1 (Fig. 4B). These cells showed the same aberrant cell shape and inter-tubule distribution as the YFP-positive cells that were negative for P450^{SCC} expression. As expected, SF1 expression in Sertoli cells in mutant animals was not affected. Mutant testes do occasionally contain YFP cells that continue to express SF1 and exhibit the cell shape and inter-tubule position indicative of fully functioning Leydig cells, probably representing *Cyp11a1*-expressing Leydig cells that did not delete both *Sf1*^{Fl} alleles. Analysis of 3βHSD expression showed a similar phenotype to P450^{SCC}, with all YFP cells in adult control testes being positive for 3βHSD but an abundance of YFP cells in mutant testes showed no detectable 3βHSD and had atypical cell shapes and/or distributions (Fig. 4B). To confirm the immunohistochemical studies, qRT-PCR was performed for *Cyp11a1*, *Cyp17a1* and *Hsd3b1* expression and the levels of these genes were found to be reduced in the mutant testes to 21%, 24% and 16% of control testes levels, respectively (Fig. 4C). The level of the Sertoli cell-specific *Sox9* gene, was unaffected in mutant testes and supports the previous finding that Sertoli cell gene expression is not disturbed (Fig. 4C). These data clearly show that the steroidogenic gene programme and cellular morphology of adult Leydig cells require SF1 in a cell-autonomous manner.

Steroidogenic cells of the adult ovary show differential SF1 dependence

The mammalian adult ovary is responsible for the production of numerous steroids that are required for female reproduction. Unlike the adult testis, several different cell types produce P450^{SCC} and allow for the production of oestrogens in the ovarian follicle or progesterone from the corpus luteum. Steroidogenic P450^{SCC}-positive cells that express detectable levels of SF1 include the

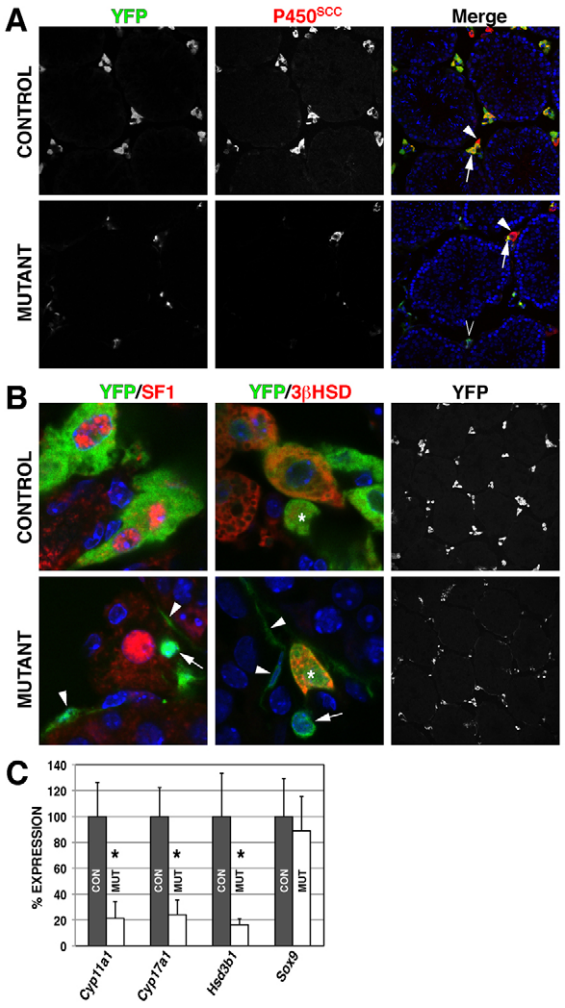

Fig. 4. Adult Leydig cells require SF1 for steroidogenesis. (**A**) Protein expression of nuclear and cytoplasmic YFP (green) and mitochondrial P450^{SCC} (red) in control and mutant adult testes (blue, DAPI). YFP-positive cells in controls are always positive for P450^{SCC} (arrow). Leydig cells not targeted by the Cre recombinase show P450^{SCC} expression but no YFP (filled arrowhead). Mutant testes also contain YFP-positive cells with no P450^{SCC} expression (unfilled arrowhead). (**B**) YFP/SF1 (left) and YFP/3βHSD (middle) protein expression in control and mutant testes. Control interstitial YFP cells show nuclear SF1 expression whereas YFP mutant Leydig cells have lost SF1 expression (arrow and arrowheads) and display peritubular (arrowheads) or interstitial (arrow) positions. Mutant Leydig cells (middle panel) lose 3βHSD expression (arrow and arrowheads) whereas non-SF1-deleted cells remain steroidogenic (asterisk). Low magnification (20×) of the YFP adult Leydig cells (right-hand panels) reveals mutant Leydig cells that are broadly distributed throughout the testes and show interstitial and peritubular positions. (**C**) Quantitative RT-PCR analyses of steroidogenic Leydig and Sertoli cell-specific genes in control (CON) and mutant (MUT) testes. *P<0.05. Error bars represent s.e.m.

