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Targeted disruption of β-catenin in Sf1-expressing cells impairs development and maintenance of the adrenal cortex

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The nuclear receptor steroidogenic factor 1 (Sf1, Nr5a1) is essential for adrenal development and regulates genes that specify differentiated adrenocortical function. The transcriptional coactivator β-catenin reportedly synergizes with Sf1 to regulate a subset of these target genes; moreover, Wnt family members, signaling via β-catenin, are also implicated in adrenocortical development. To investigate the role of β-catenin in the adrenal cortex, we used two Sf1/Cre transgenes to inactivate conditional β-catenin alleles. Inactivation of β-catenin mediated by *Sf1/Cre*^{high}, a transgene expressed at high levels, caused adrenal aplasia in newborn mice. Analysis of fetal adrenal development with Sf1/Cre^{high}-mediated β-catenin inactivation showed decreased proliferation in presumptive adrenocortical precursor cells. By contrast, the *Sf1/Cre*^{low} transgene effected a lesser degree of β-catenin inactivation that did not affect all adrenocortical cells, permitting adrenal survival to reveal age-dependent degeneration of the cortex. These results define crucial roles for β-catenin – presumably as part of the Wnt canonical signaling pathway – in both embryonic development of the adrenal cortex and in maintenance of the adult organ.

KEY WORDS: Adrenal cortex, β-Catenin, Cre-loxP, Gene knockout, Steroidogenic factor 1

INTRODUCTION

The adrenal cortex is part of the hypothalamic-pituitary-adrenal (HPA) axis that mediates the response to stress through synthesis and release of corticosteroid hormones. Although there are differences among species, the adrenal cortex in both mice and humans is initially derived from the proliferation and migration of coelomic epithelial cells and intermediate mesoderm of the urogenital ridge to form the adrenogonadal primordium (Else and Hammer, 2005; Kim and Hammer, 2007). A distinct adrenal primordium is first detected around the 8th week of gestation in humans and embryonic day 12 (E12.0) in mice (Zubair et al., 2006). After formation of the adrenal primordium, the adrenal cortex undergoes further maturation and development to form a transient fetal zone (x-zone), which is particularly well developed in the human adrenal gland, and the definitive (adult) cortex. After birth, the fetal zone regresses, while presumptive 'stem/progenitor' cells adjacent to the capsule proliferate and renew the definitive cortex through centripetal cellular repopulation (Else and Hammer, 2005; Kim and Hammer, 2007).

Analyses of humans with congenital adrenal hypoplasia and knockout mice have identified various factors required for the initial specification and subsequent development of the adrenal cortex, including the nuclear receptors Sf1 and dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (Dax1, Nr0b1); the transcriptional co-activator CREB-binding protein/p300-interacting transactivator, with ED-rich tail, 2 (Cited2); and the Pre-B-cell leukemia homeobox1 (Pbx1) (Bamforth et al., 2001; Luo et al., 1994; Moore et al., 1998; Sadovsky et al., 1995; Schnabel et al., 2003; Zanaria et al., 1994). In addition to transcriptional regulators, paracrine and morphogenic factors play key roles in the development of the adrenal cortex. Targeted disruption of *Wnt4*, a member of the 'wingless-like MMTV integration site' family of morphogens, was associated with abnormal differentiation of the definitive zone of the adrenal cortex and ectopic expression of 'adrenal-like' cells in the gonads that was attributed to abnormal migration of adrenocortical progenitor cells (Heikkila et al., 2002; Jeays-Ward et al., 2003; Val et al., 2007). Analysis of a kindred with a complex phenotype that includes renal and adrenal hypoplasia and lung abnormalities similarly implicated *WNT4* in human adrenal development (Mandel et al., 2008).

Wnt members participate in various developmental processes during embryogenesis; in adult tissues such as the skin, mammary gland, and hematopoietic and central nervous systems, Wnts function in proliferation, specification of cell fate, stem cell maintenance and differentiation (Blanpain et al., 2007; Logan and Nusse, 2004). In this manuscript, we have focused on the role of the β-catenin signaling. In the absence of Wnt ligands, the pool of β-catenin is sequestered to the cellular membrane/cell adherence junctions and the cytoplasmic concentration is maintained at low levels by ubiquitin-mediated proteolysis through a degradation complex consisting of Axin/adenomatous polyposis coli/glycogen synthase kinase 3 beta (Axin/Apc/Gsk3β). Upon binding of Wnt ligands to their respective frizzled receptors, the degradation complex is disrupted, which permits cytoplasmic and nuclear accumulation of β-catenin. Inside the nucleus, β-catenin interacts with members of the lymphoid enhancer-binding factor/T-cell factor (Lef/Tcf) family of transcription factors to activate expression of target genes. β-Catenin has also been shown to interact functionally with Sf1 to activate target genes synergistically, including *Nr0b1* (Dax1), *Inha* (inhibin-α), *Star* (steroidogenic acute regulatory protein), *Hsd3b1* (3β-hydroxysteroid dehydrogenase), *Cyp19a1* (aromatase) and *Lhb* (β-subunit of luteinizing hormone) (Gummow et al., 2003; Jordan et al., 2003; Mizusaki et al., 2003; Parakh et al., 2006; Salisbury et al., 2007). These studies raised the possibility that β-catenin also plays important roles in adrenocortical development and function. Knockout (KO) mice that are globally deficient in β-

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catenin undergo embryonic lethality during gastrulation and lack mesoderm, precluding analysis of these potential defects in adrenal development (Haegel et al., 1995).

To define the role of β-catenin in the adrenal cortex, we used the Cre-loxP transgenic strategy to conditionally inactivate β-catenin alleles in the adrenal cortex. Depending on the extent of β-catenin inactivation, these studies revealed either complete adrenal aplasia during development or defects in maintenance of the adult cortex resulting in depletion of adrenocortical cells. Thus, β-catenin plays a crucial role in development and maintenance of the adrenal cortex.

MATERIALS AND METHODS

Mice

All experiments involving mice were performed in accordance with institutionally approved and current animal care guidelines from the respective universities. The *Sf1/Cre^{high}* transgene targets high levels of expression of Cre recombinase to the urogenital ridge by E10.0 and activates a Cre-dependent reporter gene throughout the adrenal cortex (Bingham et al., 2006); other sites of expression include pituitary gonadotropes, the ventromedial hypothalamic nucleus, somatic cells of the gonads and the spleen. The *Sf1/Cre^{low}* transgene is a single-copy transgene that is expressed at lower levels in the same sites. Mice carrying the floxed β-catenin allele (*Ctnnb1^{tm2kem}*) were purchased from The Jackson Laboratory (Bar Harbor, ME); this conditional allele contains *loxP* sites flanking exons 2-6, resulting in complete inactivation upon Cre-mediated recombination (Brault et al., 2001). The Cre-dependent reporter Z/AP [*Tg(ACTB-Bgeo/ALPP)1Lbe*] was purchased from The Jackson Laboratory (Lobe et al., 1999). The LEF/Tcf-LacZ (Wnt-Gal) reporter transgenic mice were generously provided by Daniel Dufort (Mohamed et al., 2004).

Following timed matings, embryos were staged by designating noon of the day on which the copulatory plug was detected as E0.5. Correct staging was verified by appropriate morphological criteria as described (Kaufman, 1992). Genotyping for the *Sf1/Cre* transgenes and the β-catenin loxP alleles was performed on the amnion of each embryo and in adult mice, as previously described (Bingham et al., 2006; Brault et al., 2001; Truett et al., 2000). Sexes were determined by PCR analysis with primers specific for the Y-chromosome gene *Zfy* (Jeyasuria et al., 2004).

