

Wilms' Tumor Protein Wt1 Is an Activator of the Anti-Müllerian Hormone Receptor Gene *Amhr2*

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Received 20 September 2006/Returned for modification 8 November 2006/Accepted 27 March 2007

The Wilms' tumor protein Wt1 plays an essential role in mammalian urogenital development. *WT1* mutations in humans lead to a variety of disorders, including Wilms' tumor, a pediatric kidney cancer, as well as Frasier and Denys-Drash syndromes. Phenotypic anomalies in Denys-Drash syndrome include pseudohermaphroditism and sex reversal in extreme cases. We have used cDNA microarray analyses on *Wt1* knockout mice to identify Wt1-dependent genes involved in sexual development. The gene most dramatically affected by *Wt1* inactivation was *Amhr2*, encoding the anti-Müllerian hormone (Amh) receptor 2. *Amhr2* is an essential factor for the regression of the Müllerian duct in males, and mutations in *AMHR2* lead to the persistent Müllerian duct syndrome, a rare form of male pseudohermaphroditism. Here we show that *Wt1* and *Amhr2* are coexpressed during urogenital development and that the Wt1 protein binds to the promoter region of the *Amhr2* gene. Inactivation and overexpression of *Wt1* in cell lines was followed by immediate changes of *Amhr2* expression. The identification of *Amhr2* as a Wt1 target provides new insights into the role of Wt1 in sexual differentiation and indicates, in addition to its function in early gonad development and sex determination, a novel function for Wt1, namely, in Müllerian duct regression.

The Wilms' tumor suppressor gene *WT1* (*Wt1* in the mouse) encodes an essential factor regulating mammalian urogenital development. Mutations in *WT1* cause Wilms' tumor, a form of pediatric kidney cancer (11, 19), as well as a variety of human syndromes. Three of these disorders, namely, WAGR (for "Wilms tumor, aniridia, genitourinary abnormalities, mental retardation"), Denys-Drash syndrome (DDS), and Frasier syndrome, affect development of gonads and genitalia. The resulting abnormalities 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 (7, 31, 41, 47). How the reduction of *WT1* levels or mutations of the gene lead to these drastic phenotypic anomalies is still unclear.

Biochemically, Wt1 is a bona fide transcription factor harboring four zinc fingers at its C terminus. Expression of *Wt1* yields a protein family arising from usage of alternative translation initiation sites, RNA editing, and alternative splicing (reviewed in reference 16). The most important alternative splicing event leads to the insertion of three additional amino acids (KTS) between zinc fingers 3 and 4. Biochemical evidence suggests that the -KTS and +KTS isoforms of Wt1 play different roles (49). While Wt1(-KTS) unequivocally is a transcription factor, Wt1(+KTS) has been shown to be involved in RNA processing (10, 39).

In mice, inactivation of *Wt1* results in embryonic lethality and failure of kidney and gonad development (32) as well as other organ defects (22, 55–57). With respect to the gonad,

Wt1 has multiple roles. In *Wt1* knockout mice, gonad development does not proceed beyond the formation of the early gonad at embryonic day 11.5 (E11.5). One gene that mediates this early function of Wt1 is *Sfl*, encoding steroidogenic factor 1 (58). Recently, Wt1(-KTS)- and Wt1(+KTS)-specific knockout mice have been generated (21). Regarding gonad development, the -KTS variants of Wt1 are required for the survival of the gonadal primordium, whereas the +KTS forms seem primarily involved in the male sex determination pathway.

An important step after mammalian sex determination is the differentiation of the Müllerian and the Wolffian ducts. The Müllerian duct serves as the primordium of the oviduct, uterus, and upper vagina in females, whereas in males the Wolffian duct develops into the epididymis and vas deferens. In order for male sexual development to occur, the Müllerian ducts have to be eliminated. In humans failure of Müllerian duct regression leads to the development of internal pseudohermaphroditism with uterine and oviductal tissues in otherwise normally virilized males, a condition known as persistent Müllerian duct syndrome (30).

The key factor in the regression of the Müllerian ducts is the anti-Müllerian hormone (Amh) that is secreted by Sertoli cells of the testis (for a review see reference 29). As a member of the transforming growth factor β family, Amh signals through a heterodimeric receptor complex consisting of a type I and a type II serine/threonine kinase receptor. The Amh type II receptor (*Amhr2*) confers the specificity and is initially expressed in the coelomic epithelium next to the Müllerian ducts in both males and females (59). Subsequently, under the influence of Amh, the *Amhr2*-expressing cells in males undergo an epithelial-to-mesenchymal transition and migrate into the mesenchyme surrounding the Müllerian duct. There, Amh signal-

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ing leads to degeneration of the Müllerian duct via a paracrine mechanism (1, 50). *Amhr2* is also expressed in fetal and adult gonads of both sexes (4, 14, 52). The physiological function of *Amhr2* in the gonad is not yet fully understood. In contrast, the observation that mutational inactivation of *Amhr2* or *Amh* in humans and mice leads to persistent Müllerian duct syndrome (28, 36) demonstrates the biological relevance of *Amhr2* for *Amh* signaling during Müllerian duct regression.

