

# WT1 Maintains Adrenal-Gonadal Primordium Identity and Marks a Population of AGP-like Progenitors within the Adrenal Gland

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## SUMMARY

Adrenal glands and gonads share a common primordial (AGP), but the molecular events driving differentiation are poorly understood. Here we demonstrate that the Wilms tumor suppressor WT1 is a key factor defining AGP identity by inhibiting the steroidogenic differentiation process. Indeed, ectopic expression of WT1 precludes differentiation into adrenocortical steroidogenic cells by locking them into a progenitor state. Chromatin immunoprecipitation experiments identify *Tcf21* and *Gli1* as direct targets of WT1. Moreover, cell lineage tracing analyses identify a long-living progenitor population within the adrenal gland, characterized by the expression of WT1, GATA4, GLI1, and TCF21, that can generate steroidogenic cells in vivo. Strikingly, gonadectomy dramatically activates these WT1<sup>+</sup> cells and leads to their differentiation into gonadal steroidogenic tissue. Thus, our data describe a mechanism of response to organ loss by recreating hormone-producing cells at a heterotopic site.

## INTRODUCTION

Adrenal glands and gonads are crucial steroidogenic organs required to maintain body homeostasis in mammals. Consistent with their overlapping functions, the adrenal cortex and gonads share a common developmental origin, the adrenogonadal primordium (AGP). In the mouse, the AGP can be first detected as a thickening of the coelomic epithelium at approximately embryonic day (E) E9.5 (Hatano et al., 1996; Ikeda et al., 1994). By E10.5, the AGP splits into adrenal and gonadal primordia that will continue to differentiate along separate pathways. Neural crest-derived cells invade the adrenal primordium at E12.5 and will ultimately differentiate into the neuroendocrine chromaffin cells (Anderson and Axel, 1986). Encapsulation of the adrenal occurs at E14.5, in a process that is believed to involve conden-

sation of mesenchymal cells surrounding the developing organ (Mesiano and Jaffe, 1997; Uotila, 1940). However, definitive proof of this hypothesis is missing to date. After encapsulation, adrenal development proceeds with development of the definitive cortex (Zubair et al., 2008) followed by the formation of specific steroidogenic zones (glomerulosa, fasciculata, X-zone), a process that is only completed after birth.

The Wilms tumor suppressor gene 1 (*WT1*) encodes a transcriptional regulator that plays key roles during the formation of many organs (Hohenstein and Hastie, 2006). More recent work highlights an equally important role in the maintenance and repair of several tissues (Chau et al., 2011; Smart et al., 2011). The molecular biology of WT1 is complex and at least 36 different isoforms can be produced by a combination of alternative transcription start sites, alternative splicing, and RNA editing. Alternative splicing at the junction of exons 9 and 10 generates distinct isoforms that contain (+KTS) or lack (−KTS) the three amino acids KTS, which gives rise to proteins that have different biochemical and biological properties. WT1-KTS shows high affinity to DNA (Bickmore et al., 1992) and a diffuse nuclear distribution (Larsson et al., 1995) that is typical of classic transcription factors. Indeed, several studies have demonstrated the ability of WT1-KTS to directly regulate gene expression (Hohenstein and Hastie, 2006) and influence the chromatin state of target loci (Essafi et al., 2011). In contrast, WT1 +KTS isoforms preferentially bind RNA (Caricasole et al., 1996; Kennedy et al., 1996) and have been suggested to play a role in RNA metabolism (Niksic et al., 2004). In vivo evidence for distinct roles of +KTS and −KTS comes from genetic analysis in mice and mutants lacking either of the variants display distinct phenotypes (Hammes et al., 2001).

Development of the AGP requires WT1 and its direct target SF1 (Luo et al., 1994; Val et al., 2007; Wilhelm and Englert, 2002) and targeted deletion of either of these genes results in AGP apoptosis and, as a consequence, agenesis of both gonads and adrenal glands (Kreidberg et al., 1993; Luo et al., 1994; Moore et al., 1999). Whereas both WT1 and SF1 are expressed in the AGP, WT1 is switched off within the adrenal primordium soon after separation (Moore et al., 1998; Vidal and Schedl, 2000). The functional significance of this repression is presently unknown.

In the present study, we identify WT1 as an essential player in defining AGP cell identity. We show that ectopic expression of the transcriptionally active WT1 –KTS isoform is sufficient to prevent differentiation of AGP cells into steroidogenic cells by directly regulating the expression of genes such as *Gli1* and *Tcf21*. In addition, we identify AGP-like cells within the adrenal cortex that exhibit WT1 expression and maintain the ability to generate both adrenocortical and gonadal steroidogenic cells throughout life.

## RESULTS

### WT1 Repression Is Essential for Adrenocortical Development

To study the dynamics of WT1 expression in adrenal development, we first carried out comparative expression analysis at various developmental stages. At E9.5, WT1 expression was found throughout the urogenital ridge, where it largely overlapped with GATA4 (Figure 1A). Appearance of first SF1 positive (WT1<sup>+</sup>/GATA4<sup>+</sup>/SF1<sup>+</sup>) cells occurred at E9.75 (Figure 1A) and was followed by rapid downregulation of WT1 and GATA4 in the presumptive adrenal progenitor compartment (Figure 1B). By E11.5, WT1 was no longer detectable in the majority of SF1<sup>+</sup> adrenocortical progenitors (Figure 1B). Of note, rare WT1<sup>+</sup>/GATA4<sup>+</sup> cells, some of which expressed SF1, persisted within the adrenal cortex throughout development (Figure 1B, arrows; Figure S1C available online). In contrast to the adrenal gland, cells within the developing gonad continued to coexpress WT1 and SF1 until later stages (Figure 1C and data not shown).

To understand the functional significance of *Wt1* repression during adrenocortical differentiation, we generated mice that permit Cre-mediated activation of WT1 + or –KTS isoforms in a tissue-specific fashion (*Rosa26:Wt1+KTS* and *Rosa26:Wt1–KTS* lines; Figures S1A and S1B). Genetic crosses with the *Sf1:Cre<sup>high</sup>* line (Bingham et al., 2006), a transgenic line expressing high levels of Cre within the steroidogenic compartment, resulted in activation of WT1 in the developing adrenal cortex as early as E12.5 (Figure S1C). Heterozygous *Rosa26<sup>Wt1+KTS</sup>/Wt1+KTS*; *Sf1:Cre* and *Rosa26<sup>Wt1–KTS</sup>/Wt1–KTS*; *Sf1:Cre* embryos developed normal adrenal glands (data not shown). Because *Rosa26* is known to be a relatively weak promoter, we crossed the targeted allele to the homozygous state to further increase transgene expression levels. Homozygous *Rosa26<sup>Wt1+KTS</sup>/Wt1+KTS*; *Sf1:Cre* embryos (from now on called +KTS<sup>GOF</sup>) displayed no dramatic changes in adrenal architecture (Figure 1E). In contrast, *Rosa26<sup>Wt1–KTS</sup>/Wt1–KTS*; *Sf1:Cre* mice (from now on called –KTS<sup>GOF</sup>) developed highly abnormal adrenal glands of reduced size and abnormal cellular morphology (Figures 1E and S1C–S1E).

To determine if WT1 repression was necessary for differentiation of progenitors into adrenocortical cells, we examined whether the expression of the AGP marker gene GATA4 persisted in transgenic animals. Indeed, activation of WT1–KTS, but not WT1+KTS, triggered expression of GATA4 in SF1-expressing cells (Figure 1E). Expression of GATA4 was evident as early as E12.5 (Figure S1C). At this early time point, both GATA4 and WT1 were expressed homogeneously in all fetal adrenocortical (SF1<sup>+</sup>) cells of –KTS<sup>GOF</sup> mice (Figure S1C). Later in development, two subtypes of cells became apparent that

were distinguished by the levels of WT1 expression, perhaps as a result of stochastic/epigenetic factors. WT1<sup>high</sup> cells (high levels of WT1) showed ectopic activation of GATA4, but exhibited low levels of SF1 (Figure 1E, –KTS<sup>GOF</sup>, and S1C). In contrast, WT1<sup>low</sup> cells maintained strong SF1 expression and showed reduced levels of GATA4. We conclude that high levels of WT1-KTS prevent complete differentiation of AGP cells into steroidogenic adrenocortical cells.