Analysis of adrenal histology, immunohistochemistry and in situ hybridization analysis

Adrenal glands were collected at the indicated ages and fixed for 2–3 hours in 4% paraformaldehyde/phosphate-buffered saline (PBS). Tissues were dehydrated in graded ethanol solutions and embedded in paraffin before sectioning. Sections were cut at 6 μm and processed using standard procedures.

For immunohistochemical analyses, adrenal glands were processed as above and washed in Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.5). Antigen retrieval was performed by boiling rehydrated sections in 10 mM sodium citrate (pH 6.0) for 20 minutes, followed by one wash in deionized water and two washes in TBST at room temperature. Antibody staining was conducted using VECTASTAIN ABC kits and Vector Mouse on Mouse (M.O.M.) kits according to manufacturer's protocol (Vector Laboratories, Burlingame, CA). Tissue sections were blocked in antibody diluent solution for 1 hour, and then incubated overnight at 4°C with anti-β-catenin (H-102) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-tyrosine hydroxylase (1:500, Pel-Freez Biologicals, Rogers, AR) or either of two antibodies against Sf1: A (1:1000 dilution and generously provided by Dr Ken Morohashi) or B [1:1500 dilution of a rabbit antiserum raised against recombinantly expressed, full-length SF1 protein that was affinity purified as a GST fusion protein and then liberated by thrombin cleavage using standard methods (Invitrogen, Carlsbad, CA)]. The next day, sections were washed, exposed to secondary antibodies and processed for signal detection according to the manufacturer's protocol.

For X-gal staining, tissues were collected at indicated ages. The tissues were prefixed in a *lacZ* fixation solution (2.7% formaldehyde, 0.20% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40, PBS) for 10 minutes, followed by three washes in PBS. The X-gal staining was performed using the β-Gal Staining Set (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. The tissues were stained for 24 hours and then post-fixed in 4% formaldehyde/PBS solution for 1 hour. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for DNA fragmentation was performed using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol.

For in situ hybridization, embryos were collected at the indicated stages, fixed for 4 hours in 4% paraformaldehyde (PFA) and cryoprotected in 20% sucrose overnight. Embryos were embedded in OCT compound (Tissue Tek Sakura, Torrance, CA) and transverse sections were cut at 12 μm. Nonradioactive in situ hybridization analysis using digoxigenin-labeled probes was performed according to standard procedure; a specific protocol is available from the authors upon request. Probes used in this study were 3β-hydroxysteroid dehydrogenase (3β-HSD, Accession Number NM_008293, 512-1523), side-chain cleavage enzyme (Cyp11a1, Accession Number NM_019779, 132-691) and 21-hydroxylase (Cyp21, Accession Number NM_009995, 469-1554).

To examine cell proliferation in E12.5 and E13.5 embryos, the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN) was used. Pregnant mothers were injected at the appropriate stages with BrdU (B-500, Sigma-Aldrich, St Louis, MO; 50 mg/kg body weight) and embryos were harvested 1 hour later and then processed as described above. Slides containing 5 μm sagittal sections were treated according to the manufacturer's protocol with alkaline phosphatase (AP) as the detection agent, followed by NBT/BCIP visualization. The slides were then stained with anti-Sf1 antiserum B to identify adrenocortical cells. Singly- and doubly-stained nuclei were counted in serial (at least three) sections of each genotype (wild type and knockout) at each age. Statistical significance was calculated using Student's *t*-test.

Southern blotting

The *Sf1/Cre^{high}* and *Sf1/Cre^{low}* transgenic mouse lines were prepared and analyzed as previously described (Bingham et al., 2006), except that copy number was determined by ImageJ (NIH, Bethesda, MD). Briefly, the probe contains sequences from the first intron of Sf1; following digestion of genomic DNA with restriction endonucleases (*Eco*RI, *Eco*RV, *Nco*I), the probe hybridizes to DNA fragments of 4 kb (endogenous gene) and 2.6 kb (Sf1/Cre transgene), respectively. The copy number is determined by relative intensities of the signal for the endogenous gene (two copies) and that produced by the transgene (five copies for Sf1/Cre^{high} and one copy for Sf1/Cre^{low}).

Real-time PCR

Adrenal glands were removed, cleaned and snap frozen. Frozen tissues were lysed in Trizol reagent using an electric tissue homogenizer, and total RNA was prepared according to the manufacturer's protocol. Total RNA was treated with DNase (Ambion, Austin, TX) to remove residual genomic DNA and quantitated by UV spectrometry. Total RNA (1 μg) was used to synthesize cDNA using the iScript kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The final cDNA products were purified and eluted in 50 μl of Tris-EDTA buffer using PCR purification columns (QIAGEN, Hilden, Germany) or directly diluted to final volume. Primer sequences for each gene are: human placental alkaline phosphatase (*hAP*), fwd-5′ ctgctgccctccagacat and rev-5′ cgggttctcctcctcaact; *Axin2*, fwd-5′ gcaggagcctcacccttc and rev-5′ tgccagtttctttggctctt; tyrosine hydroxylase (*Th*), fwd-5′ cccaagggcttcagaagag and rev-5′ gggcatcctcgatgagact; *Sf1*, fwd-5′ acaagcattacacgtgcacc and rev-5′ tgactagcaaccaccttgcc; glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), fwd-5′ aatgtgtccgtcgtggatct and rev-5′ cccagctctccccatcacta; *Hsd3b1* (3β-Hsd), fwd-5′ cagtttgtgtcttgggcttaaca and rev-5′ gcagatcacagtgggagtga; *Cyp11b2*, fwd-5′ gcaccaggtggagagtatgc and rev-5′ gccattctggcccatttag; *Cyp21a1*, fwd-5′ gacccaggagttctgtgagc and rev-5′ tccaaaagtgaggcaggaga; *Star*, fwd-5′ aaggctggaagaaggaaagc and rev-5′ ccacatctggcaccatctta; *Actb1* (β-actin), fwd-5′ ctaaggccaaccgtgaaaadg and rev-5′ accagaggcatacagggaca.

For quantitative, real-time PCR (qRT-PCR) analyses of mRNA abundance, reactions were performed with a 2× SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) and gene-specific primers in the ABI 7300 thermocycler (Applied Biosystems, Foster City, CA). Each quantitative measurement was normalized to Rox dye as an internal standard and performed in triplicate. Transcript abundance was normalized in each