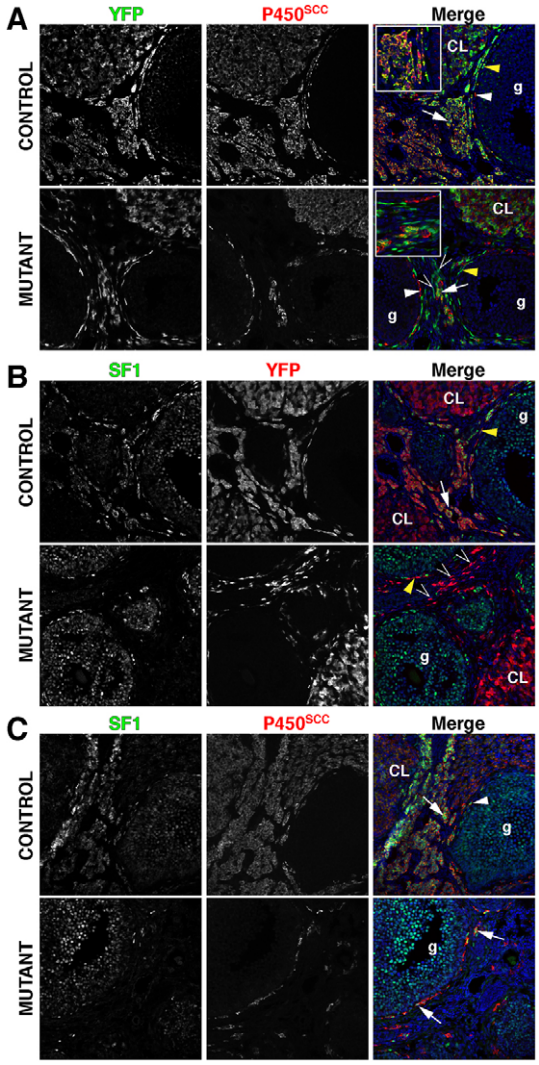

Fig. 5. Theca and stromal cells of the adult ovary require SF1 for steroidogenesis. (**A**) Protein expression of nuclear and cytoplasmic YFP (green) and mitochondrial P450^{SCC} (red) in control and mutant adult ovaries (blue, DAPI). YFP-positive cells in controls are always positive for P450^{SCC} (arrow). Double-positive theca cells (yellow arrowhead) surround the growing follicle with occasional non-Cre targeted theca cells (white arrowhead). YFP/ P450^{SCC} double positives reside in the corpora lutea (CL). Mutant ovaries contain cells with all the expression profiles described above. Additionally, YFP single positives (unfilled arrowheads) are present throughout the mutant interstitium. Mutant YFP cells show reduced P450^{SCC} and a flattened morphology (insets). YFP/P450^{SCC} cells are unaffected in mutant CL. (**B**) Protein expression of nuclear SF1 and YFP in control and mutant adult ovaries. Control theca (yellow arrowhead) and stromal (white arrow) YFP cells express SF1 but the cells of the CL do not. Granulosa (g) cells express SF1 but not YFP. Mutant YFP stromal (unfilled arrowhead) and theca (yellow arrowhead) do not express SF1, exhibit a reduced size and flattened morphology. SF1 expression in mutant granulosa cells is unaffected. (**C**) Protein expression of SF1 and P450^{SCC} in control and mutant adult ovaries. Control P450^{SCC} stromal (arrow) and theca (arrowhead) cells express SF1. P450^{SCC}-positive theca and stromal cells continue to express SF1 in mutant ovaries (arrows).

interstitial stromal cells and the theca cells, but not the P450^{SCC}-expressing cells within the corpus luteum (Fig. 5B,C). Based on our findings from the mutant testes, we decided to analyse the control and mutant ovaries in a similar manner to the mutant males to determine whether SF1 deficiency impacts P450^{SCC} cells differentially. Double immunostaining for the Cre recombinase reporter (YFP) and P450^{SCC} in adult control ovaries revealed that Cre activity was restricted specifically to the three prominent P450^{SCC} expressing cells: thecal, stromal and corpora luteal cells (Fig. 5A). Like the testis, all control YFP cells were P450^{SCC}

positive (Fig. 5A). However, occasional P450^{SCC} single positive cells were observed, suggesting that not all steroidogenic cells expressed the *Cyp11a1*-Cre transgene (Fig. 5A). In contrast to controls, mutant ovaries showed an obvious reduction in P450^{SCC} expression within the stromal and thecal compartment, although YFP expression indicated that the cells were still resident within the interstitial regions (Fig. 5A). However, we did observe YFP+/P450^{SCC}+ stromal cells in mutant ovaries, which probably represent cells that did not delete both *Sf1* conditional alleles but activated the reporter transgene (Fig. 5A). Similar to the mutant testis, YFP+/P450^{SCC}– cells in the mutant ovary had a change in cell shape, being flatter and more elongated, which was particularly obvious in the stromal compartment (Fig. 5A, inset). Strikingly, no reduction in P450^{SCC} levels was observed in the corpora luteal cells, which is consistent with the lack of SF1 in this compartment. Double immunostaining with SF1 and YFP showed that mutant ovaries had an obvious loss of SF1-positive cells in the steroidogenic stromal and theca cell compartments (Fig. 5B). Control and mutant ovaries express SF1 in the granulosa cells of the follicle, a cell population that never expresses P450^{SCC} and, as expected, SF1 level was not altered in this compartment in mutant ovaries. Consistent with previous results, YFP-positive mutant cells in positions where stromal and theca cells reside have an altered cell shape and do not co-express SF1 (Fig. 5B). SF1/P450^{SCC} co-staining showed that all mutant cells that had lost P450^{SCC} had also lost SF1 and was consistent with the mutant YFP+/P450^{SCC}– stromal and theca cells also being deficient in SF1 (Fig. 5C). Importantly, we did observe YFP+/SF1+ cells in mutant ovaries, suggesting that not all *Cyp11a1*-expressing stromal and theca cells delete both *Sf1* alleles (Fig. 5B,C). Our data shows that, in contrast to the adult testis, not all P450^{SCC}-positive ovarian cell types require SF1, as shown by the continued expression of P450^{SCC} in corpora luteal cells that have been exposed to Cre activity. However, like adult Leydig cells, the stromal and theca cells require SF1 cell-autonomously for their steroidogenic phenotype and cell morphology.