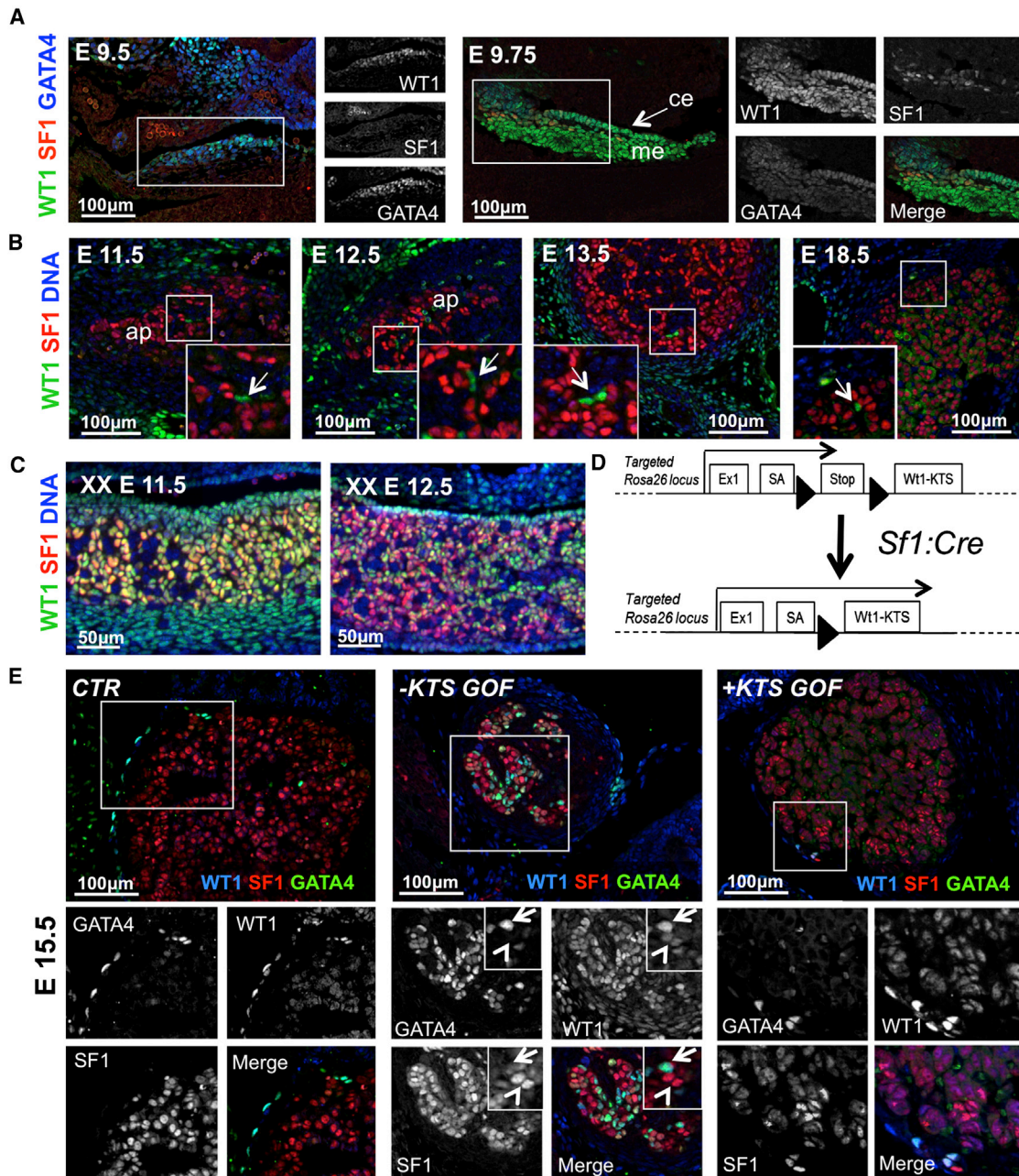
### Compensation of Hormonal Production in Adult WT1-KTS-Expressing Adrenal Glands

Adrenal glands from adult –KTS<sup>GOF</sup> animals were smaller than controls (Table S1) and displayed cortical spindle-shaped cells, effectively dividing the cortex into lobular structures (Figure 2A). In each lobule, the zonation of the gland was grossly conserved, as indicated by the expression of the general steroidogenic enzyme 3β-HSD2, and the *zona fasciculata* marker AKR1b7 (Figure S2A). The only affected adrenocortical area was the X-zone, which was dramatically reduced in –KTS<sup>GOF</sup> mice (Figure S2B). Despite the severe morphological changes, the adrenal glands from –KTS<sup>GOF</sup> animals appeared to be functional and transgenic animals showed normal circulating levels of corticosterone (Figure S2C). Expression levels of the main enzymes involved in steroidogenesis were also comparable to those found in control animals (Figure S2E). Maintenance of steroid production was likely achieved through increased ACTH levels in –KTS<sup>GOF</sup> mice (Figure S2C). ACTH is known to stimulate the expression of steroidogenic enzymes, and indeed increased cellular staining for AKR1b7 and 3β-HSD2 (Figure S2A) and 21-hydroxylase could be observed (Figure S1E) together with a mild increase in steroidogenic cell size (Figure S2D).

Immunostaining analysis revealed a similar association as observed during development, and steroidogenic SF1-positive cells expressed only very low levels of WT1 (Figure 2B). In contrast, cortical spindle-shaped cells in adult –KTS<sup>GOF</sup> animals showed strong expression of WT1 and reduced SF1 (Figure 2B). Surprisingly, we also occasionally observed spindle-shaped WT1<sup>+</sup>; SF1<sup>low</sup> cells in the adrenal cortex of wild-type animals, but in contrast to –KTS<sup>GOF</sup> mice these were found in rare wedge shape patches located in the subcapsular region (Figure 2C). +KTS<sup>GOF</sup> animals did not show a dramatic phenotype (Figure 2A) and, although low expression of WT1 was present in steroidogenic cells, only a few spindle-shaped WT1<sup>+</sup> cells could be detected and these were restricted to the subcapsular region (Figure 2B).

### –KTS<sup>GOF</sup> Animals Develop Normal Gonads and Are Fertile

The *Sf1:Cre* driver is not only expressed in adrenocortical, but also in gonadal cells (Bingham et al., 2006), and we wondered whether gonads may also be affected in this transgenic strain. In males, gonadal steroid synthesis is initiated during development, whereas females only produce sex hormones after birth. The expression pattern *p450scc*, a functional marker of steroidogenic cells, was unchanged between wild-type and –KTS<sup>GOF</sup> embryos (Figure S2F), suggesting that ectopic expression of WT1 did not interfere with steroid synthesis during development. ISH analysis for 21-hydroxylase did not reveal abnormal displacement of adrenocortical cells into the gonads of –KTS<sup>GOF</sup>



**Figure 1. Repression of WT1 Is Required to Allow Adrenocortical Differentiation**

(A) Immunostaining against WT1 (green), SF1 (red), and GATA4 (blue) in early (E9.5) and late (E9.75) AGP. WT1 and GATA4 characterize AGP cells from the earliest stages, whereas SF1 can only be detected in late AGP cells.

(B) WT1<sup>+</sup> cells (green) are detected in the mesenchyme surrounding the adrenal primordium before encapsulation (E11.5 and E12.5) and in the adrenal capsule at later developmental stages. Few WT1<sup>+</sup>, SF1<sup>-</sup> cells are also located within the adrenal cortex after encapsulation (arrow).

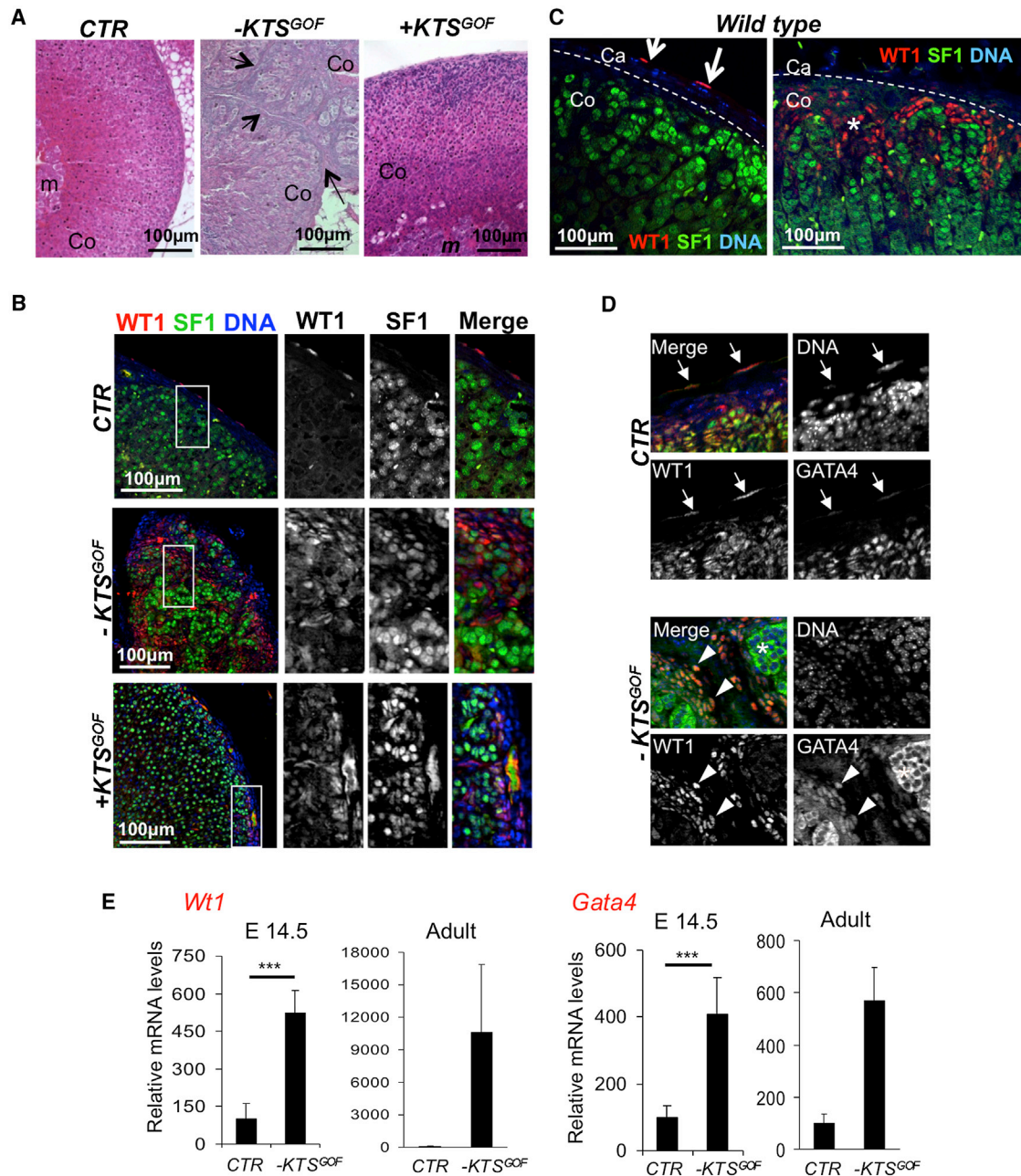
(C) Coexpression of WT1 and SF1 persists in E11.5 and E12.5 gonads until later stages.