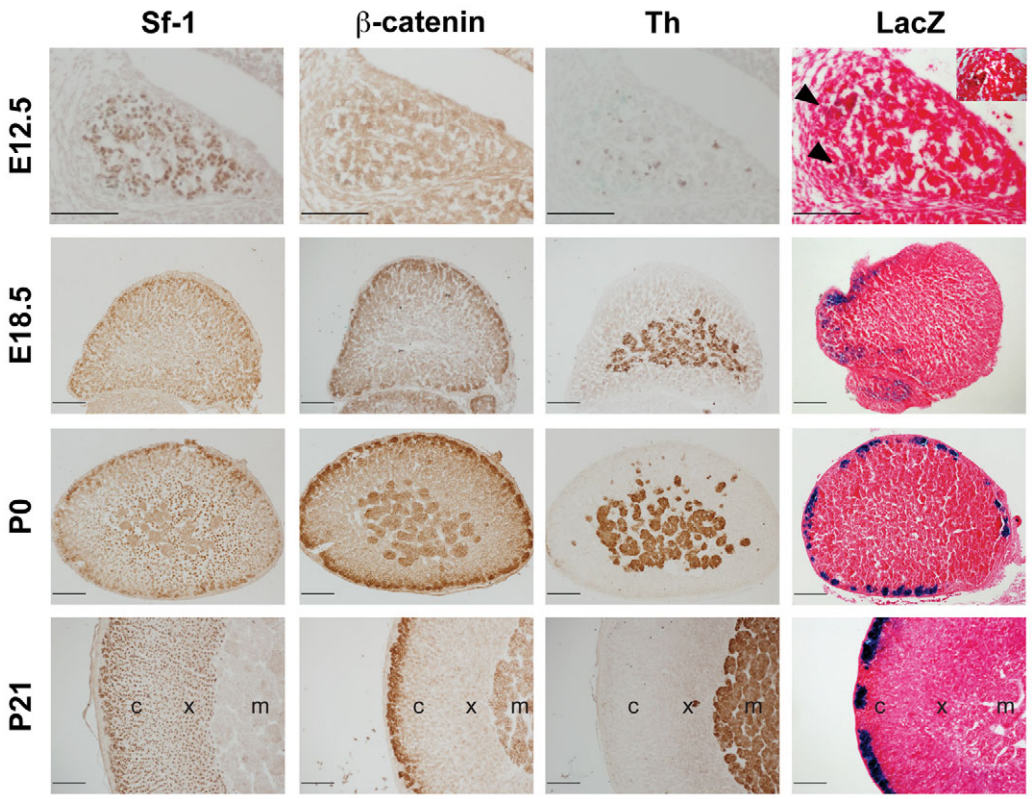


Fig. 1. Developmental profile of β-catenin and canonical Wnt signaling in the embryonic and adult adrenal glands. Immunohistochemical analyses of Sf1 (using antiserum A), β-catenin and tyrosine hydroxylase (Th) were performed as described in the Materials and methods. Colorometric determinations of *lacZ* activity, as a surrogate for active canonical Wnt signaling, in adrenal glands from Wnt-Gal mice at E12.5, E18.5, P0 and P21 were performed as described in the Materials and methods. The black arrowheads highlight *lacZ* staining in the E12.5 section. Scale bars: 100 μm. The inset in the E12.5 section shows a 40× magnification of the area stained for *lacZ*. c, adrenal cortex; x, adrenal fetal/X-zone; m, adrenal medulla.

sample to the average Ct value for mouse Gapdh and β-actin (Livak et al., 2001). For mRNA quantitation, a minimum of three samples from differing genotypes was analyzed. Statistical significance was calculated using Student's *t*-test.

Semi-quantitative determination of *lacZ* expression

To quantitate *lacZ*-positive cells in *Wnt-Gal/β-catenin*$^{F/F}$ (Wnt-Gal) and *Sf1/Cre*^{low}*/Wnt-Gal/β-catenin*$^{F/F}$ (Wnt-Gal/β-cateninKO^{low}) adrenals, we used at least three representative sections from adrenal glands stained for *lacZ* activity and counterstained with Eosin. Images were captured using an Optiphot-2 microscope (Nikon, Melville, NY) with an Olympus DP-70 camera and software system (Olympus, Hauppauge, NY). Images were further analyzed using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) and ImageJ (NIH, Bethesda, MD). Statistical significance was calculated using Student's *t*-test.

ACTH measurements

All mice were individually housed for 24 hours preceding all procedures in a low stress environment. Baseline blood samples were obtained at 09:00 hours by decapitation and collection of core-trunk blood within 30 seconds of initial mouse handling to minimize stress-induced ACTH secretion. Blood plasma was collected using the Microvette CB 300 blood collection tube (Sarstedt, Germany) and stored at –80°C prior to analysis. The ACTH analysis was conducted through Vanderbilt Hormone Assay & Analytical Services Core (Vanderbilt University, Nashville, TN). Statistical significance was calculated using one-way analysis of variance (ANOVA) and Tukey post-hoc test.

RESULTS

Canonical Wnt signaling increasingly becomes restricted to cells adjacent to the developing adrenal capsule

To define the temporal and spatial organization of canonical Wnt signaling in the developing adrenal cortex, we examined the embryonic and postnatal adrenal expression of β-catenin, as well as the expression of *lacZ* driven by the canonical Wnt/β-catenin reporter gene *Wnt-Gal* (Fig. 1). At E12.5, β-catenin and Sf1 staining overlapped throughout cells of the adrenal primoridum. By contrast, a few Sf1-negative cells in the adrenal primordium expressed tyrosine hydroxylase (*Th*), a marker for chromaffin cell precursors that will form the adrenal medulla. This close correlation between sites of Sf1 and β-catenin expression was not maintained at later stages. In adrenals at E18.5 and postnatal day 0 (P0), Sf1 expression was seen in nuclei throughout the adrenal cortex, whereas β-catenin expression was preferentially localized to the subcapsular region. This apparent restriction of β-catenin expression in only a subset of Sf1 positive cells persisted at 3 weeks after birth.

At E12.5, *lacZ* staining (indicative of canonical Wnt signaling) was seen in a few cells in the outer region of the gland, immediately adjacent to the emerging adrenal capsule. The establishment of the capsule and the restricted subcapsular localization of *lacZ* were more evident at E18.5. In the newborn adrenal gland (Fig. 1, P0), active canonical Wnt signaling, as visualized by *lacZ* expression, was seen in discrete clusters of cells at the periphery of the adrenal cortex, again immediately beneath the capsule. By 3 weeks of age, *lacZ* expression in the immediate subcapsular region was more uniform and corresponded more closely to the expression pattern for β-catenin. Moreover, the expression and the active β-catenin signaling in the fetal/X-zone are not observed. These observations were confirmed using a different Wnt-reporter strain (*BAT-Gal*, data not shown). Importantly, we observed active β-catenin signaling – as revealed by *lacZ* expression – in only a subset of subcapsular cells expressing β-catenin protein (Fig. 1). This observation is consistent with the premise that only a subset of adrenocortical cells maintains active canonical β-catenin signaling through Lef/Tcf transcription factors at any given time. Together, these data define the establishment of canonical Wnt signaling, which

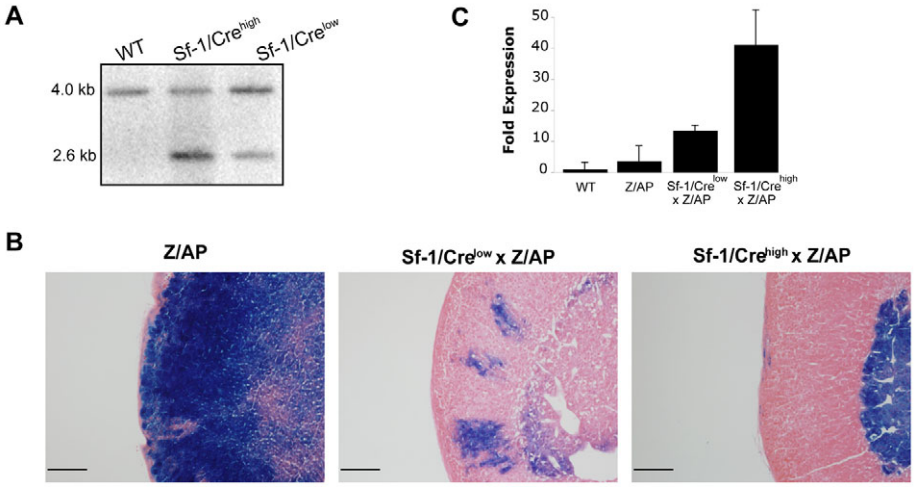


Fig. 2. Cre expression in the adult adrenal gland. (**A**) Southern blot analysis of isolated genomic DNA from wild-type, Sf1/Cre^{low}, and Sf1/Cre^{high} mice. (**B**) *LacZ* activity staining of adrenal glands from 6-week-old male Z/AP, Sf1/Cre^{low} × Z/AP and Sf1/Cre^{high} × Z/AP, as described in the Materials and methods. Scale bars: 100 μm. (**C**) Quantitative-PCR analysis of hAP expression in adrenal glands from 6-week-old male wild-type, Z/AP, Sf1/Cre^{low} × Z/AP and Sf1/Cre^{high} × Z/AP mice.

activates β-catenin dependent transcription in the presumptive adrenocortical stem/progenitor cells of the definitive cortex as it organizes under the developing capsule.