Foetal adrenal cells require SF1 for steroidogenesis

The adrenal primordium is first apparent at E10.5 in the mouse embryo (Hatano et al., 1996). However, steroidogenic cells, as detected by *Cyp11a1* expression, are not obvious until E11.5. We confirmed that the *Cyp11a1*-Cre transgene was specifically inducing recombination in P450^{SCC}-positive cells in the E14.5 foetal adrenal gland (Fig. 1A). Double immunostaining of control foetal adrenal glands showed that, like the foetal testis, all YFP cells were positive for P450^{SCC} (Fig. 6A, inset). In contrast to the foetal testis, however, we observed many YFP–/P450^{SCC}+ cells in control adrenal glands, suggesting reduced activity of the *Cyp11a1*:Cre transgene in this foetal organ (Fig. 6A). Mutant foetal adrenal glands contained YFP+/P450^{SCC}– cells, a marker combination type never observed in control adrenal glands (Fig. 6A, inset). Some of these cells showed a more elongated and flat shape, as seen in the foetal mutant testis (Fig. 5A, inset). Co-staining for YFP and SF1 showed that all YFP-positive cells in control adrenal glands express SF1 (Fig. 6B, inset). However, most mutant YFP-positive cells did not express SF1, suggesting the *Cyp11a1*:Cre transgene was deleting both *Sf1^{Fl}* alleles in the targeted adrenal cortical cells (Fig. 6B, inset). The large number of YFP+/P450^{SCC}+ cells in the mutant adrenal glands suggests that the P450^{SCC} protein has a slow decay rate, as is the case in the foetal Leydig cell. An increase in apoptosis due to the lack of SF1 could account for the relatively lower number of YFP+/P450^{SCC}– cells in the mutant foetal adrenal gland compared with the testis. However, Lysotracker staining on E14.5 adrenal glands did not show any differences between control and mutant tissue (supplementary material Fig. S2A).

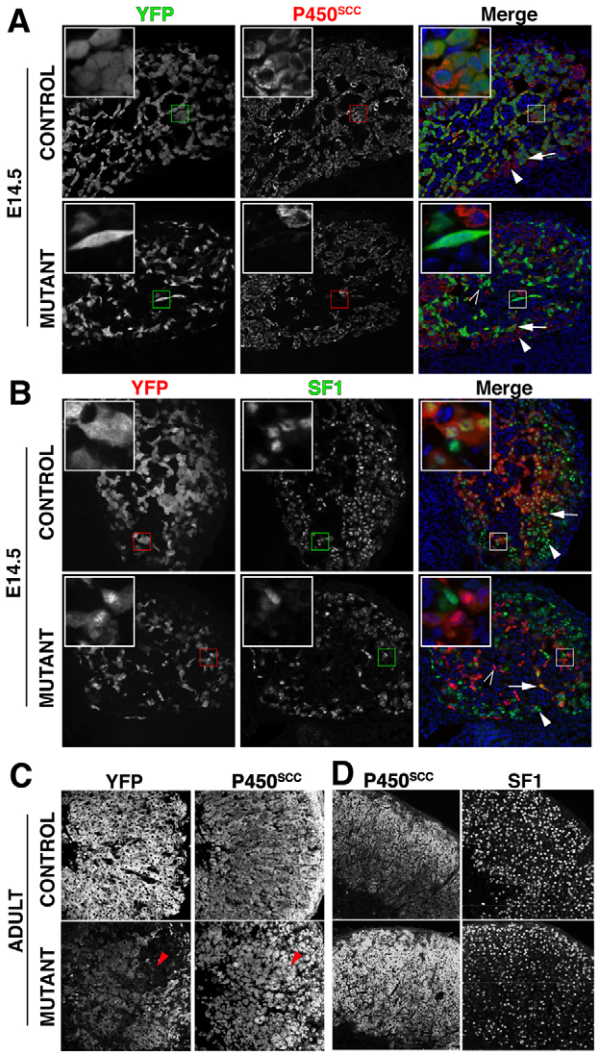

Fig. 6. Adrenal defects in *Cyp11a1*-Cre;*Sf1$^{Fl/Fl}$* mice. (**A**) Protein expression of nuclear and cytoplasmic YFP and mitochondrial P450^{SCC} in control and mutant E14.5 adrenal glands. In control adrenal glands, all YFP-expressing cells are P450^{SCC} positive (arrow and inset), but some P450^{SCC}-expressing cells have no YFP (arrowhead). Mutant adrenal glands contain cells expressing only YFP (unfilled arrowhead and inset), indicative of reduced P450^{SCC} expression. (**B**) SF1 and YFP protein expression in control and mutant E14.5 adrenal glands. Control and mutant adrenal glands exhibit cells positive for both YFP and nuclear SF1 (arrow and inset), whereas some remain negative for YFP (arrowhead and inset). Additionally, a large number of cells in mutant adrenal glands express only YFP (unfilled arrowhead and inset), indicating Cre-mediated deletion of SF1. (**C**) YFP and P450^{SCC} protein expression in control and mutant adult adrenal glands. In control adrenal glands, most cells co-express YFP and P450^{SCC}, although a few express only P450^{SCC}. P450^{SCC} expression was found throughout the cortex of adult mutant adrenal glands. Unlike the control adrenal gland, a number of P450^{SCC}-positive cells in the mutant did not express YFP (red arrowhead). (**D**) P450^{SCC} and SF1 expression in control and mutant adult adrenal glands in adjacent sections.