(D) Schematic representation of the genetic approach used to ectopically express WT1-KTS isoforms in steroidogenic cells. Black arrowheads indicate LoxP sites. An equivalent strategy was used to knock in and ectopically express Wt1+KTS isoforms.

(E) WT1 (blue) SF1 (red) and GATA4 (green) immunofluorescence on adrenal sections from CTR, -KTS<sup>GOF</sup> and +KTS<sup>GOF</sup> E15.5 embryos. Two cell populations can be distinguished in -KTS<sup>GOF</sup> adrenal cortex (insets): WT1<sup>high</sup>, which have strong GATA4 and low SF1 expression (arrows); and WT1<sup>low</sup>, which instead have reduced or absent GATA4 and strong SF1 expression (arrowheads). Please note that the cytoplasmic WT1 staining in control adrenal glands represents background noise.

me, mesonephros; ce, coelomic epithelium; and ap, adrenal primordium. See also Figure S1.





**Figure 2. Cells Ectopically Expressing WT1-KTS Are Blocked in an AGP-like State throughout Life**

(A) Haematoxylin and eosin staining of adrenals from wild-type,  $-KTS^{GOF}$  and  $+KTS^{GOF}$  adult mice. Arrows indicate capsular-like cells within the adrenal cortex of  $-KTS^{GOF}$  animals.

(B) Immunostaining against WT1 (red) and SF1 (green) on adrenals from wild-type,  $-KTS^{GOF}$  and  $+KTS^{GOF}$  adult mice shows the persistence of WT1<sup>+</sup> expressing cells within the adrenal cortex of  $-KTS^{GOF}$  and  $+KTS^{GOF}$  mice.

(C) Immunostaining for WT1 (green) and SF1 (red) on adult adrenal glands reveals the presence of WT1<sup>+</sup> cells within the adrenal capsule (left) and in rare patches located in the subcapsular cortex (asterisk, right).

(D) WT1 positive capsular and cortical cells (red) found in adult wild-type animals also express GATA4 (green). Note the cytoplasmic GATA4 signal in steroidogenic cells represents background (asterisk).

(E) RT-qPCR on adrenal glands from E14.5 embryos and adult mice. Quantification of *Wt1* and *Gata4* messenger RNAs reveals increased expression of both genes in  $-KTS^{GOF}$  adrenals. E 14.5: *Wt1*, CTR:  $100 \pm 61.85$ ,  $n = 6$ ;  $-KTS^{GOF}$ :  $522.28 \pm 89.27$ ,  $n = 6$ ; *Gata4*, CTR:  $100 \pm 35.38$ ,  $n = 6$ ;  $-KTS^{GOF}$ :  $407.20 \pm 109.67$ ,  $n = 6$ . Adult: *Wt1*, CTR:  $100 \pm 48.20$ ,  $n = 9$ ;  $-KTS^{GOF}$ :  $10,578.96 \pm 12,509.51$ ,  $n = 3$ ; *Gata4*, CTR:  $100 \pm 69.51$ ,  $n = 6$ ;  $-KTS^{GOF}$ :  $569.70 \pm 309.06$ ,  $n = 4$ . \*\*\* $p < 0.001$ . Statistical significance was determined using Student's *t* test. Mean values  $\pm$  SEM. The data presented were normalized for *Hprt1* expression.

Ca, adrenal capsule; Co, adrenal cortex; and m, adrenal medulla. See also Figure S2.

embryos and only rare adrenal-like cells were detected (Figure S2G), as has been described in wild-type testes (Val et al., 2006). Finally, gonads from adult  $-KTS^{GOF}$  animals displayed normal histology (Figures S2H and S2I) and both sexes were fertile. qPCR analysis for WT1 revealed only very mild overexpression of *Wt1-KTS* in the developing gonads (Figure S2J), which may explain the lack of a gonadal phenotype.

### WT1 Activates *Tcf21* and *Gli1*

Given the similar morphology of WT1-expressing cells in  $-KTS^{GOF}$  animals and those discovered in control animals (Figure 2C), we next asked whether they shared expression of common AGP markers. We first investigated the expression of the transcription factor GATA4 that we found coexpressed with WT1 in AGP cells, in a proportion of capsular and in few cortical cells in the developing adrenals (Figures 1A, 1E, and S1C). Indeed, GATA4 was expressed in both WT1<sup>+</sup> capsular cells and spindle-shaped cells within the adult adrenal cortex in both wild-type and  $-KTS^{GOF}$  animals (Figure 2D). RT-qPCR analysis further confirmed the association of WT1 and GATA4 expression and the expression of both genes was strongly increased in  $-KTS^{GOF}$  animals (Figure 2E).

*Gli1* and *Tcf21* encode two transcription factors expressed in capsular cells that have been recently suggested to mark adrenal progenitor cells (Huang et al., 2010; King et al., 2009; Kim and Hammer, 2007). To examine whether WT1 overlaps with the expression of these markers we performed staining against  $\beta$ -galactosidase in *Gli1:LacZ* mice (Bai et al., 2002) and RNA in situ hybridization for the *Tcf21* transcript. Indeed, expression of both genes largely followed WT1 expression in cortical and capsular cells in wild-type animals (Figures 3A–3C and 3F), albeit a proportion of GLI1-expressing cells, primarily located at the interior side of the capsule, were negative for WT1 (Figure 3A, asterisk). To test whether WT1 induces expression of *Tcf21* and *Gli1*, we next performed RT-qPCR analysis. Both *Tcf21* and *Gli1* transcripts were dramatically increased in  $-KTS^{GOF}$  adrenals (Figures 3D and 3E) and their expression was restricted to spindle-shaped WT1<sup>+</sup> cells (Figure 3F). Similarly, *Ptch1*, a downstream target of GLI1, was increased in  $-KTS^{GOF}$ , but not in  $+KTS^{GOF}$  animals (Figures S3A and S3B). In contrast, expression of *Shh*, a gene marking a population of subcapsular cells in wild-type animals (King et al., 2009; Huang et al., 2010), showed no significant change, although *Shh*-expressing cells were misplaced in  $-KTS^{GOF}$  mice (Figures S3A and S3B).

Because WT1-KTS acts as a transcriptional regulator, we next asked whether it might directly activate the promoters of *Gata4*, *Tcf21*, and *Gli1*. Primers were designed according to the presence of evolutionary conserved regions and peaks identified in WT1 ChIP-seq analysis carried out on E18.5 kidneys (Figures S3C–S3E). In vivo ChIP assays performed on adrenal glands from  $-KTS^{GOF}$  animals (Figure 3G) demonstrated WT1 binding to two evolutionary conserved regions upstream from *Tcf21* (–1.5 and –48.5 kb from the transcription start site) and an intronic region of *Gli1* (Figure 3G). We were unable to detect any enriched region in the promoter of *Gata4* (data not shown).

Taken together, these data indicate that ectopic expression of the transcriptionally active isoform of WT1 (WT1–KTS), but not WT1+KTS, in adrenal primordial cells is sufficient to prevent

AGP cells from differentiation in a process that is likely to involve the WT1 target genes *Gli1* and *Tcf21*.

### WT1<sup>+</sup> Progenitors Generate Adrenocortical Cells during Development

To better understand the relationship between WT1-expressing cells and their descendants, we next carried out cell-lineage tracing experiments using *Wt1:Cre-GFP*; *mTmG* mice (Zhou et al., 2008). Cre-induced recombination activates the green fluorescent protein and thus irreversibly labels all *Wt1*-expressing cells as well as their descendants. Consistent with a direct descent of steroidogenic cells from WT1<sup>+</sup> AGP cells (Figure 1A), the totality of the SF1<sup>+</sup> adrenal cortical cells stained positive for GFP (Figure S4A). To address whether WT1-expressing mesenchymal cells maintain progenitor features at later stages of development, we resorted to the recently generated *Wt1:Cre-ERT2* strain, which allows temporal control of Cre activity (Zhou et al., 2008; Figure 4A). Activation of Cre recombinase by tamoxifen injection at E12.5 or E14.5 resulted in labeling of a large proportion of cells within the adrenal capsule at E18.5 (Figures 4B and 4D), indicating that WT1<sup>+</sup> mesenchymal cells directly participate in the generation of this tissue. Moreover, a small proportion of GFP cells was located within the adrenal cortex and some of them expressed the steroidogenic marker SF1 (Figure 4B). Importantly, these GFP<sup>+</sup>SF1<sup>+</sup> cells had lost WT1, indicating a complete differentiation of WT1<sup>+</sup> progenitors into adrenocortical cells (Figures 4B and S4B).