Differential effects of the Sf1/Cre transgenes on adrenocortical expression of Cre recombinase

The two Sf1/Cre transgenes differ in copy number (five copies for *Sf1/Cre^{high}* versus one copy for *Sf1/Cre^{low}* as revealed by quantitative Southern blotting data in Fig. 2A) but have similar sites of expression (Bingham et al., 2006). To visually and quantitatively determine the efficiency of the Cre-mediated recombination in the adrenal cortices of the two Sf1/Cre transgenic lines, we generated mice carrying the Cre transgenes and the Cre-dependent reporter *Z/AP* (Lobe et al., 1999). These reporter mice initially express the *lacZ* reporter gene; following Cre-mediated recombination, they silence *lacZ* and express human placental alkaline phosphatase (*hAP*). As revealed by expression of *lacZ* (Fig. 2B), the recombination efficiencies of the two Sf1/Cre transgenes differed in adrenal glands at 6 weeks of age. The *Sf1/Cre^{low}* transgene mediated only partial recombination of the reporter gene, as evidenced by persistent *lacZ* staining in some cortical cells. By contrast, the *Sf1/Cre^{high}* transgene completely abolished *lacZ* expression in the adrenal cortex, suggesting that it mediates Cre-dependent recombination in the adrenal cortex in a highly efficient manner. Moreover, quantitative PCR analyses of adrenal expression of hAP confirmed the different efficiencies of Cre-mediated recombination for the two Sf1/Cre transgenes (Fig. 2C), with approximately threefold higher expression of hAP in Z/AP mice carrying the *Sf1/Cre^{high}* transgene than in those with the *Sf1/Cre^{low}* transgene. The results indicate that the Cre protein in Sf1/Cre^{low} adrenals is expressed in a lower number of cells of the cortex than that in Sf1/Cre^{high} mice.

β-catenin KO mediated by the *Sf1/Cre^{high}* transgene causes adrenal aplasia

Having characterized the relative efficiencies of the two Sf1/Cre transgenes in driving Cre-mediated recombination, we next examined their functional effect on the conditional β-catenin allele (*Ctnnb1^{tm2kem}*), crossing mice with either the *Sf1/Cre^{low}* or the *Sf1/Cre^{high}* transgene and one copy of the floxed β-catenin allele with mice that were homozygous for the floxed β-catenin allele.

Direct effects of *Sf1/Cre^{high}*-mediated β-catenin KO on β-catenin expression were examined using immunohistochemical assays with an anti-β-catenin antibody. As shown in Fig. 3A (and similar to results in Fig. 1), β-catenin at E12.5 was expressed in the wild-type adrenal primordium, as well as in other regions of the embryo (top panels). Thereafter (E14.5 and E16.5), the cells that expressed β-catenin again localized as a thin layer of cells near the subcapsular zone at the periphery of the adrenal cortex. In the *Sf1/Cre^{high}*-mediated β-catenin KO mice, by contrast, adrenal immunoreactivity for β-catenin was not detected in sections at any of these stages (Fig. 3C, bottom panels), indicating that the *Sf1/Cre^{high}* transgene caused complete ablation of β-catenin expression. These studies document that Cre recombinase driven by the *Sf1/Cre^{high}* transgene abrogates expression of β-catenin at very early stages of adrenal development.

Based on the striking effect on β-catenin expression, we next examined adrenal development at different stages, focusing both on histology (Fig. 3B) and on expression of Sf1 (Fig. 3C). At E12.5, the developing testis was visible as a group of cells under the coelomic epithelium, some of which expressed Sf1. Immediately adjacent to this gonadal precursor are cells that comprise the adrenal primordium, which also expressed Sf1 (Fig. 3B). At this early developmental stage, we observed relatively subtle histological differences between wild-type and β-catenin KO mice (Fig. 3 B), although the apparent decrease in the number of Sf1-positive cells (Fig. 3C) suggests that the adrenal primordium is already affected by the conditional β-catenin KO.

By E14.5, the β-catenin KO adrenal glands were smaller than their wild-type counterparts (Fig. 3B) and contained considerably fewer Sf1-positive cells (Fig. 3C); very similar findings were observed in sections from E16.5 embryos. In fact, many of the cells remaining in the region where the adrenal gland normally resides at E16.5 expressed *Th*, identifying them as chromaffin cell precursors derived from the neural crest. Finally, by E18.5, all remnants of an adrenal gland, including the presumptive chromaffin cells (Fig. 3B), had disappeared.

Based on the striking effect of β-catenin inactivation on adrenal structure, we also used in situ hybridization analyses to examine the effect on expression of several steroidogenic enzymes. As shown in Fig. 4, the cholesterol side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 21-hydroxylase (Cyp21) normally are expressed in a subset of cells within the adrenal primordium at E12.5, with higher expression seen at E13.5. By marked contrast, expression of Cyp11a1 and 3β-HSD was decreased relative to wild-type levels at E12.5; even greater apparent differences in expression of all three steroidogenic enzymes in β-catenin KO mice was apparent at