Mutant adult adrenal cortical cells maintain SF1 expression

As in the case of Leydig cells, adrenal cortical cells have a foetal and adult (or definitive) population. Therefore, we analysed the phenotype of the adult adrenal gland of *Cyp11a1*-Cre;*Sf1*$^{Fl/Fl}$ animals to investigate whether SF1 had a similar role to that in adult Leydig cells. Double immunohistochemistry on control animals showed that all YFP-positive cells expressed P450^{SCC} and, in contrast to foetal stages, only a few P450^{SCC}-positive cells failed to show evidence of Cre recombinase activity, confirming that the *Cyp11a1*-Cre transgene was effective in the cells that contribute to the adult adrenal gland (Fig. 6C). In the mutant adrenal gland, in contrast to all other mutant steroidogenic adult tissues that express SF1, P450^{SCC} expression was not reduced and was found throughout the cortex. YFP expression analysis revealed that some P450^{SCC}-positive cells were positive for YFP and others were not (Fig. 6C). The number of YFP-positive cells was variable and tended to occur in patches, suggesting a clonal origin (see supplementary material Fig. S2C for other examples of mutant adrenal staining). Immunohistochemistry analysis revealed that SF1 had not been lost in the mutant adrenal cortex as most cells retained the protein (Fig. 6D), which is consistent with these cells being positive for P450^{SCC} expression. Therefore, these studies suggest that lack of SF1 is incompatible with adult adrenal cortical cell development.

Characterization of SF1-deficient adult Leydig cells

The cell shape and peritubular association of many mutant adult Leydig cells suggested that the cells had transdifferentiated into a different cell type. Therefore, we investigated the expression of various markers in YFP-positive cells within the mutant testis. Like controls, mutant Leydig cells continue to express the androgen receptor (AR) (Fig. 7A). Peritubular myoid (PTM) cells express AR and smooth muscle actin (SMA), and have flat nuclei and thin cell bodies around the basement membrane of the seminiferous tubules. The cell morphology, nuclear shape and expression of AR in mutant cells raised the possibility they had taken on a PTM cell fate. Immunostaining for YFP and SMA predictably showed that control Leydig cells express YFP but do not co-express SMA (Fig. 7B). Although mutant cells exhibit numerous hallmarks of PTMs, we never observed SMA in mutant YFP cells but found them in tight juxtaposition (Fig. 7B). Vascular smooth muscle cells (VSMCs) and pericytes (PCs) have been proposed to be precursors to adult Leydig cells (Davidoff et al., 2004). To test whether the SF1-deficient Leydig cells had reverted back to a more 'primitive' developmental state that would cause them to express VSMC and PC markers, we analysed the expression of the pan-endothelial marker PECAM (CD31) (Fig. 7C). Even though mutant Leydig cells can exhibit similar cell morphology shapes to endothelial cells, we failed to detect any co-expression of YFP and PECAM (Fig. 7C). Mutant Leydig cells also lacked neurofilament heavy chain expression, which was identified as a marker of a transient intermediate state of adult Leydig cell differentiation (data not shown) (O'Shaughnessy et al., 2008).

The consistent and dramatic change in size and morphology prompted us to assess the mTOR pathway owing to its known role in regulating cell growth (Laplante and Sabatini, 2012). The serine-phosphorylated form of the ribosomal subunit S6 (RPS6) protein (P-S6) has been shown to mark cells with mTOR activation and its target S6 kinase. YFP-positive adult Leydig cells in control testes exhibited high levels of P-S6, showing that the mTOR pathway is active in this cell population in vivo (Fig. 7D). By contrast, YFP-positive cells in mutant testes are negative for P-S6 (Fig. 7D). These data show that the role of SF1 in differentiated steroidogenic cells is not restricted to regulating the transcription of steroid-producing genes. Consistent with this, we analysed by (q)RT-PCR the expression of Leydig cell markers that were not associated with the production of steroids from cholesterol, such as *Insl3*, *Hmgcs1*, which encodes the enzyme HMG CoA synthase, and *Lhcgr*, which encodes the luteinizing hormone receptor, and found that their levels were significantly reduced in the mutant (Fig. 7E). These data suggest that *Sf1* deletion leads to a loss of steroidogenic cell identity.