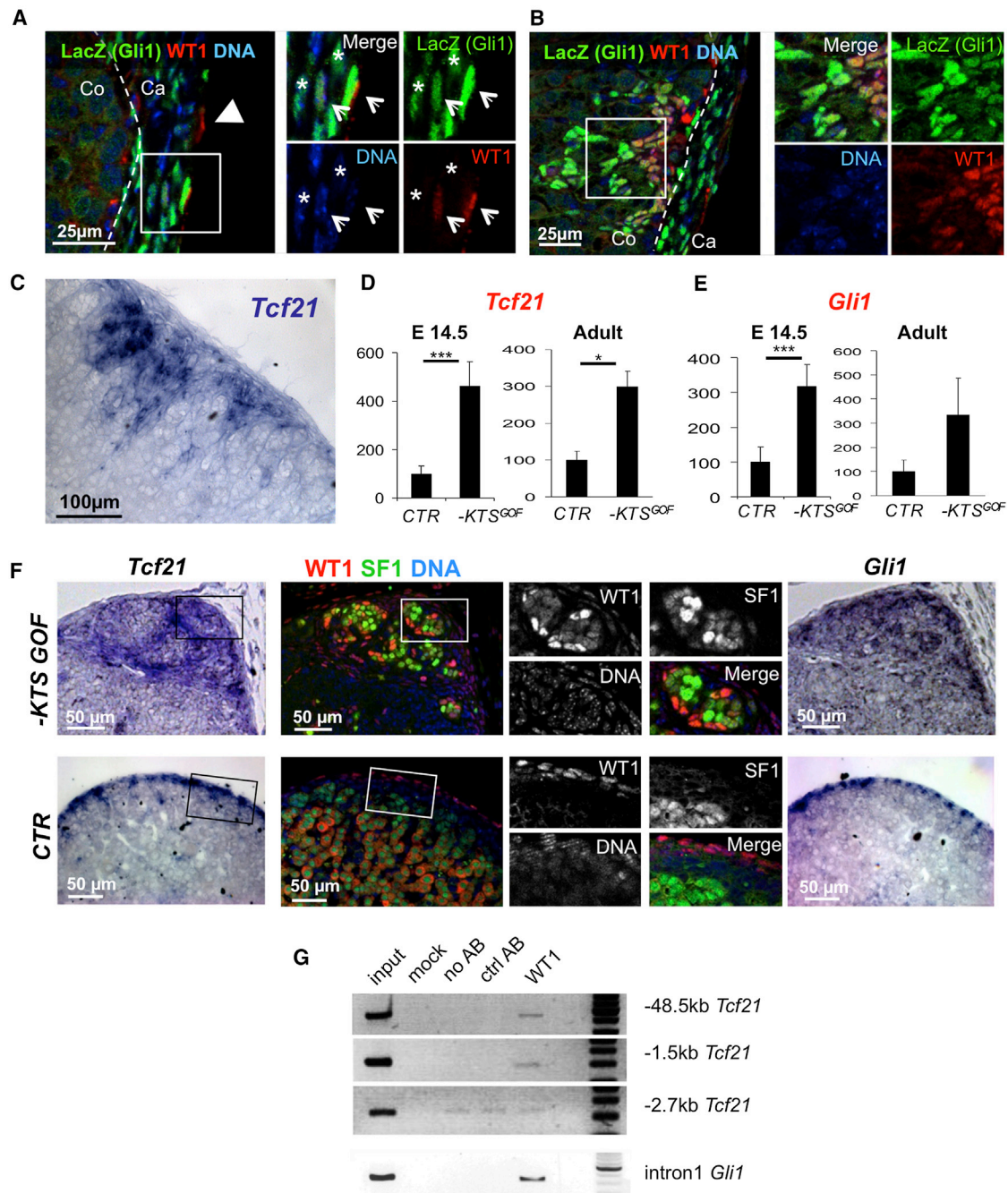
Recently King and colleagues (King et al., 2009) described a progenitor cell population residing within the adrenal capsule that expresses the transcription factor GLI1. Given the fact that we identified *Gli1* as a direct target of WT1, we decided to investigate the relationship between these two populations. Analysis of *Wt1:Cre-ERT2*; *mTmG*; *Gli1:LacZ* E18.5 embryos treated with tamoxifen at E12.5 allowed us to highlight a partial overlap of expression between the two factors (Figures S4E and S4F). Nevertheless, lineage tracing of WT1-expressing cells resulted in a substantially different pattern when compared to that obtained with *Gli1:Cre-ERT2* mice (King et al., 2009). GFP was mainly detected in the external capsule, where WT1 expression is stronger, and did not cover the entire GLI1 population (Figures S4E and S4F).

From our data, it appears that the activation of the *Wt1:Cre-ERT2* only occurs in a subpopulation of cells expressing WT1 (and thus Cre-ERT2) at high levels (Figure S4E). Cells traced by King and colleagues are likely to be WT1<sup>low</sup> or WT1-negative precursors that may have arisen from AGP cells at an early time point of development. Indeed, we could identify WT1<sup>+</sup>GLI1<sup>+</sup> cells at the interior side of the capsule, which seem to be good candidates for such a population (Figure S4E, asterisk).

### WT1<sup>+</sup> Cells Are Long-Living Progenitors Able to Generate Adrenocortical Cells throughout Life

Given the persistence of rare WT1<sup>+</sup> cells in adult adrenal glands (Figure 2C), we next examined whether they might retain progenitor properties throughout life. Tamoxifen induction at E12.5 or E14.5 revealed GFP-labeled cells within the capsule (Figures 4B and 4D; E18.5), as well as a small number of GFP patches within the cortex at E18.5 (Figures 4B and S4B–S4D). The number (Figure S4G) and size of cortical patches of GFP<sup>+</sup> cells





**Figure 3. WT1-KTS Ectopic Expression Directly Upregulates *Tcf21* and *Gli1***

(A and B) LacZ (green) and WT1 (red) immunofluorescence on adrenal sections from adult *Gli1::LacZ* mice. (A) The adrenal capsule is composed of at least three cell types that can be distinguished by the presence or absence of WT1 and GLI1 (WT1<sup>+</sup> GLI1<sup>-</sup>, arrowhead; WT1<sup>-</sup> GLI1<sup>+</sup> asterisk; WT1<sup>+</sup> GLI1<sup>+</sup>, arrows in insets). (B) WT1<sup>+</sup> adrenocortical patches are also positive for GLI1 expression.

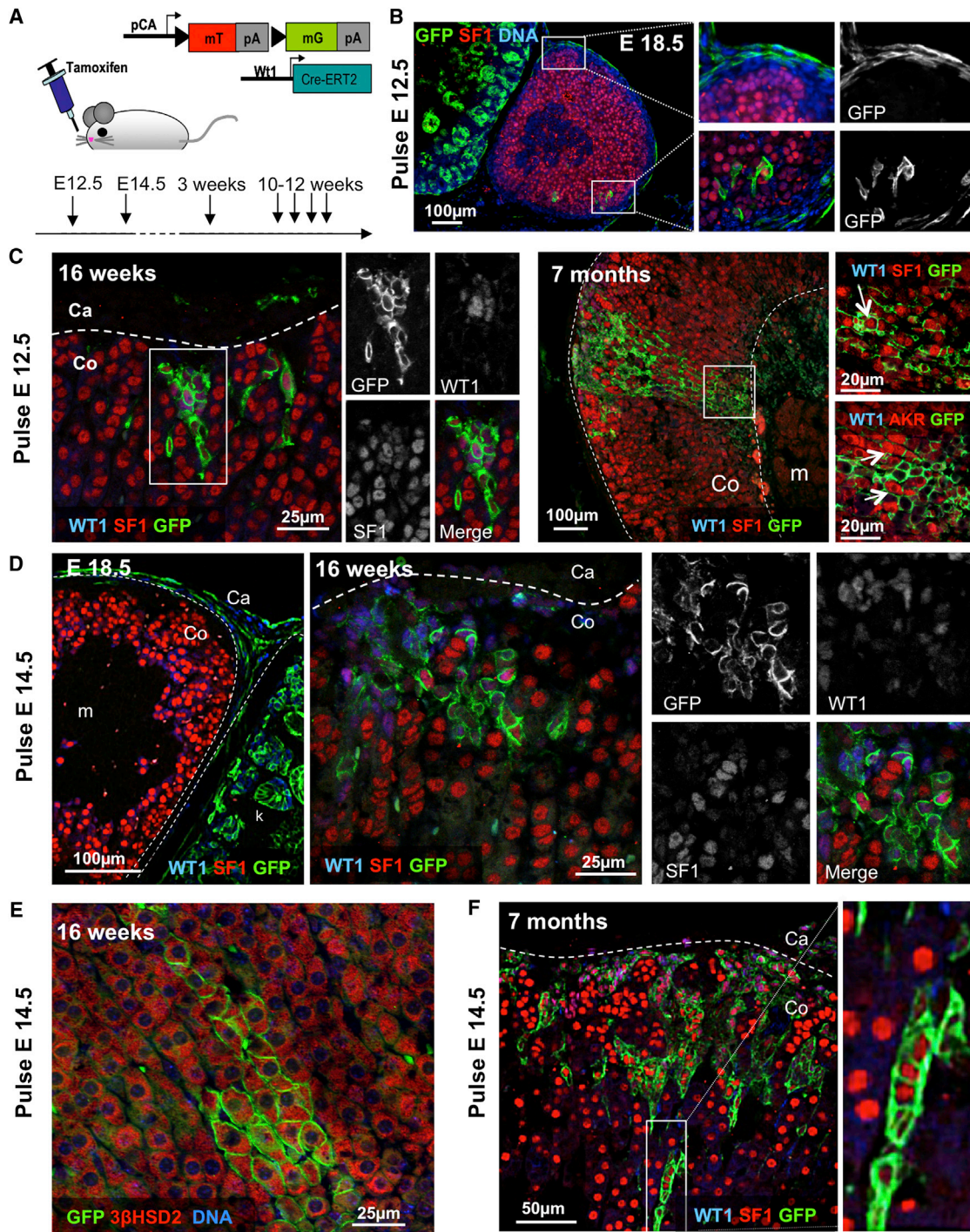
(C) *Tcf21* RNA in situ hybridization showing expression of this marker in the same cells.