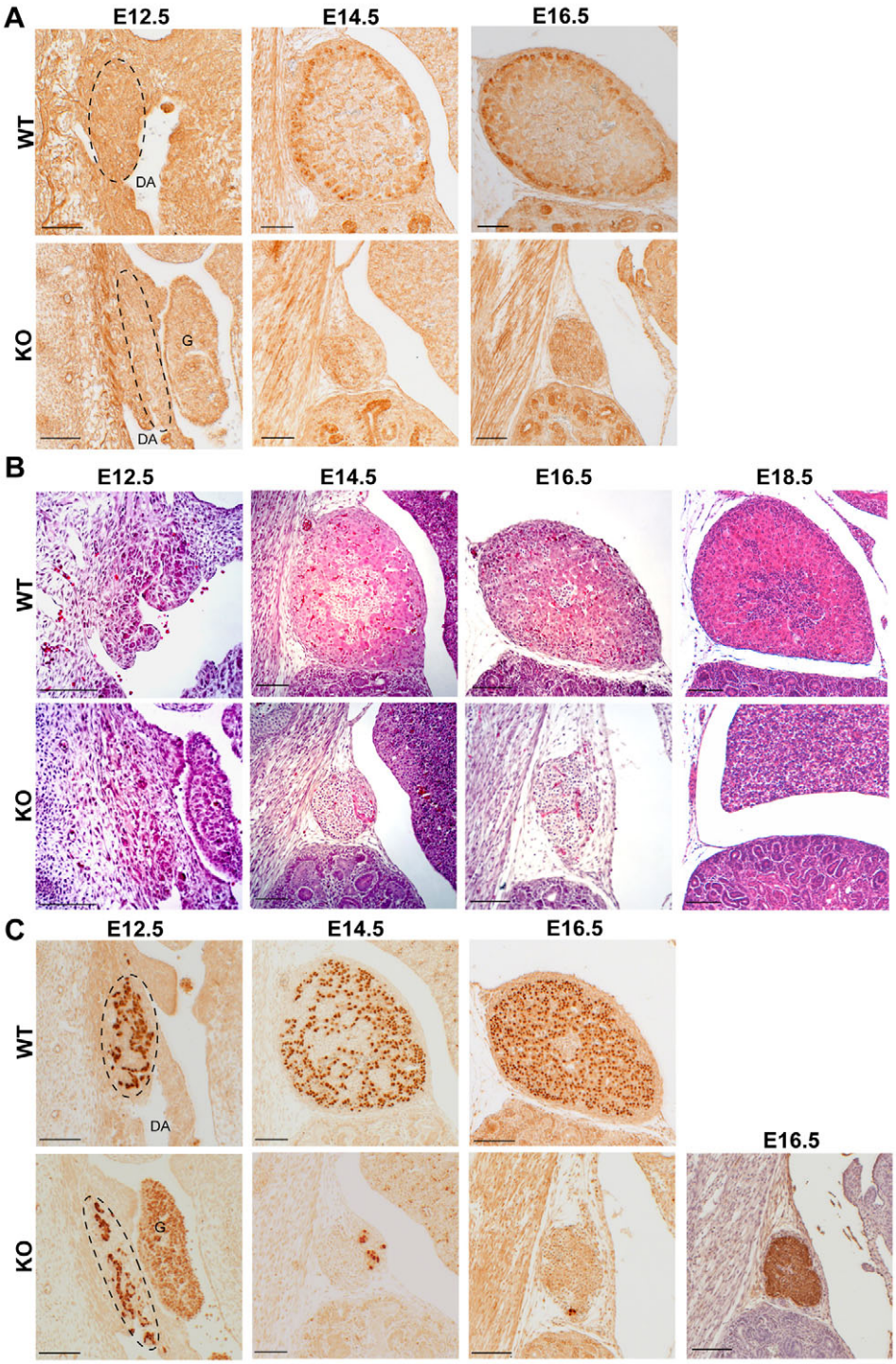


Fig. 3. Comparison of wild type and Sf1/Cre^{high}-mediated β-catenin KO embryos at different developmental stages. Embryos were harvested from timed-pregnant dams at the indicated stages and sagittal sections were processed and analyzed as described in the Materials and methods. (**A**) Immunohistochemical analysis of β-catenin expression. (**B**) Hematoxylin and Eosin (H&E) staining of sections. (**C**) Immunohistochemical analysis of SF1 expression using antiserum B against Sf1 to identify adrenocortical progenitors. The offset shows a section from an E16.5 embryo stained with an antiserum against tyrosine hydroxylase, which detects chromaffin cell precursors. DA, dorsal aorta; G, gonad. Scale bars: 100 μm.

E13.5. These studies suggest that β-catenin is directly or indirectly required for the expression of multiple components of the steroidogenic pathway in the adrenal gland from very early stages of differentiation.

Adrenal and gonadal regression in Sf1 KO mice is associated with increased apoptosis (Luo et al., 1994), whereas studies in mice with Leydig cell-specific disruption of Sf1 showed that gonadal proliferation is markedly decreased (Jeyasuria et al., 2004). We therefore used BrdU labeling to assess proliferation in mice with *Sf1/Cre^{high}*-driven ablation of β-catenin. As shown in Fig. 5, BrdU incorporation in the region of the adrenal primordium in the KO mice did not differ from that seen in wild-type mice at E12.5 but was decreased considerably at E13.5. Consistent with our previous observation that the absence of β-catenin affects adrenal development from very early stages (Fig. 3B), the total number of Sf1-expressing cells in the KO mice was significantly decreased at both time points. The decreased number of cells labeled with BrdU supports an important role for β-catenin in regulating cell proliferation in the embryonic adrenal gland. By contrast, apoptosis – as revealed by TUNEL staining for DNA fragmentation – did not differ significantly in the same region of the developing adrenal gland (data not shown). Thus,

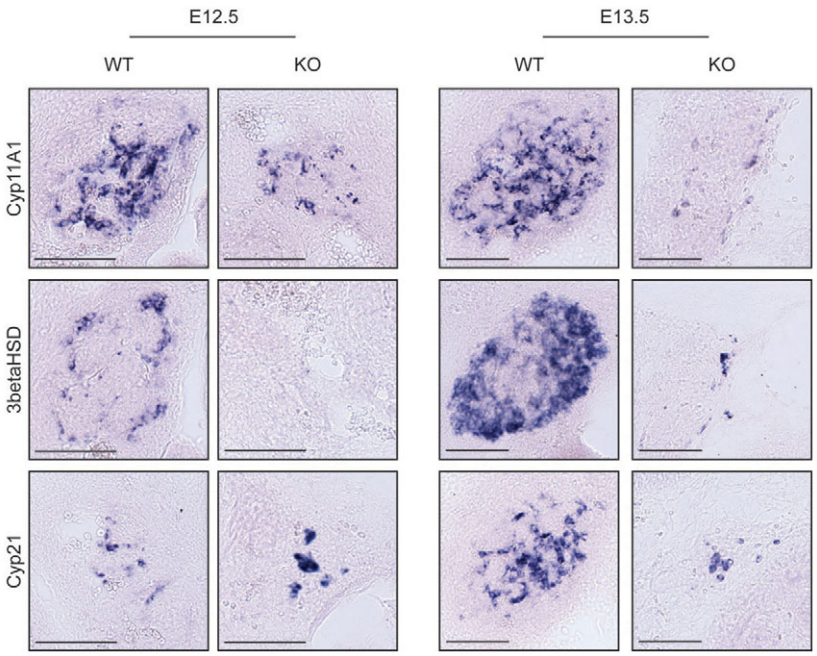


Fig. 4. Effect of Sf1/Cre^{high}-mediated β-catenin KO on adrenal expression of steroidogenic enzymes. Embryos from mice of the indicated genotypes were harvested at E12.5 or E13.5, processed as described in the Materials and methods, and used for in situ hybridization analyses. Probes included cholesterol side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 21-hydroxylase (Cyp21). Scale bars: 100 μm.

decreased proliferation apparently is the predominant factor in the adrenocortical regression seen in mice with β-catenin KO driven by the *Sf1/Cre^{high}* transgene.

Effect of β-catenin inactivation mediated by the Sf1/Cre^{low} transgene on adrenal structure and function

We also examined the effect of β-catenin disruption mediated by the *Sf1/Cre^{low}* transgene on adrenal structure (Fig. 6A). Both in analysis of the intact urogenital region (top panels) and in histological sections (bottom panels), the WT adrenal glands immediately after birth were located rostral to the kidneys. Very similar findings were seen in mice with *Sf1/Cre^{low}*-mediated KO of β-catenin. Consistent with the developmental studies described above, mice with *Sf1/Cre^{high}*-mediated KO of β-catenin exhibited complete absence of the adrenal gland (Fig. 6A). These data demonstrate that either the timing or the extent of β-catenin ablation mediated by the two Sf1/Cre transgenes differs in functionally important ways.

