The Wilms tumor suppressor WT1 regulates early gonad development by activation of *Sf1*

Dagmar Wilhelm¹ and Christoph Englert^{2,3}

Institut für Toxikologie und Genetik, Forschungszentrum Karlsruhe, 76021 Karlsruhe, Germany

In mammals, several genes including the Wilms tumor suppressor gene *Wt1*, the Lim homeobox gene *Lhx9*, and the gene encoding steroidogenic factor 1 (*Sf1*) have been implicated in the development of the indifferent gonad prior to sexual differentiation. Interactions among these genes have not yet been elucidated. Using biochemical and genetic experiments, we demonstrate here that WT1 and LHX9 function as direct activators of the *Sf1* gene. Interestingly, only the -KTS form of WT1 is able to bind to and transactivate the *Sf1* promoter. This observation is consistent with differential roles for the -KTS and +KTS variants of WT1 which have been postulated on the basis of human disorders such as the Frasier syndrome. Our data suggest a pathway in which the products of the *Wt1* and *Lhx9* genes activate expression of *Sf1* and thus mediate early gonadogenesis.

[Key Words: *Dax-1*; *Lhx9*; transcription factor; zinc finger protein; transgenic mice; sex determination]

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In contrast to many other fundamental biological processes such as the control of the cell cycle or the formation of the body axes, sex determination does not seem to be evolutionarily conserved. In fact, this process shows remarkable plasticity and, to date, there is only one gene known, *Dmrt1*, which plays a role in sex development and is conserved among *Caenorhabditis elegans*, *Drosophila*, and mammals (Raymond et al. 2000).

The first phase of sex determination in mammals is the development of the bipotential (or indifferent) gonad. The latter arises as a thickening of the ventromedial surface of the mesonephros and is visible after ~4 wk of human embryonic development and at around embryonic day 10 (E10) in the mouse (Swain and Lovell-Badge 1999). This indifferent gonad is unique among organ primordia since it has the capacity to differentiate into two different organs, ovary or testis.

Factors that are involved in the establishment of the indifferent gonad include the product of the steroidogenic factor 1 (*Sf1*) gene, the Wilms tumor suppressor WT1, and the Lim homeobox protein LHX-9. The *Sf1* gene encodes an orphan nuclear receptor that regulates the expression of several genes involved in steroidogenesis as well as gonadal development, such as members of the

Cytochrome P-450 hydroxylases, *Mis*, and *Dax-1* (Parker et al. 1999). The requirement of SF1 function for adrenal and gonadal development was demonstrated in humans and mice. A heterozygous inactivating mutation of *SF1* was reported to be associated with male to female sex reversal and adrenal failure in a 2-week-old human patient (Achermann et al. 1999). In mice, inactivation of *Sf1* does not seem to cause a phenotype in the heterozygous state, but targeting of both *Sf1* alleles results in failure of adrenal and gonadal development (Luo et al. 1994). The gonads of *Sf1*^{-/-} embryos do not develop beyond the early indifferent stage, and XY *Sf1* mutant embryos display sex reversal in that the Müllerian ducts develop into uteri, oviducts, and upper vagina. The gonadal degeneration in *Sf1*^{-/-} embryos of either sex suggests that SF1 plays a fundamental role in gonad development.

The Wilms tumor suppressor gene *WT1* (*Wt1* in the mouse) was initially identified as a gene inactivated in a subset of Wilms tumors, a form of pediatric kidney cancer (Call et al. 1990; Gessler et al. 1990). A number of additional human diseases were shown to be associated with *WT1* mutations (Englert 1998). Three of these disorders, namely the WAGR (Wilms tumor, aniridia, genitourinary abnormalities, mental retardation), Denys-Drash (DDS), and the Frasier syndrome are characterized by urogenital abnormalities. These abnormalities are variable and can range from cryptorchidism and hypospadias in male WAGR patients to streak gonads and sex reversal of internal and external genitalia in extreme DDS and Frasier cases (Riccardi et al. 1978; Pelletier et al. 1991b; Barbaux et al. 1997; Kikuchi et al. 1998; Klamt

Present addresses: ¹Institute for Molecular Bioscience, The University of Queensland, Brisbane Qld 4072, Australia; ²Biozentrum der Universität Würzburg, Lehrstuhl für Physiologische Chemie I, Am Hubland, 97074 Würzburg, Germany.

³Corresponding author.

E-MAIL cenglert@biozentrum.uni-wuerzburg.de; FAX 49-931-888-4150. Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.220102>.

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et al. 1998). A more fundamental role for WT1 in gonad development is suggested by the analysis of the *Wt1* knockout mouse. Inactivation of both *Wt1* alleles results in embryonic lethality and leads to a failure of kidney and gonad development (Kreidberg et al. 1993). A thickening of the epithelium which gives rise to the gonad primordium can be observed in E11 *Wt1*-mutant embryos, but gonad development does not progress further. The migration of germ cells, however, is not affected by *Wt1* mutation (Kreidberg et al. 1993). It is not known how WT1 contributes to early gonad development.

Expression of *Wt1*, which encompasses 10 exons, yields a protein family arising from usage of alternative translation initiation sites, RNA editing, and two alternative splicing events (I and II, reviewed in Englert 1998). Alternative splice I includes exon 5, which encodes 17 amino acids in the central part of the protein. The functional consequences of the inclusion of exon 5 have not yet been defined. Alternative splice II results from use of an alternative splice donor sequence between exons 9 and 10. Its insertion leads to three additional amino acids (the KTS sequence) that disrupt the spacing between the DNA binding zinc fingers 3 and 4. These splice variants are expressed at a constant ratio whereby the +KTS isoforms constitute the majority of WT1 proteins in a cell (Haber et al. 1991). Interestingly, in Frasier patients this balance of the WT1 splice forms is disturbed due to intronic point mutations affecting the usage of donor splice sites in intron 9 of *WT1*. This suggests that the -KTS and +KTS isoforms of WT1 might play different roles in gonad development. This hypothesis was recently confirmed by the generation of the respective mouse models (Hammes et al. 2001).

WT1 was originally viewed as a potent transcriptional repressor for which multiple targets were described (Reddy and Licht 1996). Recent experiments, however, demonstrated that WT1 can also activate target genes including the cyclin-dependent kinase inhibitor encoding gene *p21*, *Syndecan-1*, *Bcl-2*, *Dax-1*, and *Amphiregulin* (Cook et al. 1996; Englert et al. 1997; Kim et al. 1999; Lee et al. 1999; Mayo et al. 1999). In the context of sex development, *Dax-1* is of particular interest because duplication of this gene leads to XY sex reversal in humans (Bardoni et al. 1994). In mice, inactivation of *Dax-1* affects spermatogenesis, with the testes having half the normal weight (Yu et al. 1998).