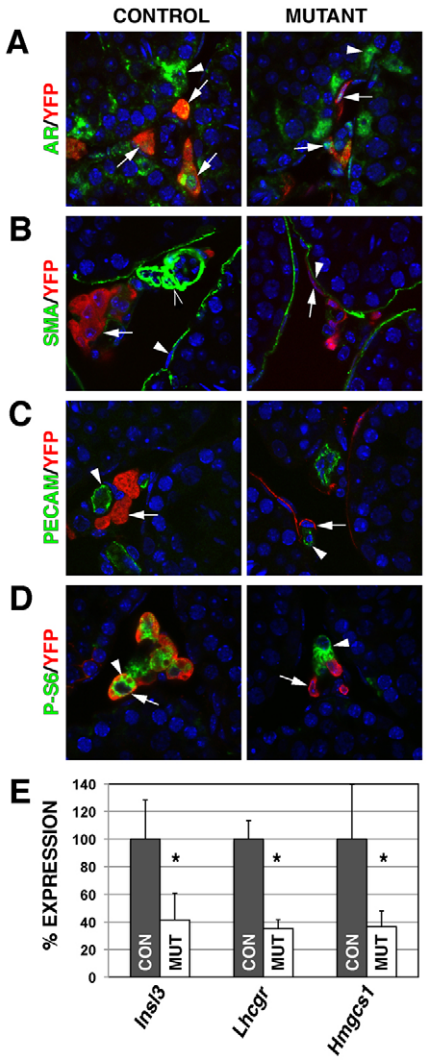

Fig. 7. Properties of SF1-deficient adult Leydig cells. (**A**) Control Leydig and Sertoli cells express nuclear-localized androgen receptor (AR) (arrows and arrowhead, respectively). Mutant Leydig cells express AR (arrows), and AR-expressing Sertoli cells (arrowhead) are unaffected. (**B**) Myoid epithelial cells (arrowhead) and endothelial cells (unfilled arrowhead) of the microvasculature express smooth muscle actin (SMA). Control and mutant Leydig cells (red, arrows) do not express SMA. (**C**) Control and mutant Leydig cells (arrows) do not express the endothelial marker PECAM (arrowheads). (**D**) Control Leydig cells express YFP (arrow) and high levels of P-S6 (arrowheads) whereas mutant Leydig cells (arrows) no longer express P-S6. A Leydig cell that escaped Cre-mediated recombination continues to express P-S6 (arrowhead). (**E**) Quantitative RT-PCR analyses of non-steroidogenic Leydig cell genes in adult control (CON) and mutant (MUT) testes. **P*<0.05. Error bars represent s.e.m.

DISCUSSION

It has been 20 years since the identification of SF1 and the proposal that it is a transcriptional activator of steroidogenic genes based on in vitro cell culture transcription assays and its in vivo expression profile. Our analysis of mutant animals provides the first in vivo genetic and molecular evidence that SF1 is necessary for the steroidogenic gene programme in foetal and adult Leydig cells, ovarian theca and stromal cells and in foetal adrenal cortical cells. All genes associated with steroid production from cholesterol that were analysed showed a consistent decrease in expression in mutant tissues. As *Sf1* deletion occurred after the targeted cells started expressing *Cyp11a1*, we are effectively investigating the role of SF1 in the maintenance of expression of steroidogenic genes within these cells. Therefore, the half life of mRNA and protein need to be considered when analysing the changes occurring in mutant tissues. Our finding that mutant foetal Leydig cells have reduced steroidogenic protein levels that progressively decrease during development argues that loss of SF1 leads to a cessation of transcriptional activation and degradation of associated transcripts and proteins over time.

The viability and reproductive ability of *Sf1* mutant animals was an unexpected phenotype. Nevertheless, this proved to benefit our analysis as it allowed us to study SF1 loss within well developed and healthy organs. A 'masculinization programming window' (MPW) exists in early foetal development, prior to the differentiation of masculinizing tissues but just after the onset of testosterone production (Welsh et al., 2008). As our model must first initiate steroidogenesis, before the loss of *Sf1* and steroid production, the normal masculinization of mutant XY foetuses suggests that sufficient levels of testosterone were present during the MPW. The phenotype of mutant adrenal glands was variable and in some cases they were found to be reduced in size (supplementary material Fig. S2C). However, all cortical cells expressed SF1 and P450^{SCC}, providing an explanation for the lack of an adrenal failure. The most likely explanation for the continued reproductive performance in both male and female Cyp11a1-cre;*Sf1*$^{Fl/Fl}$ mice is the residual steroidogenesis carried out by the Leydig, stromal and theca cells that did not have both *Sf1* alleles deleted by the Cre recombinase or that did not express the Cyp11a1-Cre transgene. A reminiscent finding was observed when Zhao et al. conditionally ablated *Sf1* in the pituitary gland (Zhao et al., 2001a). They observed a hypomorphic phenotype due to incomplete Cre-mediated recombination in the target cells. Our mutant animals produced normal levels of testosterone in the adult and exhibited no spermatogenic phenotype, although the levels of steroidogenic enzymes were reduced to ~20% of controls. This phenotype is very similar to that of mice with the *Cyp11a1* hypomorph allele (Shih et al., 2008).

We failed to detect increased apoptosis in mutant foetal adrenal glands and testes, consistent with the observation that the number of YFP-positive cells was not significantly different between wild-type and mutant organs. This differs from the apoptosis induced within the pre-steroidogenic adrenal and gonad of *Sf1*$^{-/-}$ embryos and suggests that the differentiated steroidogenic cell types do not require SF1 for survival. In contrast to most cell types in which SF1 has been studied, Leydig cells have been shown to be post-mitotic (Habert et al., 2001). One possibility is that the role of SF1 in cell survival is dependent on whether the cells have exited the cell cycle.