To explore the effects of the *Sf1/Cre^{low}*-mediated disruption of β-catenin on postnatal adrenocortical function, we examined the extent of inhibition of subcapsular canonical Wnt signaling mediated by the *Sf1/Cre^{low}* transgene. Crossing the *Sf1/Cre^{low}* transgene into the Lef/Tcf-*lacZ* (*Wnt-Gal*) reporter revealed that the field of subcapsular cells still expressing *lacZ* was diminished by ~50% in these mice (Fig. 6B), coincident with a roughly comparable decrease in the expression of the canonical Wnt/β-catenin target gene Axin2 (Fig. 6C). Therefore, the *Sf1/Cre^{low}* transgene inactivates the β-catenin gene (and hence canonical Wnt signaling) in only a subset of adrenocortical cells. This finding was supported by direct analysis of Cre-mediated recombination of β-catenin in genomic DNA samples from adrenal glands of mice with the differing genotypes (Fig. 6D). Although the presence of adrenal medullary cells can potentially influence these analyses, the cortical-specific expression of the *Sf1/Cre* transgene and lack of active Wnt signaling in the medulla makes these concerns negligible. Therefore, these data are consistent with the model that partial but not complete recombination of β-catenin occurs in the *Sf1/Cre^{low}* mice. Similarly,

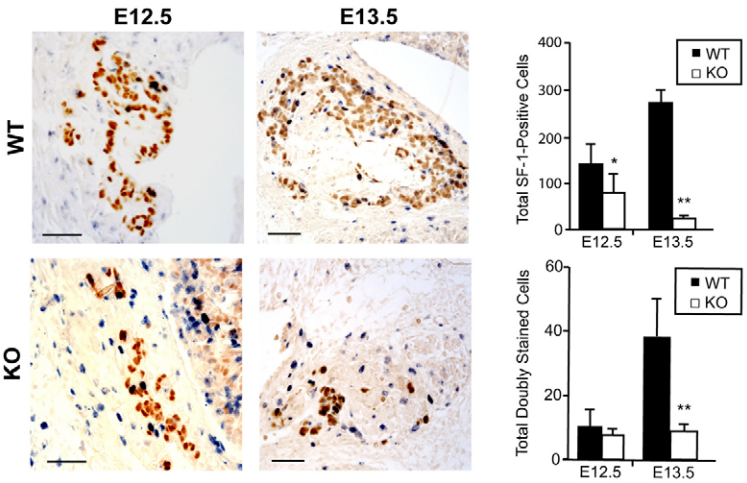


Fig. 5. Loss of β-catenin mediated by the Sf1/Cre^{high} transgene results in a decreased number of adrenocortical cells at least partly due to decreased proliferation. BrdU incorporation into wild-type and Sf1/Cre^{high}-mediated KO mice was determined at the indicated stages as described in the Materials and methods. Mice with Sf1/Cre^{high}-mediated β-catenin KO appeared to have decreased BrdU staining in the region of the adrenal primordium. The graphs show quantitation of Sf1-positive cells (top) and Sf1-positive cells that were also positive for BrdU (bottom) in E12.5 and E13.5 embryos. The number of Sf1-positive cells was decreased significantly at both E12.5 (*P<0.01 versus wild type) and E13.5 (**P<0.01 versus wild type), whereas the number of doubly labeled cells was only decreased significantly at E13.5 (**P<0.01 versus wild type). Scale bars: 50 μm.

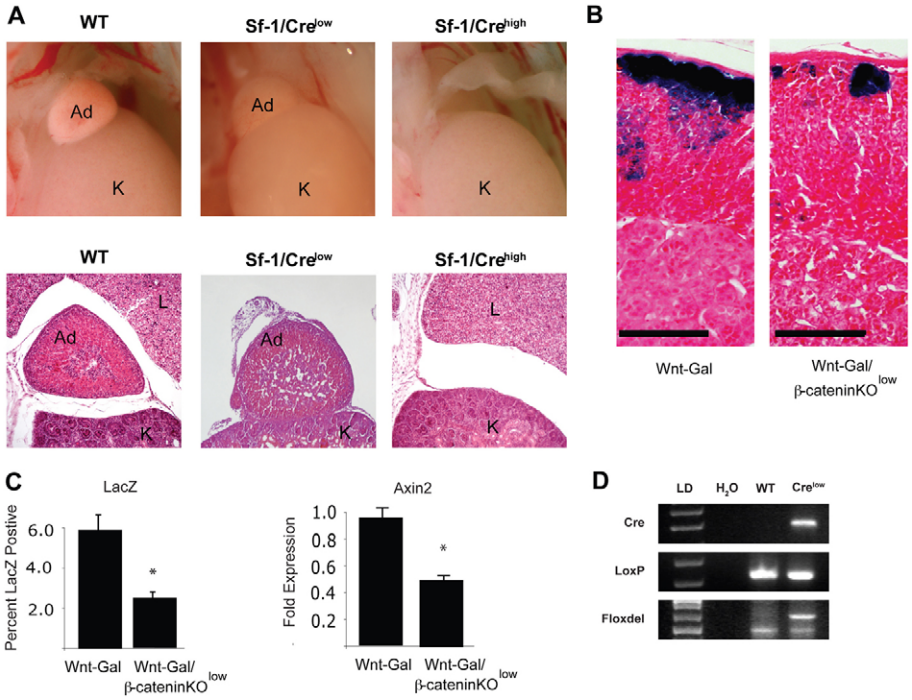


Fig. 6. Sf1/Cre^{low}-driven loss of β-catenin permits adrenal survival owing to incomplete inactivation of β-catenin. (**A**) Photograph of the urogenital region of newborn (P0) WT, Sf1/Cre^{low}- and Sf1/Cre^{high}-β-catenin KO mice. H&E staining of sagittal sections in the region of the adrenal gland from P0 wild-type mice or from mice with β-catenin KO mediated by the indicated Sf1/Cre transgenes. (**B**) *LacZ* stained sections of Wnt-Gal and Wnt-Gal/β-catenin KO^{low}, as described in the Materials and methods. Scale bars: 200 μm. (**C**) Quantitation of active canonical Wnt signaling by comparing the area of *lacZ* staining versus the total adrenal area (P<0.05 versus Cre-negative littermates). Quantitative-PCR analysis of Axin2 expression in adrenal glands from wild-type and Sf1/Cre^{low}-β-catenin KO mice (P<0.05 versus Cre-negative littermates). Ad, adrenal gland; K, kidney; l, liver. (**D**) Demonstration of recombination of the β-catenin gene in DNA samples from wild-type mice and those with β-catenin inactivation mediated by the Sf1/Cre^{low} transgene.

the presence of *lacZ*-positive cortical cells in *Sf1/Cre^{low}/Z/AP* mice (Fig. 2B) indicates that some cells in the Sf1/Cre^{low} adrenal did not undergo Cre-mediated recombination, which presumably permits survival of cells that have the potential to engage β-catenin signaling.

In order to further determine the effects of β-catenin deficiency mediated by the *Sf1/Cre^{low}* transgene, we analyzed adrenocortical structure and steroidogenic capacity in these mice at different time-points. At 15 weeks of age (Fig. 7), the adrenal glands of wild-type mice and those with β-catenin KO mediated by the *Sf1/Cre^{low}* transgene had comparable histology and Sf1 expression. In addition, the adrenal cortex at this stage maintained some degree of subcapsular β-catenin staining, presumably in cells that escaped Cre-mediated recombination of β-catenin (Fig. 7). At 30 weeks of age, we began to observe histological thinning and disorganization of the adrenal cortex in a subset of the KO mice. As shown in Fig. 6, the width of the adrenal cortex in mid-adrenal sections was markedly reduced in 50% of *Sf1/Cre^{low}* β-catenin KO mice (n=6) relative to the wild-type glands (n=6). By 45 weeks of age, all mice with *Sf1/Cre^{low}*-mediated β-catenin KO (n=4) exhibited histological disorganization and thinning of the adrenal cortex (Fig. 7). The adrenal cortex in these mice also appeared to have a decreased number of Sf1-positive cells, revealing the importance of β-catenin in adult adrenocortical organ maintenance. Thus, although β-catenin staining was still seen in some cells, the partial deficiency of β-catenin mediated by the *Sf1/Cre^{low}* transgene eventually led to striking changes in the adrenal cortex, presumably secondary to depletion of at least some of the population of adrenocortical stem/progenitor cells. These findings argue that there is a cumulative effect of the *Sf1/Cre^{low}*-mediated depletion of β-catenin activity over time that ultimately affects adrenocortical maintenance in all mice of this line.