An additional factor recently shown to control development of the early gonad is the product of the Lim homeobox gene *Lhx9*. In *LHX9*-deficient mouse embryos, a discrete gonad does not form and *Sf1* expression is reduced to minimal levels (Birk et al. 2000). It is unclear whether *LHX9* regulates *Sf1* directly or indirectly.

Although a number of genes have been shown to play a role in the various stages of gonad development and sex determination, a sex development pathway has not yet emerged. In particular, it is still unclear which interactions exist among the genes required for development of the early indifferent gonad. We have sought to define the role of the Wilms tumor protein WT1 in early gonad development by studying changes in gene expression as-

sociated with *Wt1* inactivation. As a model system, we used the *Wt1* knockout mouse. We demonstrate here that expression of the *Sf1* gene in the early gonad is WT1-dependent. In biochemical experiments, we identified and characterized four WT1 binding sites in the *Sf1* promoter. These binding sites are located within a promoter fragment which is sufficient to activate reporter gene expression in the gonad of transgenic mice in a WT1-dependent manner. In addition, our experiments show that another transcription factor, the product of the *Lhx9* gene, also binds to the *Sf1* promoter and activates *Sf1* expression. Our data suggest a very fundamental and early role for WT1 in gonad development and begin to define a hierarchy among the genes involved in early sex development.

Results

Sf1 is not expressed in *Wt1*^{-/-} gonads

To analyze differences in gene expression between normal and *Wt1*^{-/-} gonads, we isolated RNA from the gonads of wild-type and mutant embryos at days 10.5 and 11.5 of development. At these timepoints, the gonad is still undifferentiated, and RT-PCR analysis at both stages yielded identical results. As shown in Figure 1a, expression of *Sox9* and *Lhx9* was not significantly affected by the inactivation of *Wt1*. In contrast, expression of *Dax-1* and *Sf1* was totally absent in mutant animals compared to wild-type controls. For *Dax-1*, regulation by WT1 was suggested previously but not shown to occur in vivo (Kim et al. 1999). For *Sf1*, this is the first indication that this gene is regulated by WT1. To determine whether WT1, which is expressed in the urogenital ridge from day E9 on (Armstrong et al. 1992), is involved in the initiation and/or maintenance of *Sf1* expression, we performed a time-course experiment (Fig. 1b). *Sf1* expression, which could first be observed at embryonic stage 9.5, was dependent on WT1 at this and all later stages examined. This suggests that WT1 is required for the initiation of *Sf1* expression. We next wanted to confirm the RT-PCR data by an independent experimental approach and used in situ hybridization analysis. Complete absence of *Sf1* expression in *Wt1*^{-/-} gonads at day 11.5 of development was also observed by in situ hybridization (Fig. 1c). To demonstrate the presence of an undifferentiated gonad at this stage of development, we used the marker *Lhx9*, which is only expressed in cells of the gonad and not in structures of the underlying mesonephros (Birk et al. 2000). Although mutant gonads were smaller than their wild-type counterparts, *Lhx9* expression could clearly be observed in both cases. Thus both approaches demonstrate a complete absence of *Sf1* expression in the early gonad of *Wt1*^{-/-} embryos.

A 674-bp Sf1 promoter fragment is sufficient to activate reporter gene expression in the gonad in vivo

To study whether the effect of the WT1 protein on *Sf1* expression was direct or indirect, we first had to identify

Figure 1. Analysis of gene expression in gonads from wild-type and *Wt1*-mutant embryos. (a) RT-PCR analysis using RNA from paired gonad/mesonephros complexes of wild-type and *Wt1*^{-/-} embryos isolated at day 10.5 of embryonic development. The expected fragment sizes are 432 bp (*Wt1*), 335 bp (*Sox9*), 495 bp (*Lhx9*), 522 bp (*Dax-1*), 242 bp (*Sf1*), and 721 bp (*Actin*). M, 100 bp DNA marker. (b) RT-PCR analysis as in a performed with RNA from different stages of development. (c) In situ hybridization analysis using radioactively labeled RNA probes: 7- μ m transverse paraffin sections of wild-type (left) and *Wt1*-mutant embryos (right) at stage E11.5 were hybridized with antisense probes against *Sf1* (top) and *Lhx9* (bottom). For orientation, the respective bright field images are shown in insets. Both sections on the right are from the same embryo. Analysis shown in a, b, and c was done with male embryos.

the region in the *Sf1* promoter which is responsible for the expression of *Sf1* in the gonad. It was shown earlier that a 90-bp fragment of the *Sf1* promoter is sufficient to activate a reporter gene in steroidogenic cells (Woodson et al. 1997). To include additional *cis*-acting elements which might be required for *Sf1* regulation in vivo, we used a DNA fragment encompassing nucleotides -590 to +85 of the *Sf1* promoter which was cloned in front of the *LacZ* gene. With the use of this construct (named *Sf1-blue*), transgenic mice were generated. Of seven potential founder animals, three expressed and passed on the transgene. In all three lines, β -Gal activity was observed in the gonads of E11.5 embryos. Expression levels differed significantly and ranged from low to very high (Fig.

2a). In addition to expression in the gonad, all lines showed β -Gal activity along the spinal cord. Sectioning of the embryos revealed staining of dorsal root ganglia as well as the ventrolateral region of the spinal cord (see Fig. 6c). These expression domains are presently unexplained, as neither *Sf1* nor *Wt1* has been reported to be expressed in these areas. Weak staining was also observed in the hindbrain region. This staining was also observed in nontransgenic controls and probably reflects

Figure 2. Analysis of mice transgenic for the *Sf1-blue* construct. The construct used harbors nucleotides -589 to +85 from the murine *Sf1* promoter region followed by the *LacZ* gene carrying an NLS at its 5' terminus. (a) X-gal staining of nontransgenic (upper panel) and transgenic embryos which express the transgene at low (middle panel) or high levels (bottom panel) are shown in a side (left) and top view (right). All animals were at stage E11.5, and the staining pattern and intensity were independent of the sex of the animals. The arrowhead and the dotted line demarcate the weakly stained cells in the gonads of the low-expressing line. (b) Colocalization of SF1 and β -Gal activity in an *Sf1-blue* embryo. X-gal stained nontransgenic (left) and transgenic (highly expressing, right) E11.5 embryos were processed into 7- μ m transverse paraffin sections and then treated with an anti-SF1 antibody. SF1-positive cells appear dark purple. For better orientation, the area containing stained cells in the right panel is demarcated by a dotted line. gr, gonadal ridge; ms, mesonephros.

ectopic reporter gene expression due to cryptic elements present in the *LacZ* gene (Moore et al. 1998). No transgene expression was seen in other organs normally expressing *Sf1* such as the adrenal glands and the hypothalamus. Given the consistent β -Gal activity in the gonad of all animals examined thus far, we conclude that a 674-bp fragment of the 5' region of the *Sf1* gene contains regulatory elements which are sufficient for expression in the gonad *in vivo*.