The only gonadal steroidogenic cell type independent of SF1 function for the maintenance of steroidogenesis is the corpora luteal cell. Low levels of SF1 have been previously reported in corpora lutea; however, we failed to detect staining above background in control and mutant animals. The related nuclear hormone receptor LHR-1 (NR5A2), which can activate steroidogenic gene transcription in vitro, has been shown to be expressed in corpora luteal cells (Falender et al., 2003; Hinshelwood et al., 2003), suggesting that it could be the primary steroidogenic regulator in this cell population. Unfortunately, *Nr5a2*$^{-/-}$ mice are embryonic lethal and corpora lutea are missing from *Amhr2*$^{tm3(cre)Bhr}$;*Nr5a2*$^{Fl/-}$ ovaries owing to ovulation defects, preventing the functional assessment of this related molecule in this third steroidogenic cell type of the ovary (Duggavathi et al., 2008). The use of our new Cyp11a1-Cre strain in combination with the *Nr5a2* conditional allele could test this model.

Cell lineage marker studies in the mouse have suggested that the definitive or adult adrenal cortical cells are derived from the foetal population and appear prior to birth (Zubair et al., 2008; Wood and Hammer, 2011). The mutant adrenal gland showed a similar dependence on SF1 for steroidogenic gene expression at E14.5 to the other tissues analysed. However, in the adult adrenal cortex, SF1 and P450^{SCC} expression were maintained in most cells, including the YFP-positive cells. These data suggest that that the only cells that were able to give rise to adult cortical cells were those in which either the *Cyp11a1*-Cre transgene was not expressed or in which Cre recombinase had failed to delete both *Sf1* alleles. The patchy nature of P450^{SCC} expression in some mutant adrenal glands (see supplementary material Fig. S2B) is consistent with the model of clonal expansion of SF1-retaining cells. These results also suggest that steroidogenic *Cyp11a1*-expressing cells can serve as precursors of adult cortical cells. As we were not able to detect a significant increase in apoptosis during foetal stages, the fate of the *Sf1*-deleted cells was not clear. Careful analysis of the adult adrenal gland revealed a few YFP-positive cells that did not express P450^{SCC} at the border between the cortex and medulla, particularly in virgin females (supplementary material Fig. S2C). This pattern was reminiscent of the *lacZ* expression observed in adult transgenic mice containing the *Sf1* foetal adrenal enhancer driving this marker (Zubair et al., 2006). These cells also had a more elongated shape, as seen in *Sf1*-deleted cells of the other organs.

An unexpected finding from these studies was the dramatic morphological cell shape changes and loss of expression of most Leydig cell markers in the mutant testis. Even though the cell shape and position of the mutant cells in the adult testis resembled endothelial or PTM cells, our marker studies ruled out a transdifferentiation process to these cell fates taking place. A second possibility is that *Sf1* deletion triggered a de-differentiation programme to a more progenitor-like state. Adult Leydig cells originate from non-steroidogenic stem cells during the prepubertal phase (Habert et al., 2001). However, YFP cells in the mutant adult testis did not consistently express the stem cell markers c-kit or leukemia inhibitory factor receptor (Ge et al., 2006) suggesting that these cells had not acquired a stem or precursor cell fate (data not shown). A third possibility is that these cells represent a regressed or involuted state, as observed with foetal Leydig cells that extinguish their steroidogenic activity in the maturing pubertal testis (Habert et al., 2001). Control foetal Leydig cells are permanently marked by YFP in our experimental design and should continue to express YFP, even if they lose their steroidogenic activity (e.g. loss of *Cyp11a1* expression), change their shape and/or reposition themselves in the intertubular space. Careful analyses of adult testes from control Cyp11a1-Cre mice with the R26R^{YFP} Cre reporter showed that all YFP interstitial cells were steroidogenic and suggests that the non-steroidogenic foetal Leydig cells are removed.

Our study highlights the all-encompassing role of SF1 in the gonad and adrenal gland, where it is required for their initial

development and also serves as a major regulator of the differentiated steroid-producing cells within these organs. In steroidogenic cells, our data show that it not only regulates the expression of genes involved in steroid synthesis but also controls cell morphology, cell signalling and the expression of non-steroidogenic markers of these cells.

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The authors declare no competing financial interests.