To explore the mechanism of this postnatal adrenocortical depletion, we performed TUNEL staining to assess DNA fragmentation, which is indicative of apoptosis (Fig. 7). Although the adrenal glands in the *Sf1/Cre^{low}* β-catenin KO mice at 15 weeks were relatively intact histologically, an increase in TUNEL staining in the adrenal cortex was consistent with increased cell death via apoptosis occurring at this time. The marked increase in TUNEL staining in the adrenals of *Sf1/Cre^{low}* β-catenin KO mice at 30 weeks (Fig. 7) indicates that the loss of β-catenin in the adrenal cortex progressively contributes to loss of adrenocortical tissue *via* apoptosis. We also observed increased TUNEL staining in the adrenal medulla of 30-week old *Sf1/Cre^{low}* β-catenin KO mice (Fig. 7), consistent with the known roles of the cortex in maintaining medullary function.

Following the observation of cortical thinning and disorganization in 50% of conditional β-catenin KO mice at 30 week, we stratified these mice into two groups (histological failure versus no histological failure) and analyzed these mice in more detail with regards to adrenal size, ACTH levels and steroidogenic enzyme expression. We predicted that mice with histological failure would have smaller adrenal glands with a compensatory elevation in ACTH levels with or without a decrease in steroidogenic enzyme expression. As shown in Table 1, the KO mice with clear histological failure (n=3) have a significant reduction in adrenal mass compared with both wild-type and the KO mice without evidence of histological failure, consistent with the observed stochastic rate of cortical depletion. In addition, these mice have significantly elevated basal ACTH levels compared with wild type

Table 1. Analysis of adrenal weight and basal level ACTH measurement of 30-week-old Sf1/Cre^{low}-β-catenin knockout mice

	Adrenal mass (normalized)	Basal ACTH (pg/ml)
Wild type	$1.31\times10^{-4}\pm1.83\times10^{-5}$	64.29±31.16
KO: cortical decline	$9.88\times10^{-5}\pm5.73\times10^{-6}$*	168.77±80.90*
KO: non-cortical decline	$1.35\times10^{-4}\pm1.99\times10^{-5}$	59.58±38.07

The mice were separated into wild type, KO-histological failure and KO-non-histological failure groups. The mass of adrenal glands and the whole mouse were obtained at time of euthanasia. The adrenal mass was normalized to the total body weight (*P<0.05 versus wild type and KO non-histological failure). The basal levels of ACTH were measured as described in the Materials and methods (*P<0.05 versus wild type and KO non-histological failure).

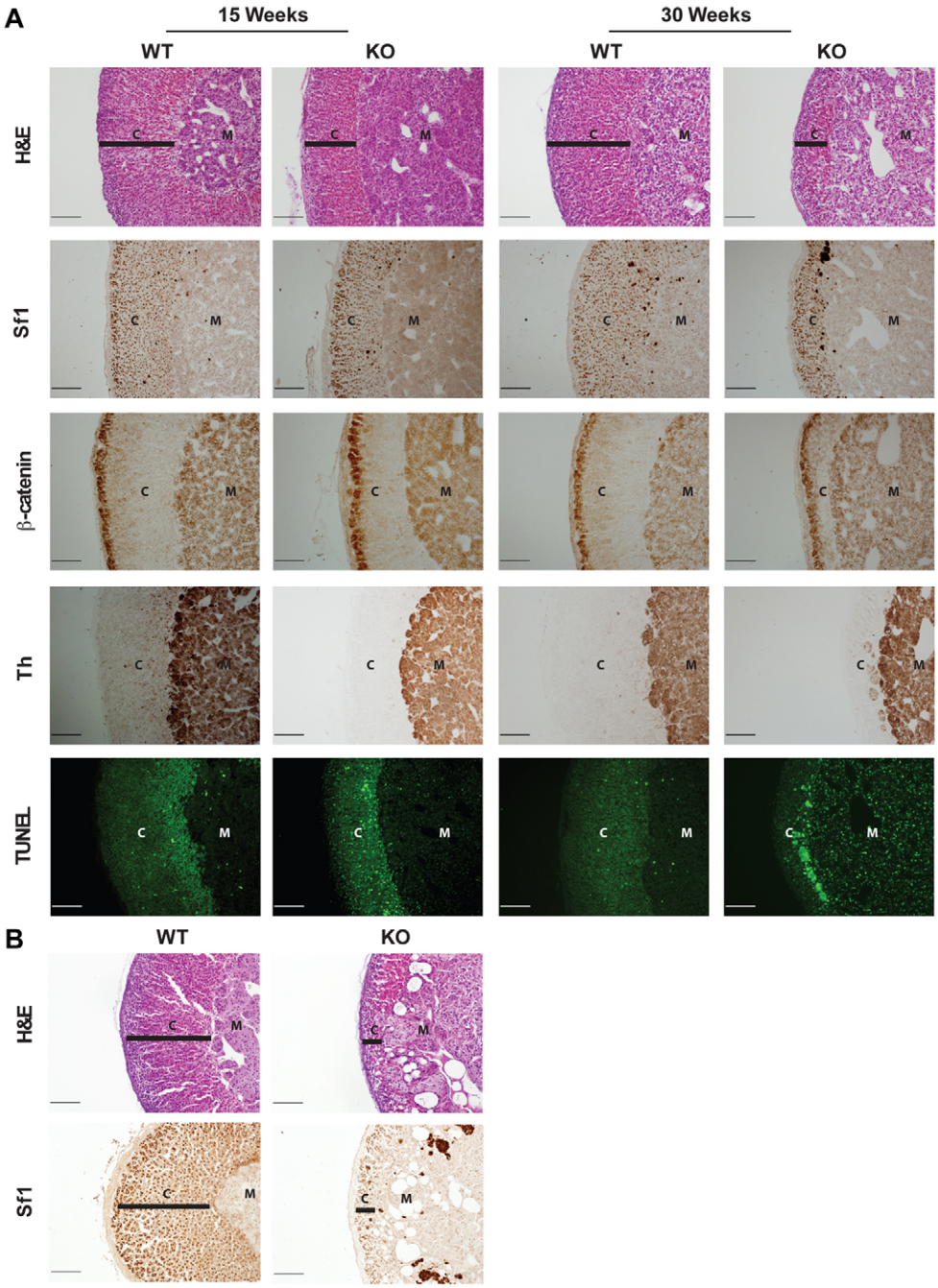


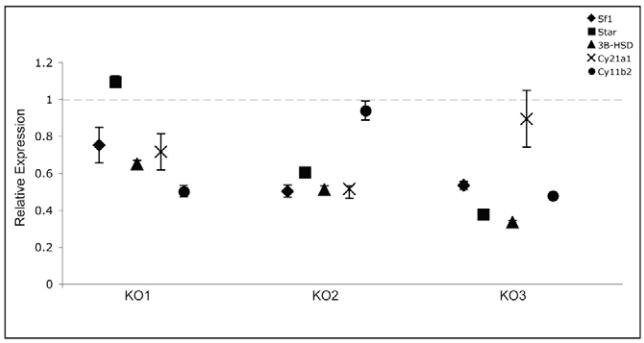
Fig. 7. Progressive decline of adrenocortical volume in Sf1/Cre^{low}-mediated β-catenin KO mice. (**A**) Histological analysis of the adrenal glands from wild-type and Sf1/Cre^{low}–mediated β-catenin KO mice at different ages. Adrenal glands were isolated from mice of the indicated genotypes as described in Materials and methods, and processed for immunohistochemical detection of Sf1 (antiserum A), β-catenin and tyrosine hydroxylase (Th). TUNEL assay was conducted on the section as described in the Materials and methods. (**B**) H&E staining and Sf1 immunohistochemistry (antiserum A) of adrenal from wild type and Sf1/Cre^{low}-mediated β-catenin KO mice at 45 weeks of age. The black bar highlights the adrenal cortex. Scale bars: 100 μm. C, adrenal cortex; M, adrenal medulla.