To assess whether the same cells which express the *Sf1* gene endogenously also display β -Gal activity, we performed a double labeling experiment. We used an anti-SF1 antiserum to analyze sections from nontransgenic and transgenic embryos which had been stained with X-gal. As described previously (Ikeda et al. 1994), *Sf1* is expressed in the genital ridges of E11.5 embryos (Fig. 2b, left). This expression overlapped significantly with the expression pattern of the transgene; that is, almost every cell which was positive for SF1 also showed β -Gal activity (Fig. 2b, right). Thus with regard to gonadal *Sf1* expression at the timepoint examined, the 674-bp *Sf1* promoter fragment used here seems to reflect the endogenous expression.

Characterization of WT1 binding sites within the *Sf1* promoter

To determine whether the WT1 protein is able to bind to the *Sf1* promoter, we performed a *DNase I* footprinting analysis. We made use of an internal *Bst*XI site at position -210 of the *Sf1* promoter and separated the 674-bp fragment into a distal (P5) and a proximal subfragment (P3). Our analysis revealed protection from *DNase I* digestion of four regions by the WT1(-KTS) protein but not by GST alone or WT1(+KTS) (Fig. 3a). One protected region (nucleotides -325 to -304, *WB1*) was located within the distal fragment, and three regions (nucleotides -206 to -182, *WB2*; nucleotides -83 to -66, *WB3*, and nucleotides -49 to -25, *WB4*) were identified within the proximal fragment. The distal region encompassed an almost perfect WT1-response element (*WTE*) which had been previously defined as a high-affinity WT1 binding site (Nakagama et al. 1995). The sequence of the *WTE* is GC GTGGGAGT, whereas the *Sf1* promoter harbors the element GGGTGGGAGT (nucleotides -309 to -318 in antisense orientation). The region centered on nucleotide -74 harbored an E-box, whereas the most proximal potential WT1 binding site overlapped in part with a GA-rich element (Fig. 4d). Both elements were previously shown to be essential for *Sf1* promoter activity in steroidogenic cells (Woodson et al. 1997).

To confirm these sequences as WT1 binding sites, we performed electrophoretic mobility shift assays (EMSAs). Using labeled duplex oligonucleotides, WT1(-KTS) was shown to bind to all four sequences. In each case, introduction of a triple point mutation (Fig. 3c) abolished binding of WT1(-KTS) to the respective element. WT1(+KTS) as well as a DDS patient-derived mutant form of WT1(-KTS) with an arginine to tryptophane ex-

change at position 394 (Pelletier et al. 1991a) did not bind any of the probes (Fig. 3b; data not shown). Mutation of *WB1* within the distal *Sf1* promoter fragment and *WB2* to *WB4* within the proximal fragment completely abolished WT1 binding. This indicates that there are no additional WT1 binding sites present within the *Sf1* promoter fragment. Alignment of the four sites demonstrates the presence of a common GTGGG motif, which can also be found in the *WTE* sequence (Fig. 3c).

Binding of LHX9 to the *Sf1* promoter

The absence of *Sf1* expression in the gonads of *Wt1*^{-/-} embryos demonstrates that WT1 is required for the activity of the *Sf1* gene. It was recently shown that in gonadal tissue from embryos lacking the *Lhx9* gene, only minimal *Sf1* expression can be detected although the *Wt1* gene is transcribed (Birk et al. 2000). This suggests that WT1 is necessary but not sufficient to activate *Sf1* expression. The almost complete absence of *Sf1* expression in *Lhx9*^{-/-} embryos prompted us to examine whether the *Lhx9* gene product is able to bind to and activate the *Sf1* promoter. Incubation of the distal *Sf1* promoter region with recombinant GST-LHX9HD protein (C-terminus including the homeodomain) revealed protection of nucleotides -304 to -290 from *DNase I* digestion (Fig. 4a). In the proximal promoter fragment, no protection could be detected. The potential LHX9 binding site is immediately adjacent to the most distal WT1 binding site and harbors the sequence CGCTAA CAAGCCGC (Fig. 4d). EMSA experiments using oligonucleotides demonstrated that the homeodomain of LHX9 is sufficient for binding of the protected sequence (Fig. 4b). A mutated sequence CGCTCACCAGCCGC was no longer bound by LHX9. Incubation of a labeled fragment encompassing the WT1 and the LHX9 binding sites with both recombinant proteins and subsequent EMSA analysis revealed the formation of an additional complex (Fig. 4c). This complex could be observed using the homeodomain of LHX9 as well as with the full-length protein (data not shown). This indicates that at least *in vitro*, both proteins can bind the *Sf1* promoter simultaneously.

WT1(-KTS) and LHX9 activate the *Sf1* promoter

After having identified four WT1 binding sites and one LHX9 binding site within the *Sf1* upstream region, we sought to determine whether both proteins could transactivate the *Sf1* promoter in transfection experiments. For this analysis we used two different cell types, the murine Sertoli cell line TM4 and human U2OS-derived *Wt1*-inducible cell lines (Englert et al. 1995). Since both systems yielded virtually identical results, only those generated with the TM4 cells are shown. Expression analysis of these cells demonstrated absence of WT1 and SF1 at the protein level as well as absence of *Lhx9* mRNA. Cotransfection of *Wt1*(-KTS) expression constructs with the 674-bp *Sf1* promoter fragment (SF1P, Fig. 5a) resulted in a moderate twofold activation of lu-