Supplementary material
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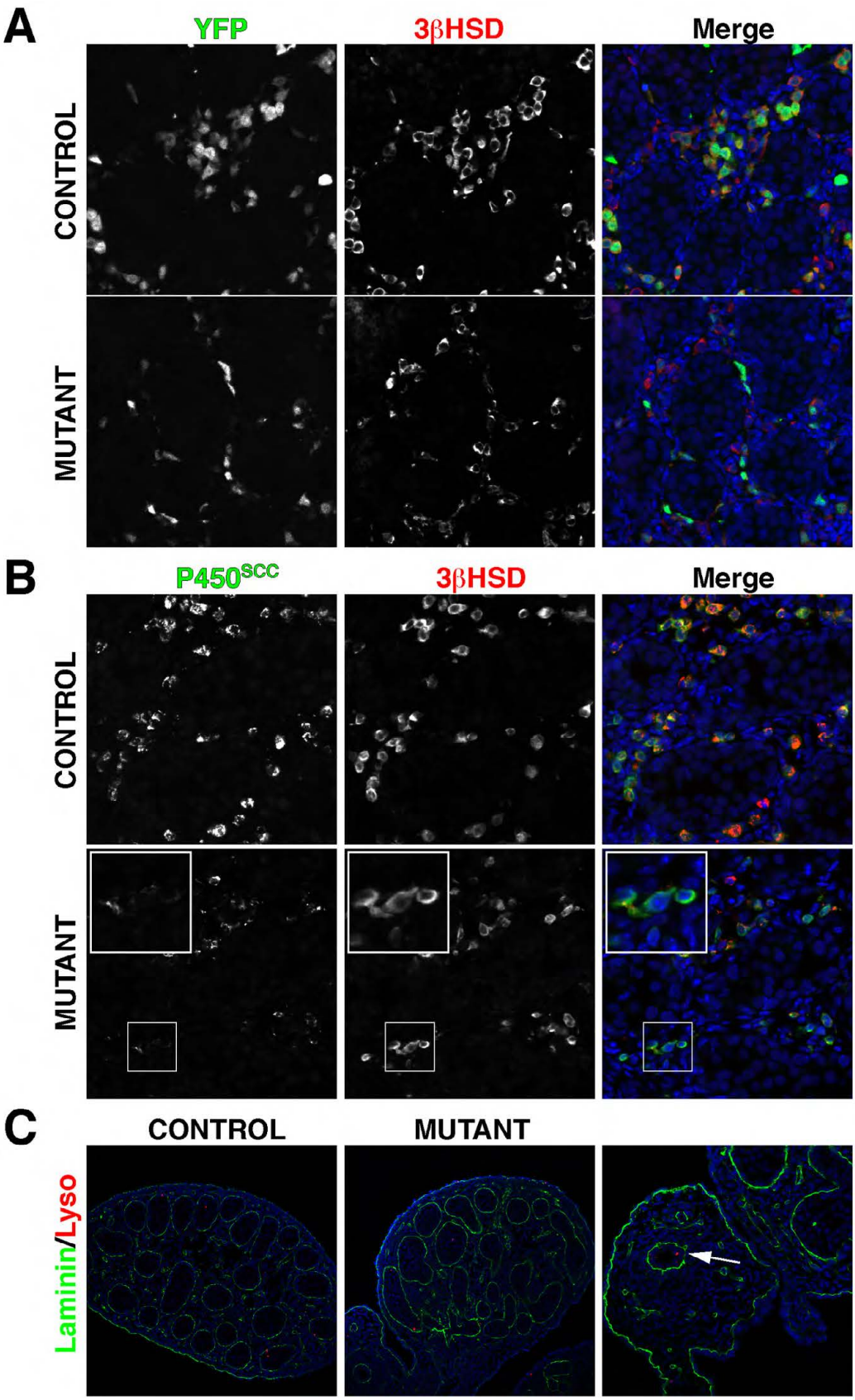

Fig. S1. 3βHSD expression and cell death analysis in E14.5 foetal *Cyp11a1*-Cre;*Sf1*$^{Fl/Fl}$ testes. (**A**) Protein expression of YFP (green) and 3βHSD (red) as detected by co-immunofluorescence in control and mutant E14.5 testes. Interstitial YFP-positive cells in controls are always positive for 3βHSD. These cells exhibit YFP expression throughout the nuclear and cytoplasmic compartments with strong non-uniform endoplasmic reticulum-localized 3βHSD that is devoid from the nucleus. Mutant testes continue to express 3βHSD in a large number of cells. (**B**) Protein expression of P450^{SCC} (green) and 3βHSD (red) in control and mutant E14.5 testes. Control interstitial P450^{SCC} cells co-express 3βHSD whereas mutant Leydig cells exhibit a notable loss of P450^{SCC} but continue to express high levels of 3βHSD (inset). (**C**) Apoptosis, detected by Lysotracker (red) is not altered in mutant foetal testes. Laminin (green) identifies the testis cords (left two panels) and control mesonephric ducts (far right panel) that are known to have cells undergoing apoptosis at this developmental time point (arrow).