(*n*=6) and with KO mice without histological failure (*n*=3). Last, the expression of a panel of steroidogenic genes in individual KO mice with histological failure was routinely decreased compared with mean values in wild-type mice (Fig. 8). These qPCR studies also supported the apparent decrease in immunohistochemical detection of Sf1 described above. The reciprocal elevation in ACTH and reduction in cortical size with concordant decrease in steroidogenic enzyme expression is consistent with developing adrenal failure.

DISCUSSION

The complete absence of adrenal glands in mice with efficient disruption of β-catenin driven by the *Sf1/Cre^{high}* transgene unequivocally establishes the essential role of β-catenin in mouse adrenocortical development. This effect is qualitatively different than that seen in *Wnt4* KO mice (Heikkila et al., 2002), perhaps owing to potential roles of Wnt4 in additional noncanonical Wnt signaling (Brisken et al., 2000; Stark et al., 1994; Vainio et al., 1999), to the activation of the canonical pathway in the adrenal gland by additional Wnt ligands, or to Wnt-independent actions of β-catenin (Hino et al., 2005; Taurin et al., 2006).

Regardless, β-catenin can be included in the small group of transcriptional regulators, including Sf1, Dax1, Wt1 Pbx1 and Cited2, the deficiency of which causes complete adrenal absence. Although further studies will be needed to define the molecular mechanisms of β-catenin signaling in adrenocortical cells, the reported synergy between β-catenin and Sf1 suggests that these two genes may interact to regulate the expression of crucial target gene(s) whose expression is essential to stimulate adrenocortical



	Gene	Relative Expression w/ S.E.M.
KO1	Sf1	0.75 ± 0.10
	Star	1.09 ± 0.03
	3β-HSD	0.65 ± 0.02
	Cyp21a1	0.71 ± 0.10
	Cyp11b2	0.50 ± 0.03
KO2	Sf1	0.50 ± 0.03
	Star	0.60 ± 0.00
	3β-HSD	0.51 ± 0.02
	Cyp21a1	0.51 ± 0.02
	Cyp11b2	0.94 ± 0.05
KO3	Sf1	0.53 ± 0.02
	Star	0.38 ± 0.01
	3β-HSD	0.33 ± 0.01
	Cyp21a1	0.89 ± 0.15
	Cyp11b2	0.48 ± 0.01

Fig. 8. Analysis of differentiated adrenocortical markers. The quantitative PCR was performed on adrenal cDNA from the 30-week-old wild-type and Sf1/Cre^{low}-β-catenin KO-histological failure mice as described in the Materials and methods.

proliferation and/or inhibit apoptosis. Of interest, proposed target genes of both β-catenin and Sf1 include a number of genes that regulate proliferation, providing plausible candidates for this co-regulation (Doghman et al., 2007; Gummow et al., 2003; Jordan et al., 2003; Mizusaki et al., 2003; Parakh et al., 2006; Salisbury et al., 2007). Moreover, mutations and/or amplification of both Sf1 and β-catenin have been linked to adrenocortical tumorigenesis in humans, again suggesting that these two genes play key roles in adrenocortical cell proliferation in vivo (Figueiredo et al., 2005; Tissier et al., 2005). Sf1 exhibits marked dose-dependent effects on growth, as revealed by the impaired adrenal development seen in mice with Sf1 haploinsufficiency (Beuschlein et al., 2002; Bland et al., 2004; Bland et al., 2000). By contrast, haploinsufficiency for β-catenin apparently is compatible with normal adrenocortical function, as we observed no obvious adrenal phenotype in mice carrying the *Sf1/Cre^{high}* transgene and one conditional β-catenin allele. Given our model that these transcriptional co-regulators cooperate in adrenocortical organogenesis, the basis for their differing dose dependence is an important area for further investigation.

Our direct analyses of immunoreactive β-catenin and indirect analyses of β-catenin-dependent transcription using the Wnt-Gal reporter revealed that some adrenocortical cells retaining immunoreactive β-catenin in the *Sf1/Cre^{low}*-mediated β-catenin KO mice did not activate β-catenin-dependent gene expression. Thus, β-catenin activates downstream events of its canonical signaling pathway in only a subset of adrenocortical cells where it is expressed. The precise mechanisms that convey competence for β-catenin-mediated transcription to a subset of cells expressing the gene remain to be determined, as does the relative distribution of β-catenin protein between the cytoplasmic and nuclear compartments. It is tempting to speculate that other transcription factors/co-regulators also are permissive for β-catenin-dependent transcription and that the expression of these factors 'marks' a specific population of cells within the adrenal cortex. Further studies will be needed to identify these putative co-regulators and to define just how they may interact with β-catenin to affect the pool of adrenocortical progenitors.

The presence of cells that express *Th* in the region of the adrenal gland argues strongly that the common sympathoadrenal precursors can differentiate into chromaffin cells, despite the marked depletion of Sf1-expressing adrenocortical cells. Although cell culture studies suggested an obligatory role for steroid hormones in the differentiation of these precursors into chromaffin cells (Anderson, 1993), studies with Sf1 KO mice demonstrated that the complete loss of steroidogenic adrenocortical cells was compatible with the differentiation of sympathoadrenal precursors into cells that exhibited several characteristics of chromaffin cells (Gut et al., 2005). Although we have not explored the function of these cells in detail, they apparently disappear from the region of the adrenal gland by E18.5, arguing that the adrenal cortex plays important roles in supporting their continued survival. The enhanced TUNEL staining in the postnatal adrenal medulla of mice with *Sf1/Cre^{low}*-mediated β-catenin KO (Fig. 6) is consistent with this model.

The Sf1/Cre transgenes are expressed in the anterior pituitary gland (Bingham et al., 2006), and defects in pituitary expression of corticotropin are associated with impaired development of the adrenal cortex. However, the Sf1/Cre transgenes are not expressed in pituitary corticotropes, and even complete absence of corticotropin does not cause agenesis/aplasia of the adrenal gland. Moreover, ACTH levels in the *Sf1/Cre^{low}* β-catenin KO mice with histological failure were higher than those in wild-type mice and KO mice without clear histological failure, suggesting a primary defect in adrenal function. Although defining the effects of Sf1/Cre-mediated disruption of β-catenin in other sites such as the anterior pituitary, ventromedial hypothalamic nucleus and gonads is an important area for future studies, the finding that surviving adrenal cells are those that have not inactivated the floxed *lacZ* Cre reporter (Fig. 2) argues that these are cell-autonomous adrenal effects rather than the result of external perturbations. Thus, it is extremely unlikely that the phenotype observed here reflects secondary effects on the adrenal cortex due to disruption in other sites.

The available data from other tissues suggest the importance of canonical Wnt signaling in the development and maintenance of organ systems (Dessimoz et al., 2005; Huelsken et al., 2001; Reya and Clevers, 2005; Rulifson et al., 2007; Zechner et al., 2003). For example, Wnt signaling in hair follicles is localized to the stem cell population. Within the adrenal cortex, active canonical signaling is seen in the developing adrenal primordium from E12.5. As the definitive cortex subsequently emerges, Wnt signaling increasingly becomes restricted to the subcapsular area of the cortex, coincident with the organization of the surrounding capsule. Although definitive studies identifying the capsular and subcapsular cells as the bona fide adrenocortical niche/stem-progenitor unit are lacking (Kim and Hammer, 2007), the data presented here indicate that canonical Wnt signaling in these cells is crucial for the development of the definitive cortex and maintenance of the adult gland.

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