Figure 3. Identification of WT1 binding sites within the *Sf1* promoter. (a) *DNase I* footprint analysis of the *Sf1* promoter (nucleotides –589 to +85). A *Bst*XI site at position –210 served to define a proximal (P3) and a distal promoter fragment (P5) which were used to characterize WB1 and WB3/WB4, respectively. For WB2, a fragment encompassing nucleotides –350 to –50 was used. The fragments were 5'-labeled, incubated with recombinant GST (control), GST-WT1(–KTS), and GST-WT1(+KTS) protein and subjected to partial *DNase I* digestion. Sequencing reactions were performed using the same fragments as for the footprinting experiment. Four regions protected by GST-WT1(–KTS) are indicated by boxes. Four hypersensitive sites are marked by asterisks. (b) EMSA analysis of *Sf1* promoter fragments using oligonucleotides constituting the WB1 site (left) as well as the distal (P5) and proximal (P3) *Sf1* promoter fragments (right) as probes. Labeled oligonucleotides and fragments were incubated with recombinant GST, GST-WT1(–KTS), and GST-WT1(+KTS) protein as well as a mutant form of GST-WT1(–KTS) harboring a point mutation at position 394. The mutated WB1 probe contains three point mutations (described in c). The same experiments were done using probes WB2 to WB4. In the case of the P5 as well as the P3 fragment, all potential WT1 binding sites (WB1 to WB4) were mutated (P5mut, P3mut). (c) Alignment of the four WT1 binding sites within the *Sf1* promoter. At the top, the high-affinity WT1 binding site (WTE) (Nakagawa et al. 1995) is indicated. Sequences represent oligonucleotides which were used to characterize the WT1 binding sites in an antisense (WB1, WB2, and WB4) or sense (WB3) orientation. The asterisk marks the single mismatch between the WB1 site and the WTE. Exchange of the underlined nucleotides into thymidine residues abolishes WT1-binding. The GTGGG motif which all the WT1 binding sites have in common is boxed and shaded.

ciferase activity (Fig. 5b). Introduction of point mutations into any of the WT1 binding sites resulted in an increase in WT1-mediated activation (shown here for WB3). This suggests that in this system, WT1 can also exert some repressive function. The +KTS form of WT1 did not have any effect in this assay. To identify the sites which mediated the activation, we used the distal and proximal *Sf1* promoter fragments independently. Whereas the proximal subfragment encompassing sites WB2, WB3, and WB4 did not significantly stimulate reporter gene activity upon *Wt1* cotransfection (data not shown), the distal fragment (P5) harboring WB1 was ac-

tivated by WT1(–KTS) fivefold. The next series of experiments was therefore performed using the reporter construct harboring the distal fragment only. Cotransfection of this construct with an *Lhx9* expression vector resulted in a 2.5-fold activation in the absence of WT1. Together with WT1(–KTS), however, LHX9 led to eightfold activation. This result shows that at least in the system employed here, WT1 and LHX9 bind simultaneously and have an additive effect in activating the *Sf1* promoter fragment. Mutational analysis of the WT1 binding site and the LHX9 binding site in the distal promoter fragment demonstrated that both sites function indepen-

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Figure 4. Identification of an LHX9 binding site within the *Sf1* promoter. (a) *DNase I* footprinting analysis of the distal *Sf1* promoter fragment (nucleotides -589 to -210). The DNA fragment was end-labeled, incubated with recombinant GST (control), GST-WT1(-KTS), and GST-LHX9HD protein and subjected to partial *DNase I* digestion. A sequencing reaction was performed using the same fragment as for the footprinting experiment. The regions protected by WT1(-KTS) and LHX9 are indicated by boxes. (b) EMSA analysis of the LHX9 binding site using wild-type and mutant oligonucleotide probes. (c) EMSA analysis of an *Sf1* promoter fragment encompassing nucleotides -589 to -284. The asterisk marks a complex present only when WT1(-KTS) and LHX9HD are used simultaneously. (d) Sequence of the murine *Sf1* 5'-flanking region. Nucleotide numbers, the positions of the E-box, and the GA-rich element as well as the position of the transcriptional start site refer to a published promoter sequence (Woodson et al. 1997). WT1 binding sites as revealed by *DNase I* footprinting and EMSA analysis are highlighted by gray shading. A WTE-like element around position -314 is boxed. The LHX9 binding site is shaded in light gray.

dently, because mutation of the WT1 binding site did not affect the activation by LHX9, and vice versa (Fig. 5b). Interestingly, we found a consistent repression of the WT1-mediated activation of fragment P5m2 (harboring a

mutation of the LHX9 binding site) upon cotransfection of *Lhx9*. Finally, the introduction of point mutations into all four WT1 binding sites within the complete 674-bp fragment led to a complete loss of promoter activity upon *Wt1*(-KTS) cotransfection. This confirms the conclusion from the EMSA experiments and suggests the absence of additional functional WT1 binding sites within this region.

To examine whether expression of the endogenous *Sf1* gene could be induced by *Wt1* overexpression, we performed an RT-PCR analysis in the *Wt1*(-KTS)-inducible U2OS cell line. Upon induction of *Wt1*, however, no activation of *Sf1* expression could be detected (data not shown). This is in line with the published observation indicating that *Wt1* expression is not enough to stimulate the endogenous *Sf1* promoter (Birk et al. 2000) and can most likely be explained by the absence of cofactors.

Expression of LacZ in the gonad of *Sf1*-blue mice is WT1-dependent

To examine whether *LacZ* expression observed in the gonad of *Sf1*-blue transgenic mice requires the activity of WT1, we performed two types of experiments. First, we bred transgenic animals into the *Wt1*-mutant background. Upon crossing of transgenic males which were heterozygous for the *Wt1* mutation with a *Wt1*^{+/-} female, embryos of different genotypes were obtained and assayed for β-Gal activity at stage E11.5 of development. Whereas transgenic embryos without or with one mutated *Wt1*-allele displayed β-Gal activity in the gonads, X-gal staining of the gonads was almost completely lost in transgenic *Wt1*^{-/-} littermates (Fig. 6a). Ectopic *LacZ* expression in the spinal cord, however, was maintained in *Wt1* knockout animals. Upon characterization of additional transgenic animals, we noted a difference in β-Gal activity between wild-type and heterozygous *Wt1*-embryos. Indeed, subsequent analysis of littermates revealed a dependence of the gonad expression domain but not the *LacZ* expression along the spinal cord on the *Wt1*-gene dosage; that is, in all cases examined, heterozygous *Wt1* embryos showed much weaker staining of the gonads compared to wild-type littermates (Fig. 6b). This seemed to be caused by a lower expression level per cell as well as by a smaller number of cells which expressed the transgene (Fig. 6b). At this stage, β-Gal activity was independent of the sex of the respective animal. Given the absence of any phenotypic differences between *Wt1*^{+/-} and wild-type animals, the decrease in *LacZ* transgene expression in *Wt1*^{+/-} and *Wt1*^{-/-} animals is not a consequence of loss of a specific cell population but rather a direct result of the reduction of WT1 activity. This gene dosage effect was observed in all three *Sf1*-blue lines.