(D and E) RT-qPCR showing increased *Tcf21* (D) and *Gli1* (E) expression in adrenal glands of E14.5 embryos or adult -KTS<sup>GOF</sup> mice. E14.5: *Tcf21*, CTR: 100 ± 33.29, n = 4; -KTS<sup>GOF</sup>: 461.52 ± 102.07, n = 6; *Gli1*, CTR: 100 ± 42.58, n = 4; -KTS<sup>GOF</sup>: 317.34 ± 63.02, n = 6. Adult: *Tcf21* CTR: 100 ± 110.91, n = 20; -KTS<sup>GOF</sup>: 298.77 ± 123.12, n = 8; *Gli1*, CTR: 100 ± 147.46, n = 10; -KTS<sup>GOF</sup>: 334.15 ± 305.52, n = 4. \*\*\*p < 0.001, \*p < 0.05 Statistical significance was determined using the Student's t test. Mean values ± SEM. The data presented were normalized for *Hprt1* expression.

(F) WT1 (red) and SF1 (green) immunofluorescence on consecutive sections to *Tcf21* (left) or *Gli1* (right) RNA in situ hybridization on E18.5 adrenal glands, shows overlap between WT1 and the RNA for the two markers in both CTR and -KTS<sup>GOF</sup>. Please note that the cytoplasmic WT1 staining in control adrenal glands is background.

(G) ChIP analysis on -KTS<sup>GOF</sup> adrenal glands shows binding of WT1-KTS to the proximal promoter of *Tcf21* and to an intronic region of *Gli1*. A region located -2.7kb upstream of *Tcf21* with background amplification is shown as a negative control.

Ca, adrenal capsule; and Co, adrenal cortex. See also Figure S3.



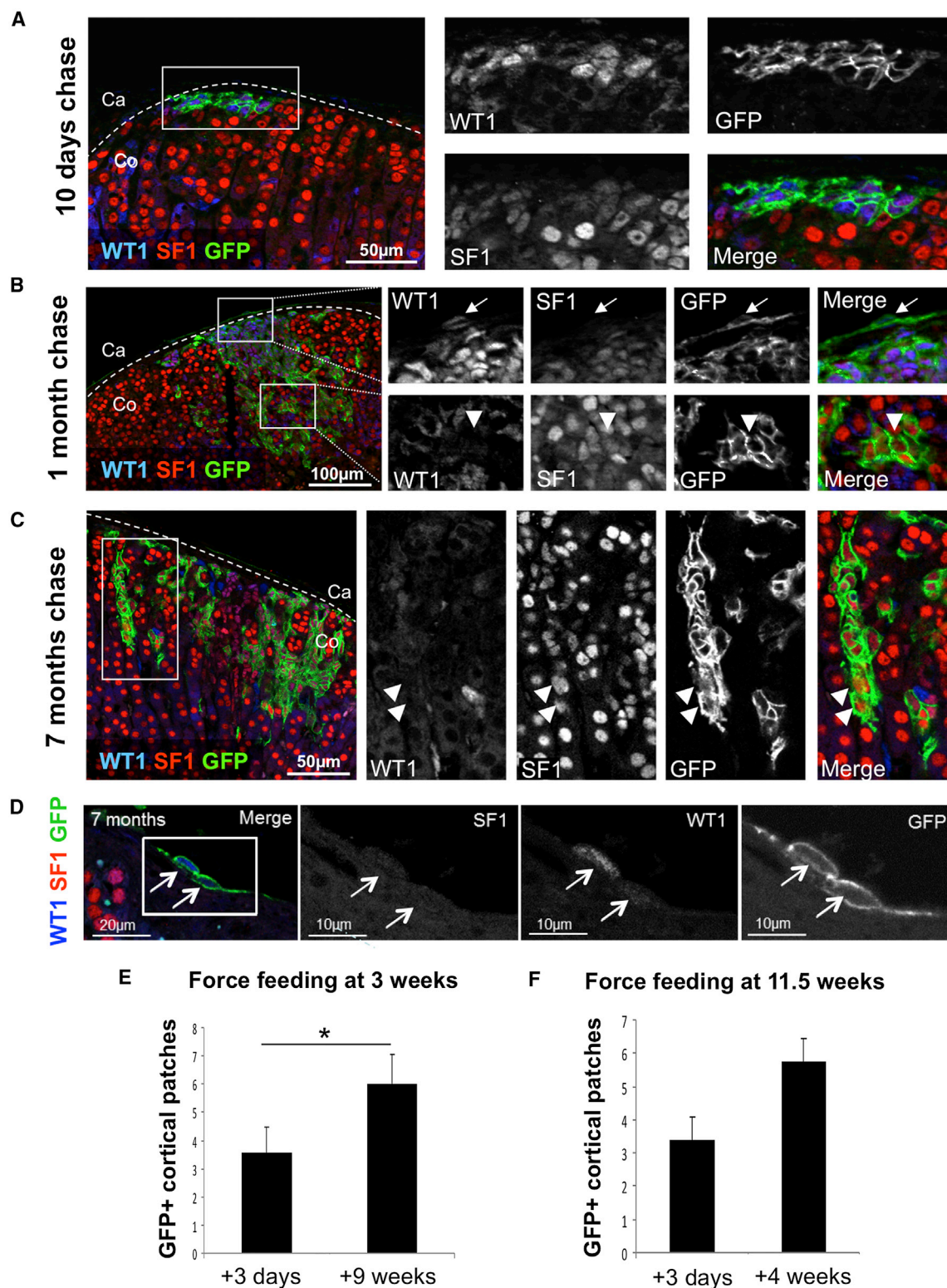
**Figure 4. WT1-Expressing Cells Are Progenitors Able to Differentiate into Steroidogenic Cells**

(A) Schematic representation of the cell lineage tracing experiments performed on *Wt1:Cre-ERT2; mTmG* mice or embryos indicating the time point when tamoxifen was administered.

(B–F) WT1 or DNA (blue), SF1, 3βHSD2, or AKR1b7 (red) and GFP (green) immunofluorescence on samples of adrenal glands from *Wt1:Cre-ERT2; mTmG* mice of different ages treated with tamoxifen at E12.5 (B and C) or E14.5 (D–F). During development, the majority of GFP+ cells are localized within the capsule (B and D, E18.5). Postnatal patches of WT1+, GFP+ cells expand under the adrenal capsule and few cells acquire a steroidogenic phenotype.

Co, adrenal cortex; Ca, adrenal capsule; and m, adrenal medulla. See also Figure S4.





**Figure 5. WT1<sup>+</sup> Cells Maintain the Ability to Generate Subcapsular Patches and to Differentiate into Steroidogenic Cells throughout Life**  
(A–D) Immunostaining against WT1 (blue), SF1 (red), and GFP (green) on adrenal glands from *Wt1:Cre-ERT2; mTmG* mice treated with four consecutive doses of tamoxifen between 10 and 12 weeks of age and collected after 10 days (A), 1 month (B), and 7 months (C and D) after the last administration. Almost all GFP<sup>+</sup> cells also express WT1 and SF1 (A–C). GFP<sup>+</sup> SF1<sup>+</sup> WT1<sup>−</sup> cells can be detected in a subset of patches (B and C). GFP<sup>+</sup> WT1<sup>+</sup> SF1<sup>−</sup> capsular cells can be detected within the adrenal capsule up to 7 months after force-feeding (D).

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significantly increased over time with some of the wedge-shaped patches spanning the entire adrenal cortex (Figures 4C and 4F). Interestingly, a small proportion of cells, generally located at the tips of the wedges, lost WT1 and acquired steroidogenic features, as evidenced by the expression of high levels of SF1, AKR1b7, and  $\beta$ HSD2 (Figures 4C, 4E, and 4F).

To further investigate the role of WT1<sup>+</sup> cells in adrenal homeostasis, we performed time course experiments in adult animals (tamoxifen induction at 3 or 10–12 weeks of age; Figure 4A). GFP<sup>+</sup> cells were detected in the subcapsular region as early as 3 days after tamoxifen induction. Over time, patches significantly increased in size (Figures 5A–5C) and number (Figures 5E and 5F), although they remained a small proportion of the adrenal cortex in all samples analyzed. Staining for the endothelial marker PECAM did not reveal an overlap with GFP-positive cells (Figures S5C and S5D), suggesting that WT1<sup>+</sup> progenitors do not give rise to endothelial cells within the adrenal. Instead, we observed a small proportion of GFP<sup>+</sup> cells that lost WT1 expression and acquired the morphology of differentiated steroidogenic cells and strong SF1 staining (Figures 5B and 5C, arrowheads).