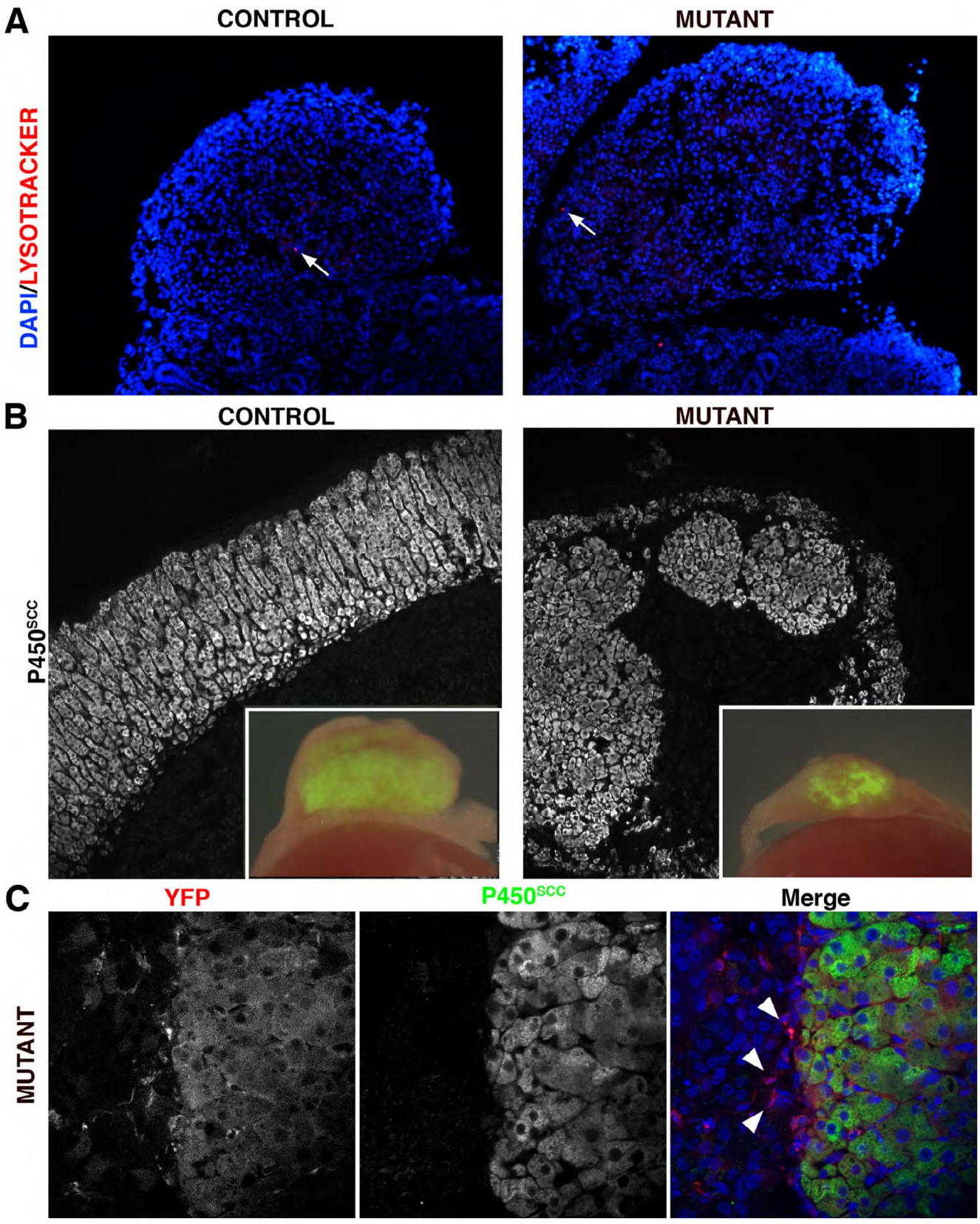

Fig. S2. In-depth analysis of adrenal defects in *Cyp11a1*-Cre;*Sf1*$^{Fl/Fl}$ mice. (**A**) Apoptosis, detected by Lysotracker (red), is not altered in mutant adrenal glands compared with controls (arrows). (**B**) Most adult mutant adrenals appeared morphologically normal, and were indistinguishable from controls. However, a small number were hypoplastic and showed disorganization within the cortex, demonstrated both by immunofluorescence of P450^{SCC} and by whole-mount analysis of YFP expression in freshly dissected glands (inset). (**C**) Double-fluorescence immunohistochemistry for YFP (red) and P450^{SCC} (green) in adult adrenal glands. In female mutants, but never in controls, a population of YFP-positive, P450^{SCC}-negative cells was present at the cortex-medulla boundary (arrowheads). This cell population exhibited a small, flat morphology.

Table S1. qRT-PCR primer sequences

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
Cyp11a1	AAAGACCGAATCGTCCTAAACC	CTTGATGCGTCTGTGTAAGACT
Cyp17a1	GATCTAAGAAGCGCTCAGGCA	GGGCACTGCATCACGATAAA
Hsd3b1	CAGGCCTCCAATAGGTTCTG	GTTGTCATCCACACTGCTGC
Sox9	AGTACCCGCATCTGCACAAC	TACTTGTAATCGGGGTGGTCT
Lhcgr	TCAGGAATTTGCCGAAGAAAGAACAG	GAAGTCATAATCGTAATCCCAGCCACTG
Insl3	TGGTCCTTGCTTACTGCGATCT	CCTGGCTATGTCATTGCAACA
Hmgcs1	TTCAAAGGAAGTGACCCAGG	GGTCTGATCCCCTTTGGTG
Rps2	CTGACTCCCGACCTCTGGAAA	GAGCCTGGGTCCTCTGAACA

Table S2. Antibodies used in immunofluorescence studies

Antibody	Supplier	Method	Working dilution
3β-HSD (rabbit)	Generous gift, Prof. Ian Mason (University of Edinburgh MRC Centre for Reproductive Health, UK)	IF-TSA	1/2000
AR (rabbit)	Millipore, 06-680	IF-TSA	1/250
c-Kit (rabbit)	Cell Signaling, 3074	IF	1/200
GFP (rabbit)	Invitrogen, A6455	IF	1/500
		IF-TSA	1/2000
Ki67 (rat)	Dako, M7249	IF	1/25
		IF-TSA	1/200
Laminin (rabbit)	Sigma, L9393	IF	1/400
LIFR (rabbit)	Santa Cruz, sc-659	IF	1/100
$P450^{SCC}$ (rabbit)	Millipore, AB1244	IF	1/200
		IF-TSA	1/1000
p-S6 (rabbit)	Cell Signaling, 4857	IF-TSA	1/500
PECAM (rat)	BD Pharmingen, 553370	IF	1/50
SF-1 (rabbit)	Generous gift, Prof. Ken Morohashi (Kyushu University, Japan)	IF	1/200
		IF-TSA	1/2000
SMA (mouse)	Generous gift, Dr David Robertson (Breakthrough Toby Robins Breast Cancer Centre, London, UK)	IF	1/4000

IF, immunofluorescence performed using a fluorescently labelled secondary antibody;
F-TSA, immunofluorescence performed using the TSA system.