To investigate whether WT1 acts directly on the *Sf1* promoter fragment, we generated transgenic mouse lines using the *Sf1*-blue construct in which all four WT1-binding sites had been mutated. This construct was named *Sf1*-white. Six independent lines were characterized, which all showed X-gal staining along the spinal cord, as

Figure 5. Activation of the *Sf1* promoter by WT1 and LHX9. (a) Schematic representation of the luciferase reporter constructs used. Positions of the four WT1 binding sites, the LHX9 binding site (LB), as well as the *Bst*XI site are indicated. The promoterless reporter vector pGL3basic was used as a backbone. Point mutations are indicated by asterisks. (b) Activation of the *Sf1* promoter by WT1 and LHX9. TM4 cells were cotransfected with different *Wt1* and *Lhx9* expression plasmids and the respective reporter constructs. Results are given as relative activation of the reporter by the expression constructs compared to the empty vector. Data represent the mean and standard deviation of three independent experiments.

did the *Sf1-blue* mice. In contrast to the latter, however, no expression of *LacZ* could be observed in the gonads of the *Sf1-white* embryos (Fig. 6c). We conclude from these experiments that the WT1 protein is responsible for the activity of the 674-bp *Sf1* promoter fragment in vivo.

WT1^{-/-} embryos show no signs of sexual development

The targeted inactivation of *Sf1* leads to death of the embryos by postnatal day 8 and is characterized by a lack of adrenal and gonad development (Luo et al. 1994). Despite the failure of gonadal organogenesis, XY and XX *Sf1* mutant mice have female internal genitalia including oviducts, uteri, and vagina. Homozygous deletion of *Dax-1* does not interfere with gonad development per se but leads to a progressive degeneration of the testicular epithelium resulting in male sterility (Yu et al. 1998). Normally *Wt1*^{-/-} embryos die between stage E13.5 and E15.5, but we recently demonstrated that crossing the *Wt1* mutation into a different mouse background (MF1) prolongs the lifespan of mutant animals until birth (Herzer et al. 1999). Prompted by our observation that WT1 regulates both *Sf1* and *Dax-1* in vivo, we examined the development of the internal genitalia on serial sections

of wild-type and *Wt1* mutant embryos at stages E13.5 and E18.5. We detected no signs of internal reproductive organs of either sex in *Wt1*^{-/-} embryos; that is, in addition to the lack of gonad development, no derivatives of the Müllerian duct (uterus, oviducts, upper vagina) or the Wolffian duct (seminal vesicles, epididymis, vas deferens) were seen (data not shown). This indicates that in terms of sex development the phenotype associated with the inactivation of *Wt1* is more severe than the one caused by deletion of *Sf1* or *Dax-1*. This observation is in agreement with our molecular analysis indicating that the *Wt1* gene is located upstream of both *Dax-1* and *Sf1*.

Discussion

A large number of genes regulated by WT1 have been characterized (Menke et al. 1998). In these cases, WT1 can function as both a repressor and an activator of transcription. The different WT1 targets have mostly been identified on the basis of the similarity of promoter sequences with WT1 binding sites and subsequent transient transfection experiments. There has not been a good correlation, however, between the results of transient transfections and the regulation of the respective

Figure 6. *LacZ* expression in the gonad of *Sf1-blue* mice is WT1-dependent. (a) Transgene expression is severely reduced in *Wt1*^{-/-} embryos. *Sf1-blue* mice were crossed into the *Wt1*-mutant background. X-gal staining was performed using embryos of different genotypes at stage E11.5 of two different *Sf1-blue* mouse lines (top, intermediate expressing line; bottom, highly expressing line). Insets show the isolated gonadal and mesonephric structures. (b) Transgene expression depends on the *Wt1* gene dosage. A comparison of X-gal stained wild-type and *Wt1*^{+/-} whole embryos (upper panel) and transverse sections (lower panel) of an *Sf1-blue* line (intermediate expressing line) is shown. (c) Analysis of mice transgenic for the *Sf1-white* construct which harbors nucleotides -589 to +85 from the murine *Sf1* promoter region with triple point mutations in each of the four WT1 binding sites followed by the *LacZ* gene carrying an NLS at its 5' terminus. Transgenic embryos at stage E11.5 were stained with X-gal (upper panel) and subsequently cut in 7-μm sections (lower panel). For comparison, a section of an X-gal-stained *Sf1-blue* embryo is shown. All animals shown in this figure are heterozygous with regard to the transgene.

endogenous promoter sequences (Englert et al. 1995; Thate et al. 1998). Large-scale expression profiling with RNA from *Wt1*-inducible cells has been used to search for WT1 targets, and *Amphiregulin* was identified as a gene activated by WT1 (Lee et al. 1999). In no case, however, has the *Wt1* knockout mouse been used to verify the physiologic significance of an interaction. To identify genes mediating the function of WT1 during gonad development, we analyzed gene expression in the early gonad of wild-type and *Wt1*-mutant embryos. Our analysis shows that the expression of the *Dax-1* and *Sf1* genes depends on the presence of WT1. This extends the earlier observation that *Dax-1* is activated by WT1 (Kim et al. 1999) and shows that this interaction is also relevant in vivo.

With *Sf1* we have identified the first physiologic WT1 target which mediates its function in early gonad development. Our data suggest that WT1 is required for the initiation of *Sf1* expression, because even at the earliest timepoint where *Sf1* expression can be observed (E9.5; Ikeda et al. 1994, our data), it is absent in *Wt1*-mutant embryos. In addition to in situ and biochemical analyses, the strongest support for *Sf1* being a genuine WT1 target comes from in vivo experiments using transgenic mice. Crossing of mice carrying a *LacZ* reporter gene under the control of an *Sf1* promoter fragment into a *Wt1*-mutant background revealed a dependence of transgene expression on WT1 activity. This dependence was sensitive to *Wt1* gene dosage as shown by a comparison of transgenic wild-type and *Wt1*^{+/-} littermates. No difference between wild-type and heterozygous *Wt1*-animals has been reported to date, and all available evidence points to normal development of the gonads in *Wt1*^{+/-} animals (Kreidberg et al. 1993; our data). Thus, reduction of transgene expression associated with *Wt1* heterozygosity is not a consequence of degeneration of the gonads and can best be explained by a direct effect of WT1 on the *Sf1* promoter. This is corroborated by our observation that an *Sf1* promoter construct with mutated WT1 binding sites is not able to drive reporter gene expression in the gonads.