Thus, WT1<sup>+</sup> cells in adult adrenals represent a progenitor cell population able to differentiate into adrenocortical steroidogenic cells throughout life. Interestingly, GFP-positive cells that retained WT1 expression could be identified within the adrenal capsule as late as 7 months after tamoxifen injection (Figure 5D), suggesting that they represent a long-lived cell population.

#### WT1<sup>+</sup> Cells Generate Gonadal-like Cells within the Adrenal Cortex upon Gonadectomy

WT1<sup>+</sup> adrenocortical and AGP cells appear to share a significant number of features and we wondered whether they might have retained gonadal potential in the adrenal gland throughout life. Gonadectomy has been shown to increase the appearance of subcapsular spindle-shaped cells (called “A cells”) and to promote their differentiation into gonadal-like cells (called “B cells”) capable of sex hormone synthesis (Bielinska et al., 2003; Woolley et al., 1952, 1953), as a consequence of the increase in gonadotropins (Bielinska et al., 2005; Kero et al., 2000).

To address whether WT1<sup>+</sup> cells can differentiate into gonadal steroidogenic cells, we subjected adult male and female mice (*Wt1-CreERT2*; *mTmG*) to gonadectomy shortly after *mTmG* activation (Figure 6A). Strikingly, 10 weeks after gonadectomy, we observed a dramatic expansion of GFP<sup>+</sup> cells invading the adrenal cortex (Figure 6B). Quantification also showed a rapid increase in the number of GFP<sup>+</sup> patches as early as 7 days after gonadectomy in males (Figures S6C and S6D). In nongonadectomized mice, the majority of GFP<sup>+</sup> cells are incapable of steroidogenesis (Figures 4 and 5), as evidenced by the absence of the steroidogenic enzymes *p450scc* and *21-hydroxylase* (Figures S5A and S5B). In contrast, the majority of GFP<sup>+</sup> cells in gonadectomized mice had lost WT1 expression and showed high levels of SF1 (Figure 6C) and GATA4 (Figure S6A). More-

over, GFP<sup>+</sup> cells expressed the general (both adrenal and gonad) steroidogenic marker *p450scc* (Figure 6D), indicating that a massive process of differentiation had taken place. Interestingly, the same cells lacked the adrenocortical marker *21-hydroxylase* (Figure 6D), but expressed the gonadal-specific genes *CYP17* and *Lhr* (Figures 6E–6G). Importantly, no *CYP17* and *Lhr* staining was found in sections from nongonadectomized control animals (Figures 6E and 6F), indicating that gonadectomy oriented these GFP<sup>+</sup> cells toward a gonadal steroidogenic fate.

Thus, WT1<sup>+</sup> cells are able to respond to hormonal changes following gonadectomy by differentiating into sex hormone-producing “B cells” (Bielinska et al., 2005; Kero et al., 2000). More generally, our results suggest that WT1<sup>+</sup> cells represent a progenitor population that maintains AGP-like features throughout life and as such have the ability to differentiate into steroidogenic cells of either adrenal or gonadal lineages.

## DISCUSSION

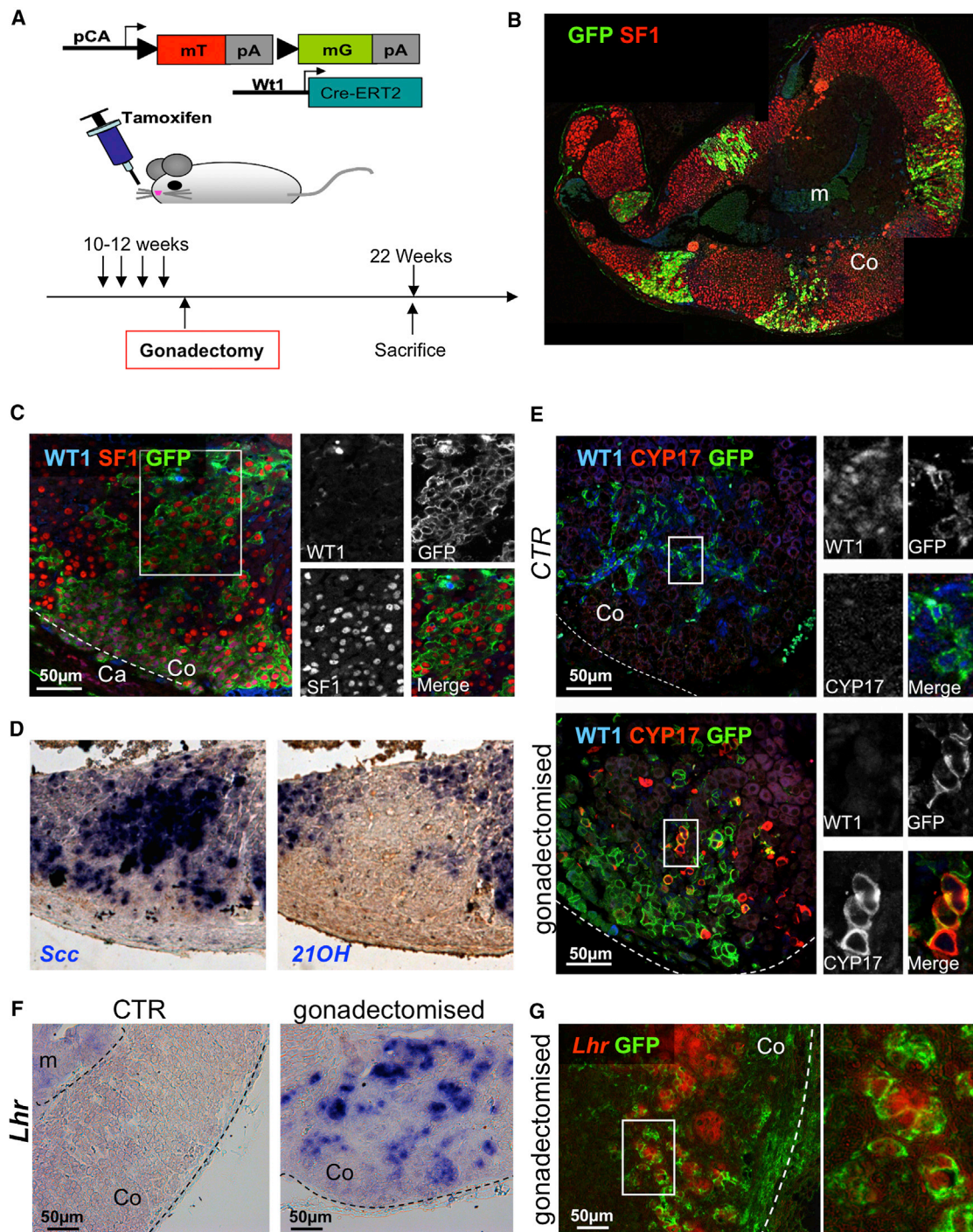
#### High Levels of WT1 Expression Are Incompatible with Differentiation into Steroidogenic Cells

The central role of WT1 in adrenogonadal development has been established by a series of loss-of-function studies and is well documented in the literature (Kreidberg et al., 1993; Moore et al., 1999). Prior to activation of SF1 (E9.5), early AGP precursors express both WT1 and GATA4 (Figure 1A). WT1 plays a major role in this tissue by directly regulating expression of the steroidogenic factor *Sf1* (Wilhelm and Englert, 2002) probably through association with CITED2 (Val et al., 2007). After separation, and upon activation of *Sf1*, adrenocortical cells lose WT1 before they differentiate into true steroidogenic cells.

Our gain-of-function analysis lends further support to the hypothesis that WT1 needs to be repressed for steroidogenesis to occur. Indeed, ectopic expression of high levels of WT1 effectively blocked the differentiation process. Interestingly, while all adrenal progenitor cells in *-KTS<sup>GOF</sup>* mice express WT1, two different types of cells that differ in WT1 expression levels could be distinguished. Although the molecular reason for this differential expression is presently unclear and may involve stochastic events, the outcome is highly instructive: while WT1<sup>low</sup> cells develop adrenocortical features including high levels of SF1 and fully differentiate into steroidogenic cells, WT1 expression in WT1<sup>high</sup> cells has crossed a certain threshold and as a consequence, these cells express GATA4, *Gli1*, and *Tcf21*; have low levels of SF1; and fail to differentiate. This results in a composite organ containing adrenal steroidogenic cells and undifferentiated adreno-gonadal precursors.

On the molecular level, our data reveal a direct regulation of *Gli1* and *Tcf21* by WT1, two genes that are upregulated in AGP-like cells in *-KTS<sup>GOF</sup>* animals. The finding that TCF21 can bind and inhibit the *SF1* promoter (França et al., 2013; Tamura et al., 2001) provides a molecular explanation for the observed SF1 repression, although we cannot exclude that additional,

(E and F) Quantification of the number of GFP<sup>+</sup> patches within the adrenal cortex of *Wt1:Cre-ERT2*; *mTmG* mice treated with tamoxifen at 3 weeks (E) or 11.5 weeks (F) of age. The number of GFP<sup>+</sup> clusters increases significantly from  $3.56 \pm 2.70$  (N = 9) to  $6.00 \pm 2.35$  (N = 9), 3 days and 9 weeks after force-feeding at 3 weeks of age, respectively. No significant increase in the number of GFP<sup>+</sup> clusters is detected in animals force-fed at 11.5 weeks, from  $3.38 \pm 2.00$  (N = 8) to  $5.75 \pm 1.98$  (N = 8). \*p < 0.05. Statistical significance was determined using the Student's t test. Mean values  $\pm$  SEM. Co, adrenal cortex; and Ca, adrenal capsule. See also Figure S5.