In this study we focused on a 674-bp fragment of the *Sf1* promoter encompassing elements which had been shown to activate reporter gene expression in steroidogenic cells (Woodson et al. 1997). Given the size of the fragment, it seems unlikely that it is able to mimic full endogenous *Sf1* expression. Nevertheless, this fragment activated *LacZ* expression in the indifferent gonad at stage E11.5 of transgenic mouse embryos, suggesting that it harbors elements which are sufficient to direct *Sf1* expression in the gonad in vivo. Our analysis of the interaction between WT1 and the *Sf1* promoter fragment suggests that this interaction is direct. Binding of WT1 to the *Sf1* promoter in vitro occurs via four binding sites which all share the motif GTGGG. This sequence can also be found in the high-affinity WT1 binding site GC GTGGGAGT (Nakagama et al. 1995). From our analysis and previous data, one can therefore derive the consensus Pu(G/C)GTGGGPuG for a WT1 binding site.

With regard to its transcriptional activity, it is inter-

esting to note that while WT1 acts as an overall activator of the *Sf1* promoter, in the transient transfection system employed here, WT1 also exerted some repressive activity. Whereas WT1 activated the 674-bp *Sf1* promoter fragment or subfragments thereof, mutation of any single WT1 binding site resulted in an even higher activation level. This is reminiscent of the *PDGF A-chain* promoter, where occupancy of multiple binding sites by WT1 was associated with transcriptional repression, and binding to a single site led to activation (Wang et al. 1993). At this point it seems appropriate to point out that the extrapolation of observations generated by using in vitro systems like transient transfection experiments to the situation in vivo must be made with great caution. This is also exemplified by the fact that whereas small *Sf1* promoter fragments could be activated by WT1 in the transient transfection system, expression of the endogenous *Sf1* gene could not be achieved by *Wt1* expression alone. This is probably due to the lack of additional factors. Nevertheless, our results confirm earlier observations about the bipotential nature of the WT1 protein (Reddy et al. 1995) and suggest that the amount of available WT1 protein is critical in determining the transcriptional activity of WT1.

An additional protein which influences *Sf1* expression is the product of the *Lhx9* gene (Birk et al. 2000). Our biochemical analysis shows that LHX9 can bind directly to the *Sf1* promoter. The binding site encompasses the motif TAACAA, which upon mutation to TCACCA can no longer be bound by LHX9. To our knowledge this sequence, which shows similarity but is not identical to any of the presently known LIM homeodomain gene targets (Jurata and Gill 1998), is the first LHX9 binding site described. Interestingly, all four WT1 as well as the LHX9 binding sites are conserved between the mouse and the human *Sf1* promoter (Woodson et al. 1997). The interaction between WT1, LHX9, and the *Sf1* promoter is reminiscent of the situation described for the *Mis* promoter. For normal *Mis* expression to occur, the activity of two transcription factors, SF1 and SOX9 is required. Whereas SOX9 plays an essential role in the initiation of *Mis* transcription, SF1 has a more modulatory role in regulating transcript levels (Arango et al. 1999). Given the proximity of the WT1 and the LHX9 binding site within the distal *Sf1* promoter sequence, one could speculate that, as in the case of SOX9 and SF1 (De Santa Barbara et al. 1998), the two factors might interact with each other. This could lead to a reciprocal sequestration and might explain the repressive effect of LHX9 overexpression on WT1-mediated transactivation of a promoter fragment with a mutated LHX9 binding site.

A fascinating aspect of the biochemistry and biology of the WT1 protein is the existence of the various splice forms. In particular, the -KTS and the more prevalent +KTS forms, differing only by a 3-aa insertion in the zinc finger region (Haber et al. 1991) have led to many speculations about their different roles in the etiology of diseases. Are there different roles for the WT1(-KTS) and the WT1(+KTS) form in sex development? The answer is clearly, yes. First, patients with an altered ratio of these

particular splice forms show abnormalities in urogenital development and function (Barboux et al. 1997; Kikuchi et al. 1998; Klamt et al. 1998). Second, it is the -KTS form of WT1 which is able to bind to and synergize with SF1 in the activation of the *Mis* gene (Nachtigal et al. 1998). Third, our analysis shows that it is again only the -KTS form which is able to bind to and transactivate the *Sf1* promoter. Based on these findings, we would predict a more fundamental role for the -KTS form compared to the +KTS variant in gonad development. This was indeed recently confirmed by the generation of mouse lines which express exclusively either the +KTS or the -KTS isoform of WT1 (Hammes et al. 2001). The analysis of the animals revealed a requirement of the -KTS variants for the survival of the gonadal primordium, whereas the +KTS forms seem primarily involved in the male sex determination pathway. Interestingly, *Wt1* null mice display a more severe gonadal phenotype than those with deletion of the WT1(-KTS) splice form. This might be explained by a partial compensation of one splice form by the other.

How can one interpret the role of WT1 as a transcription factor in the context of gonad development? Previous work (Kreidberg et al. 1993) and our own observations demonstrate that in the absence of WT1, the gonad develops only to an early timepoint of the indifferent stage. After E11.0-E11.5, significant differences between wild-type and mutant gonads become visible, and two days later no remnant of the gonad can be observed in *Wt1* mutant animals. Thus, the activity of WT1 is required for the gonad to develop from an early into a late bipotential stage. Our data suggest that one critical mediator of WT1's function in early gonadogenesis is the *Sf1* gene. Together with LHX9, WT1 binds to and activates the *Sf1* promoter, leading to the expression of this key regulator of gonadogenesis. Interestingly, in terms of growth control, the activation and not the repression function of WT1 seems to be the critical transcriptional activity (English and Licht 1999). Our experiments also confirm that the *Dax-1* gene is downstream of WT1. As suggested earlier, this regulation could be direct (Kim et al. 1999). However, it was demonstrated that *Dax-1* can be regulated by the SF1 transcription factor (Kawabe et al. 1999), and it thus remains possible that the activation of *Dax-1* by WT1 is indirect. The role of *Dax-1* in early gonad formation has not been firmly established. The inactivation of *Wt1* has more marked phenotypic consequences than deletion of either *Dax-1* or *Sf1* alone (Luo et al. 1994; Yu et al. 1998). Although it is conceivable that simultaneous deletion of *Sf1* and *Dax-1* from the germline results in a more severe phenotype, it seems more likely that WT1—also given its broader expression domain—regulates additional genes during urogenital development. These could be genes which are activated or repressed by WT1. The identification of additional critical target genes of WT1 which mediate its role in urogenital development as well as the characterization of the cofactors involved will eventually lead to a better understanding regarding the function and mechanism of this pivotal transcription factor.

Materials and methods

Mouse lines

A *Wt*^{+/-} breeding pair was obtained from The Jackson Laboratory and maintained on a C57BL/6J background. Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated E0.5. Genotyping was done by PCR using primers *Wt1*-pgk (CTACCGGTGGATGTGGAATGTGT), *Wt1*-shared (TCCCGAACAATTTACCTTGAATC), and *Wt1*-wt (AGCCTAACTTGGGGCTTATCTCC) in a single tube. The product generated from the wild-type allele comprises 140 nucleotides, and that from the mutant allele 200 nucleotides. Presence or absence of the Y chromosome was analyzed by PCR using *Zfy*- and *Sry*-specific primers as described (Hogan 1994).

RT-PCR analysis

Tissue comprising the trunk (at stages E8.5 and E9.5) or mesonephros and gonad (at stages E10.5 and E11.5) was isolated from mouse embryos of different genotypes. Polyadenylated RNA was then prepared using Dynabeads (Dyna) according to the manufacturer. The RNA was resuspended in a final volume of 10 μ L (per embryo). First-strand cDNA synthesis was done at 45°C for 1 h using 5 μ L of the RNA preparation with SuperScript II (GIBCO BRL) in the presence of 100 ng of oligo-dT15 primers in a volume of 20 μ L. A parallel reaction without reverse transcriptase (RT) was performed in each case. The cDNA was diluted 1:5 in water, and 5 μ L of the RT reaction was used for PCR analysis. A positive control and several negative controls (no template, no RT) were included in each experiment. PCR was done at an annealing temperature of 55°C using 35 cycles for all primer pairs except for *Actin* (20 cycles). Each RT-PCR was repeated at least three times with RNA from different embryos.

Primer pairs used were *Wt1*-5' (GGGGAAGCTTCGCCCATGGGTTCCGACGTGCGGGAC) and *Wt1*-3' (GGATGGTAGGCTGGCTCTCCAG), *Sox9*-5' (GCCGACTCCCCACATTCCTCC) and *Sox9*-3' (GCCGTAAGTCCAGTGTAGGTG), *Lhx9*-5' (ACGTTCTAGAAGCTCTGGGAGTGGACATCG) and *Lhx9*-3' (ACGTGGGCCCTTAGAAAAGTTTCGTTAAGGTAGT), *Dax1*-5' (GTCAAGTACTTGCCCTGCTTCCA) and *Dax1*-3' (GATGAATCTCAGCAGGAAAAGGGC), *Sf1*-5' (GTGAAATTCCTGAACAACCACA) and *Sf1*-3' (GTCTGCTTGGCCTGCAGCAT), as well as *Actin*-5' (AGAGGTATCCTGACCCTGAAGTACC) and *Actin*-3' (CCACCAGACAACACTGTGTTGCGAT).

RNA in situ hybridization

In situ hybridization using serial transverse sections (7 μ m) of paraformaldehyde-fixed and paraffin-embedded, staged mouse embryos were performed according to standard procedures (Hogan 1994; Belo et al. 1997). A 265-bp fragment derived from the 3'-untranslated region of the mouse *Sf1* cDNA (+3481 to +3746) and a 1133-bp fragment from the coding region of mouse *Lhx9* (+4 to +1137) was cloned by PCR using cDNA from urogenital ridges of C57BL/6J embryos into the pGEM-T easy vector (Promega). Constructs were sequenced and used for in vitro transcription of antisense and sense control of ³⁵S-labeled RNA probes. Additional hybridizations with identical results were performed with a second nonoverlapping *Sf1* probe comprising the entire coding region.

Luciferase reporter assays

An *Sf1* promoter fragment encompassing nucleotides -589 to +85 (Woodson et al. 1997) was generated by PCR using genomic

C57BL/6J DNA as a template and cloned into the *SacI* and *BglIII* sites of the pGL3basic luciferase reporter vector (Promega). An internal *BstXI* site at position -210 was used to define a distal and a proximal promoter element. The respective subfragments were generated by PCR and cloned into the *SmaI* site of pGL3basic. For the introduction of mutations, the QuikChange kit (Stratagene) was used. The integrity of all constructs was verified by sequencing. The *Lhx9* expression construct was made by RT-PCR using polyadenylated RNA from urogenital ridges of C57BL/6J mouse embryos (E11.5) followed by cloning of the PCR product (+4 to +1137) into the *EcoRI* and *ApaI* sites of RCMV (Invitrogen). The vector had previously been modified so that the second *EcoRI* site (+1744) was deleted. In addition, two complementary oligonucleotides had been inserted between the *HindIII* and *EcoRI* sites constituting an HA-epitope tag. *Wt1* expression constructs encompassing the entire *Wt1* coding region (with and without the KTS insertion) were cloned by PCR using *Wt1* cDNAs (Haber et al. 1993) as templates into the *HindIII* and *XbaI* sites of the unmodified RCMV vector.

The murine Sertoli cell line TM4 (obtained from the European Collection of Cell Cultures, ECACC) was grown in DMEM supplemented with 10% FCS. Two cell lines derived from human osteosarcoma U2OS cells (UB27 and UD28) were grown in DMEM supplemented with 10% FCS, 1 μ g/mL tetracycline, and antibiotics. The generation of UB27 and UD28 cell lines harboring inducible *Wt1*(-KTS) and *Wt1*(+KTS) alleles, respectively, has been described (Englert et al. 1995). Cells were split at 1.2×10^5 cells per well into 6-well dishes and transfected 24 h later with 0.6 μ g of reporter plasmid, 0.7 μ g of expression plasmid, and 0.1 μ g of internal control plasmid encoding *Renilla* luciferase (with a total of 2 μ g of plasmid DNA per well) using SuperFect (QIAGEN) as described by the manufacturer. UB27 and UD28 cells were kept in tetracycline-containing medium, that is, *Wt1* expression was repressed until transfection was performed. Reporter gene activity was determined 48 h after transfection using the Dual-Luciferase system (Promega). Values were normalized for transfection efficiency and with respect to the effects of the expression constructs on the empty pGL3basic reporter plasmid.