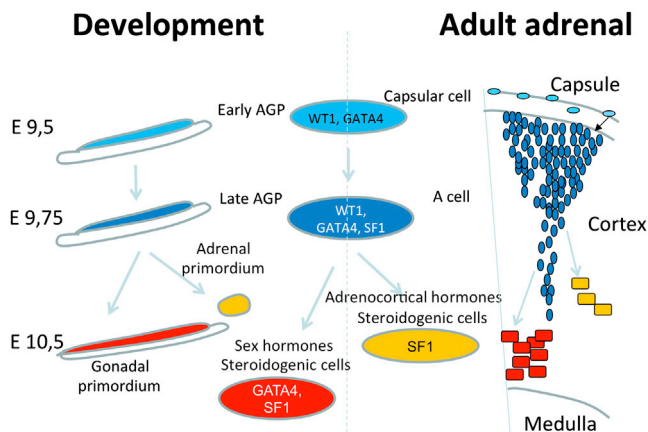
**Figure 6. WT1<sup>+</sup> Cells Generate Cells with Gonadal Characteristics in the Adrenal Cortex upon Gonadectomy**

(A) Schematic representation of the experimental design.

(B–G) Adrenal glands from mice sacrificed 10 weeks after gonadectomy, or nongonadectomized control. Gonadectomy induces massive invasion of the adrenal cortex by GFP<sup>+</sup> cells, derived from WT1<sup>+</sup> cells (B). Higher magnification shows that the majority of GFP<sup>+</sup> cells have lost WT1 expression, whereas SF1 is present (C). (D) RNA in situ hybridization shows that cells expressing cytochrome *P450<sub>scc</sub>* do not express the adrenocortical-specific marker *21-hydroxylase*. Please note that the three stainings in (C) and (D) were performed on consecutive sections, thus representing the same area. (E) CYP17 (red), a marker of cells producing sex hormones (gonadal cells) appears in some cells within the adrenal cortex of gonadectomized mice, but is never found in control animals. Coexpression of CYP17 and GFP in the same cells indicates that they are directly derived from WT1-expressing cells. (F and G) *LH* receptor RNA in situ hybridization shows expression of this gene in gonadectomized animals (F), where it colocalizes with GFP<sup>+</sup> cells (G).

Co, adrenal cortex; Ca, adrenal capsule; and m, adrenal medulla. See also Figure S6.





**Figure 7. Schematic Representation of the Fate of WT1<sup>+</sup> Progenitors during Development and Adulthood**

Our data expose clear parallels between mechanisms that drive adrenogonadal development and the differentiation of AGP-like cells in the adult adrenal cortex. Progenitor cells are characterized by WT1 and GATA4 expression. WT1 prevents differentiation by regulating expression of key genes such as *GLI1* and *TCF21*. Suppression of WT1 is thus a key step to allow steroidogenic differentiation both during development and or adult AGP-like cells. Upon gonadectomy, AGP-like cells respond by differentiating into gonadal steroidogenic cells.

*Tcf21*-independent mechanisms may also contribute to the observed phenotype. Consequently, WT1 appears to have a dual function in the development of the adrenal cortex. First, it is required for the activation of *Sf1* thus determining the steroidogenic primordium. At the same time it limits the amount of *Sf1* expression via the activation of *Tcf21*, thus effectively inhibiting full-blown differentiation toward the steroidogenic lineage (Figure 7). This second process probably occurs in a population of cells, which will give rise to the adrenal capsule. Thus, WT1 acts as a molecular switch between steroidogenic or capsular identity playing a major role in regulating organ homeostasis.

In gonads, coexpression of WT1 and intermediate levels of SF1 persist within Sertoli cells until later stages, where they contribute to the expression of the nonsteroidogenic hormone AMH (Nachtigal et al., 1998). Remarkably, also in gonads steroidogenesis is restricted to cells that lose WT1 expression and show high levels of SF1: the Leydig cells in testes and theca cells in ovaries (Morohashi and Omura, 1996). We therefore hypothesize that high levels of WT1 are more generally incompatible with the steroidogenic program. Consistent with this notion, induced deletion of *Wt1* in Sertoli cells leads to an increase in the number of steroidogenic (Leydig) cells (Gao et al., 2006), possibly by trans-differentiation. *-KTS<sup>GOF</sup>* mice do not show a gonadal phenotype, but the levels of WT1 expression from the *Rosa26* promoter appear to be extremely low. Further studies with stronger promoters would be needed to evaluate the effect of WT1 overexpression in gonadal steroidogenic cells.

#### WT1 Marks a Progenitor Cell Population within the Adrenal Gland

After separation from the AGP, adrenal development proceeds through a series of well-defined steps: (1) invasion of the adrenal primordium by chromaffin cells that will eventually form the adre-

nal medulla (Anderson and Axel, 1986); (2) encapsulation of the adrenal primordium (Mesiano and Jaffe, 1997; Uotila, 1940); and (3) development of the definitive cortex (Zubair et al., 2008). Our cell lineage tracing experiments provide direct evidence for a mesenchymal origin of the adrenal capsule. In *Wt1:Cre-ERT2; mTmG* embryos that were induced at E11.5 and E12.5—a time point when WT1 is absent from the adrenal cortex, but expressed within mesenchymal cells surrounding the forming adrenal gland—the majority of capsular cells were found to be GFP positive. These data indicate that a significant proportion of cells of the adrenal capsule are derived from condensing WT1<sup>+</sup> mesenchymal precursors.

The lineage tracing experiments carried out in this study also allows us to draw important conclusions regarding the formation of the steroidogenic lineage. Using the constitutive *Wt1:Cre* line, all steroidogenic cells were GFP positive, indicating that an early progenitor population—the AGP—must have expressed WT1. In contrast, activation of *Wt1:Cre-ERT2* at later stages (E11.5 and onward) did not result in a significant number of labeled steroidogenic cells, suggesting that these cells are derived from a population of progenitors that has lost WT1 expression. The recently identified GLI1<sup>+</sup> cell population (King et al., 2009; Huang et al., 2010) is likely to fulfill this role. Our immunofluorescent analysis identified four cell populations defined by the expression pattern of WT1 and GLI1 in the developing adrenal (Figures S4E and S4F): WT1<sup>+</sup> GLI1<sup>+</sup> capsular, WT1<sup>+</sup> GLI1<sup>−</sup> capsular, WT1<sup>+</sup> GLI1<sup>−</sup> cortical, and WT1<sup>−</sup> GLI1<sup>+</sup> capsular cells. We thus propose that this last population constitutes the main steroidogenic progenitors that give rise to the definitive cortex.

While not the major source of steroidogenic cells during development, WT1<sup>+</sup> adrenal cells maintain the ability to differentiate into steroidogenic cells throughout life. Interestingly, the number of cortical GFP<sup>+</sup> patches in *Wt1:Cre-ERT2; mTmG* mice increases over time. Whereas early on patches are probably generated by expansion of WT1<sup>+</sup> cells located within the cortex at the time of activation, in adult life other progenitors must be responsible for their generation. Three lines of evidence suggest the adrenal capsule as a source of these cells: (1) the adrenal capsule forms a rich reservoir of WT1<sup>+</sup> cells; (2) capsular cells share many of the properties of “A cells,” including the expression of WT1, GATA4, *Tcf21*, and *Gli1*; and (3) GFP<sup>+</sup>/WT1<sup>+</sup> patches are always found in the subcapsular area and are in direct contact with the capsule.

#### Invading Cells Maintain AGP-like Properties throughout Life

Invasion of the subcapsular region by spindle-shaped cells (“A cells”) is a frequent event in aged mice (Woolley et al., 1952, 1953). Our data identify WT1<sup>+</sup> progenitors that are probably situated within the capsule, as the source of these cells. Interestingly, AGP-like cells have the ability not only to differentiate into adrenocortical, but also into gonadal steroidogenic lineages upon gonadectomy. As such, they share features of adrenogonadal progenitors. Indeed, their molecular signature appears to largely overlap with that of AGP cells as they express WT1, GATA4, and SF1.

The process of differentiation of capsular cells into adrenocortical or gonadal-like cells appears to recapitulate normal development (Figure 7). WT1<sup>+</sup> capsular cells coexpress GATA4,

both of which are also found in pre-AGP cells (Figure 1A; E9.5). During the process of differentiation into “A cells”, low levels of SF1 expression, a specific marker of the AGP, are acquired (Figure 1A; E9.75; Hatano et al., 1996). The final step of differentiation involves loss of WT1 and an increase of SF1 expression that in turn activates the steroidogenic program.

“A cells” are generated in control and gonadectomized mice. In control mice, “A cells” remain quiescent and only very few cells terminally differentiate into adrenocortical cells and express the steroidogenic markers SF1, AKR1b7, and 3 beta HSD. In contrast, gonadectomy induces proliferation and differentiation of “A cells” toward the steroidogenic gonadal lineage, as demonstrated by expression of CYP17 and the LH receptor. Differentiation is likely to be a direct response to the increased levels of luteinizing hormone (LH) that are induced after removal of the gonads (Bielinska et al., 2003, 2005; Johnsen et al., 2006; Kero et al., 2000). The increased production of “A cells” and their differentiation into gonadal cells can therefore be interpreted as an attempt of the mammalian body to balance hormone homeostasis.