Electrophoretic mobility shift assays

EMSA analysis was performed using recombinant, bacterially expressed GST fusion proteins of the zinc finger region of WT1 with or without the KTS insertion, a Denys-Drash mutant harboring an exchange of Arg at position 394 to a Trp residue (Pelletier et al. 1991a), full-length LHX9 (GST-LHX9), and the C-terminus including the homeodomain of LHX9 (aa 215-378, GST-LHX9HD). Recombinant plasmids were constructed using the vector pGEX-KG (Guan and Dixon 1991).

Sf1 promoter fragment probes were cut out of the respective pGL3 reporter constructs and gel-purified. Oligonucleotide probes harboring the LHX9 binding site had the following sequences: CCCACCCAGTCGCTAACAAGCCGCTGCCTATC (wild type) and CCCACCCAGTCGCTCACCAGCCGCTGCCTATC (mutant). Fragments as well as oligonucleotide probes were labeled using [γ -³²P]ATP and T4 polynucleotide kinase for 1 h at 37°C and subsequently purified using Nick columns (Amersham). Binding reactions were performed in a final volume of 30 μ L in binding buffer (100 mM KCl, 1 mM MgCl₂, 10 μ M ZnSO₄, 10 mM Tris, pH 7.5, 4% glycerol, 0.1% Triton X-100, 1 mg/mL BSA, 1 μ g of poly(dIdC)/poly(dAdT), 0.5 mM DTT). Reaction mixtures containing 20 ng of purified protein were preincubated for 10 min at room temperature followed by the addition of 3000 cpm of end-labeled probe. After a 30-min incubation at room temperature, DNA-protein com-

plexes were resolved by electrophoresis on 4% polyacrylamide gels (containing 2.5% glycerol) at 150 V for 2 h in 0.5× TBE buffer.

Footprinting analysis

The distal and proximal *Sf1* promoter fragments were generated by PCR using one nonlabeled and one ³²P-labeled primer (distal fragment: P5s, GGGAAGCTTCACACCCTTAGCCCAGCA GT, P5as, CAGAGGCCAAGGCACTGGAG; proximal fragment: P3s, CTCCAGTGCCTTGGCCTCTG, P3as, TTTTGGATCCTCCAGGCCTCAGGTAGGGC). For the characterization of *WB2*, a PCR-generated fragment encompassing nt -350 to -50 was used. In each case, a 5'- as well as a 3'-labeled fragment was analyzed. PCR fragments were gel-purified, and probes (10,000 cpm) were incubated with 50 µg of GST, GST-WT1(-KTS), GST-WT1(+KTS), and GST-LHX9HD, respectively, in binding buffer (100 mM KCl, 1 mM MgCl₂, 10 µM ZnSO₄, 10 mM Tris, pH 7.5, 4% glycerol, 0.1% Triton X-100, 1 mM DTT) for 30 min at room temperature. Samples were treated with RQ1-DNase I (Promega) in a total reaction volume of 100 µL, and digestion was terminated after 3 min by adding 90 µL prewarmed (37°C) stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 µg/mL yeast RNA). DNA fragments were phenol/chloroform-extracted, ethanol-precipitated, and separated by electrophoresis on a 6% polyacrylamide sequencing gel in 1× TBE buffer. The sequence of the protected region was determined by alignment with a sequencing reaction using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham) with the primer which was labeled in the respective footprinting reaction.

Generation and analysis of transgenic mice

Mice carrying the *LacZ* gene under the control of a fragment of the murine *Sf1* promoter were generated by standard pronuclear injection into fertilized eggs (Hogan 1994) and maintained on a C57BL/6J background. The construct *Sf1-blue* was made by PCR reaction on murine genomic DNA as a template using the primers SF1pr/as (TTTTGGATCCTCCAGGCCTCAGGTA GGGCA) and SF1pr/s (GGGAAGCTTCACACCCTTAGC CCAGCAGTC). The primers harbor nt -589 to -570 and +66 to +85 of the *Sf1* promoter sequence (Woodson et al. 1997) and carry a *Bam*HI and a *Hind*III site, respectively. The PCR product was cloned into the pGEM-T easy vector (Promega), released with *Hind*III and *Nco*I, and ligated into the vector pSKT-NLS-*LacZ*. This vector provides the *LacZ* gene carrying a nuclear localization signal at the N terminus (Bonnerot et al. 1987). For the introduction of mutations, the QuikChange kit (Stratagene) was used. All four WT1-binding sites within the *Sf1* promoter fragment were successively mutated by using the oligonucleotides described in the legend of Figure 3. The resulting construct was named *Sf1-white*. The integrity of all constructs was verified by sequencing.

For the *Sf1-blue* mice (wild-type *Sf1*-promoter), a total number of 20 (in the case of the *Sf1-white* mice [mutated *Sf1*-promoter], nine) transgenic mice were generated, and in both cases lines derived from seven males were established and characterized. In each case, one of the founder animals did not produce offspring. Whereas two founders of the *Sf1-blue* did not pass on the transgene and one did not show any transgene expression, in the remaining three lines, transgene expression could be observed in the gonad. All six remaining mouse lines with the mutated *Sf1-white* construct expressed the transgene in the spinal cord but not in the gonad. In each line, expression of the transgene was analyzed using at least 15 transgenic embryos.

Mice were genotyped by PCR using primers SF1pr/s3 (GATC GGACAGGGCCAGTTTC) and LacZrev (AGACCATTTCATCA TCCGCACC). Transgene expression was assayed by X-gal staining of embryos following standard procedures (Hogan 1994). After completion of the color reaction, the embryos were washed 3 times in PBS and post-fixed in 4% paraformaldehyde for 30 min, washed in PBS, and transferred to 80% glycerol/PBS for storage and photography. Alternatively, samples were dehydrated, embedded in paraffin, and cut into 7-µm transverse sections. Sections were dewaxed, rehydrated, and stained with eosin. For the immunohistochemical assays, a rabbit anti-SF1 antibody (1:200 dilution) was used as primary antibody, and the sections were treated as described (Ikeda et al. 2001). The immunoreactivities with biotinylated secondary antibody (Vector) were detected using the VIP substrate kit (Vector).

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Dagmar Wilhelm and Christoph Englert

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