Interestingly, a similar mechanism may be at work in gonads of children that suffer from congenital adrenal hyperplasia. Lack of glucocorticoid production in these patients disrupts the negative feedback regulation of adrenocorticotrophic hormone (ACTH) secretion. The resulting high levels of ACTH induce so-called adrenal rests within gonadal tissue that have features of adrenal steroidogenic cells (Cabrera et al., 2001; Stikkelbroeck et al., 2001). It is tempting to speculate that AGP-like cells also persist in gonads that, upon hormonal challenge, can be activated to differentiate into adrenal steroidogenic precursors. Alternatively, these cells are derived from developmental progenitors as suggested by Val et al., 2006.

Based on the above findings, we propose that WT1<sup>+</sup> capsular cells in the adrenal gland might represent a reserve stem/progenitor cell population with adrenal progenitor features that is mobilized only after severe loss of steroidogenic cells has occurred. The fact that the mammalian body responds by inducing differentiation of WT1<sup>+</sup> progenitors into gonadal cells at a heterotopic site (the adrenal gland) is an impressive feat that exemplifies the remarkable adaptation of the mammalian body to stress.

In conclusion, we identified WT1, and more specifically its –KTS isoforms, as an essential player in maintaining AGP cells in the undifferentiated state. Although vital for adrenal and gonadal development, its repression is a key step for differentiation of adrenocortical steroidogenic cells. As a further demonstration of this, undifferentiated AGP-like cells that maintain WT1 are found in the adrenal cortex throughout life, where they maintain the ability to generate both steroidogenic cell types.

## EXPERIMENTAL PROCEDURES

### Generation of the *Rosa26:Wt1*<sup>+</sup> and –KTS Mice

*Rosa26:Wt1*–KTS knockin mice were generated by homologous recombination in ES cells as previously described (Hammes et al., 2001). The cDNA encoding WT1–KTS was cloned into pENTR11 (Invitrogen) and introduced into the *Rosa26*–DEST vector (Hohenstein et al., 2008), by in vitro recombination using the enzyme LR clonase II (Gateway Technology, Invitrogen). The generated targeting vector was linearized with KpnI and electroporated into 129-derived murine ES cells. G-418 resistant clones were then screened

with Southern blot using a 5′ end probe and double-checked with a neomycin probe.

Three positive clones for the construct were microinjected into C57BL6 blastocysts. Chimeric male founders were crossed with C57BL6 females to achieve germ-line transmission of the targeted allele.

### Mice

All animal work was conducted according to national and international guidelines. The *Sf1:Cre*; *mTmG*, *Wt1:Cre-ERT2* and *Gli1:LacZ* mouse lines were previously described (Bai et al., 2002; Bingham et al., 2006; Muzumdar et al., 2007; Zhou et al., 2008). Cell lineage tracing experiments were carried out by force-feeding the mice with 4 mg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich), per 20 g of body weight. Cre activation during development was obtained by a single tamoxifen administration into pregnant females carrying either E 12.5 or E 14.5 embryos. Cre activation in adult mice was obtained by administering tamoxifen twice at 3 weeks (with 3-day intervals) of age or twice a week for 2 weeks starting at 10 weeks of age.

Bilateral gonadectomy was performed on 3- and 12-week-old *Wt1:Cre-ERT2*; *mTmG* animals 3 days after tamoxifen administration. Animals were monitored until they fully recovered and were sacrificed at different time points.

### Quantification of GFP-Positive Patches

For quantification of the number of GFP<sup>+</sup> patches, *Wt1:Cre-ERT2*; *mTmG* animals were treated with a single dose of tamoxifen and sacrificed at different time points. Both adrenal glands were fixed, cut, and every section was stained with anti-GFP and WT1 antibodies. Quantification was performed by microscope observation.

### Immunofluorescence and Histological Analysis

For immunofluorescence experiments, tissues were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and paraffin-embedded. Then 7 μm thick sections were rehydrated, boiled in a pressure cooker for 2 min with Antigen Unmasking Solution (Vector Laboratories), and blocked in a PBS solution containing 10% normal donkey serum (Jackson ImmunoResearch) and 3% BSA. All primary antibodies were applied overnight at 4°C at the concentration listed in the Supplemental Experimental Procedures. Secondary antibodies were diluted 1:400 and applied at room temperature for 1 hr. WT1 was detected using a biotinylated secondary antibody, followed by streptavidin-Cy3 conjugated (Sigma-Aldrich).

GFP immunohistochemistry after RNA in situ hybridization was performed without antigen retrieval. The primary GFP antibody was detected with a POD-conjugated secondary antibody. DAB staining was performed using the Vector DAB substrate kit for peroxidase (cat. n. SK 4100) following manufacturer instructions.

For histological analysis, adrenal glands or gonads from embryos or adult mice were fixed overnight in Bouin solution (Sigma Aldrich) or 4% paraformaldehyde, were progressively dehydrated, and were embedded in paraffin-embedding media. Then 7 μm thick sections were then stained with hematoxylin and eosin.

### RNA In Situ Hybridization

Tissues were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and paraffin-embedded. Then 7 μm thick sections were hybridized with *Tcf21*, *Gli1*, *Ptch1*, *Shh*, *P450scc*, *21-hydroxylase*, or *LH* receptor probes (details on request) according to previously described protocols (Comai et al., 2010). The *Tcf21* probe was PCR amplified from embryonic kidney cDNA using the primers *Tcf21* S and *Tcf21* AS. The PCR fragment was cloned into the pCR11 vector (Invitrogen). The *Lhr* probe was PCR amplified from adult mouse cDNA ovary using the primers LUPub Fr and LUPub Rev. pCR2.1 containing the *Gli1* in situ probe was kindly provided by M. Studer (INSERM U636, Université de Nice-Sophia Antipolis).

### RT-qPCR

RNA was extracted from E14.5 or adult adrenal glands using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) in combination with random primers. The cDNA obtained was then used as a template for



quantitative PCR carried out using the TaqMan Master Kit (Roche) and a Light Cycler 1.5 (Roche). Expression levels were normalized for *Hprt1*. Primers (see primers list in the [Supplemental Experimental Procedures](#)) were designed on Roche Universal ProbeLibrary website.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were conducted as described in Weinmann and Farnham 2002, with minor modifications. Adrenal glands from *Rosa26<sup>WT1-KTS/WT1-KTS</sup>; Sf1:CRE* adult mice were dissected and dissociated in 20 mM HEPES pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% SDS, 0.125 M glycine, and 0.5 mg/ml phenylmethylsulfonyl fluoride (PMSF) and nuclei were released by mechanical stress. Chromatin from nuclei was sheared by sonication to an average of 400–600 bp in 20 mM HEPES pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.4% SDS, and 0.5 mg/ml PMSF. Fifteen micrograms of antibody directed against WT1 (C19, Santa Cruz) or Dicer (Santa Cruz) (used as a control) was incubated with chromatin in 20 mM HEPES pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.8% Triton X-100, 0.1% SDS, and 0.5 mg/ml PMSF. Controls included a mock reaction consisting of a chromatin sample in buffer and with no antibody. Protein A Sepharose (Sigma) was used to precipitate chromatin IgG complexes. Protein A was washed in series; three times with 20 mM HEPES pH 7.4, 1 mM EDTA, 500 mM NaCl, 0.8% Triton X-100, 0.1% SDS, and 0.5 mg/ml PMSF; three times with 20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP40, 0.5% deoxycholate, and 0.5 mg/ml PMSF; and twice in 20 mM Tris pH 8.0, 1 mM EDTA, and 0.5 mg/ml PMSF. Chromatin was eluted with 50 mM NaHCO<sub>3</sub> and 1% SDS. Protein-DNA crosslinks in chromatin were reversed by heating at 65°C for 6 hr, followed by phenol chloroform extraction and ethanol precipitation. DNA was resuspended in 20  $\mu$ l of water, and 0.5  $\mu$ l was used in each PCR reaction.

### Hormone Measurements

Hormone measurements were performed as previously described (Sahut-Bar-nola et al., 2010).

### Cell Size Measurement

Cell size in wild-type and *-KTS<sup>GOF</sup>* adrenal glands was measured using the program “cell profiler” (BROAD Institute).

### Statistical Analysis

Statistical analysis was performed according to the Student's t test. Error estimates values are expressed as SEM.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2013.09.003>.

### AUTHOR CONTRIBUTIONS

R.B. and A.S. designed the project. R.B. carried out all experiments, if not otherwise stated. V.P.I.V. contributed to immune-histological experiments and RNA in situ hybridization. F.R. and R.B. generated the *Rosa26:Wt1-KTS Rosa26:Wt1+KTS* strains. V.P.I.V. analyzed the gonadal phenotype in *-KTS<sup>GOF</sup>* mice. F.R. performed gonadectomies. M.C. performed ChIP experiments. I.S.-B. performed hormone dosage on *WT1<sup>GOF</sup>* mice. P.H. provided the backbone for the construction of the targeting construct. W.T.P. provided the *WT1-Cre* and *WT1-CreERT2* strains. A.M. provided important intellectual input and helped analyze the gain-of-function phenotype. R.B. and A.S. wrote the manuscript, and all authors provided editorial input.

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