To gain deeper insights into the role of *Wt1* during sexual development, we set out to identify *Wt1* target genes mediating its role during this process. To this end we have employed *Wt1* knockout mice and cDNA microarray methodology and have identified the *Amhr2* gene as a novel *Wt1* target. We show here that *Wt1* and *Amhr2* are coexpressed, that the *Wt1*(-KTS) protein binds to specific sites in the promoter region of the *Amhr2* gene, and that inactivation and overexpression of *Wt1*(-KTS) are followed by immediate changes in *Amhr2* expression. The identification of *Amhr2* as a *Wt1* target contributes to our understanding of *Wt1*'s role in sex differentiation and indicates a novel function for *Wt1* in Müllerian duct regression.

MATERIALS AND METHODS

Mouse lines. For this study, mice carrying a *Wt1* knockout allele on an Mf1 background (22) and Mf1 wild-type mice were used. Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated E0.5. Embryos from E10.5 to E12.5 were staged by counting the number of tail somites (ts), with 8 ts corresponding to E10.5 and 18 ts to E11.5 (20). Genotyping was performed as described before (58).

All mouse experiments were performed at the animal facility of the institute. The animals were housed under a cycle of 12 h of light and 12 h of darkness with free access to standard mouse chow and tap water. All experimental procedures complied with the rules of the German Animal Welfare Law and were approved by the local authorities. This is in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

Cell culture. The mesonephric mouse cell line M15 and the murine cell line of Sertoli origin TM4 (both obtained from the European Collection of Cell Cultures) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal calf serum (FCS). The human osteosarcoma-derived cell lines UB27 and UD28, which harbor *Wt1* alleles that can be controlled by the *tet* system (17), were grown in DMEM containing 10% FCS, 0.5 mg/ml G418, 1 µg/ml puromycin, and 1 µg/ml tetracycline to suppress *Wt1* expression. For the induction of *Wt1* expression, the cells were washed once with phosphate-buffered saline and twice with DMEM containing 10% FCS.

RNA isolation. Total RNA from urogenital ridges was isolated and reverse transcribed as described previously (8). Total RNA from eukaryotic cell lines was isolated using an RNeasy Mini kit (QIAGEN) after homogenization with QIAshredder spin columns (QIAGEN) and eluted in a total volume of 80 µl RNase-free water. Seventeen microliters of RNA was treated with RQ1 RNase-Free DNase (Promega) before reverse transcription (RT).

Microarray analysis. For microarray analysis, urogenital ridges (mesonephros plus gonad) of five (four female, one male) *Wt1*^{+/+} and *Wt1*^{-/-} embryos with 11 to 12 tail somites were pooled before RNA isolation. The microarray analysis was performed by using CodeLink UniSet Mouse 20k microarray slides (Amersham Biosciences, now part of GE Healthcare). Both samples were measured three times. Data analysis and quality control were performed using CodeLink Expression Analysis software.

Real-time RT-PCR. Quantitative real-time RT-PCR analysis was basically performed as described previously (8). The following primers were used: for *Amhr2*, CCCAACATCCCCTCCACTT and GCTGAAAGGCAGTTCTCTGG; for *AMHR2*, GGACCCTACTCAACCACAA and ACAGAGCAGAGCCAAAGAG; for *Lhx9*, CAGCAGCCTTATCCACCTTC and TATCAACACCCCATTCTCC; for *Wt1*, AGTTCCCAACCATTCCTTC and TTCAAGCTGGGAGGTCATTT; for *Actb*, TGTTACCAACTGGGACGACA and GGGGTGTTGAAGGTCCTCAAAA; for *ACTB*, ACTGGACGACATGGAGAAA and AGC ACAGCCTGGATAGCAAC; and for *Gapdh*, AACTTTGGCATTGTGG AAGG and ACACATTGGGGGTAGGAACA. All primer pairs for RT-PCR covered at least one intron. PCR primers were designed using the Primer3 program (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Expression

levels were determined in one plate for all samples simultaneously and normalized to the corresponding amounts of *Actb* (beta-actin) cDNA measured within the same plate. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (34).

RNA in situ hybridization. In situ hybridizations on 10-µm paraffin sections and whole-mount in situ hybridizations using digoxigenin-labeled (Roche) riboprobes were performed as described previously (33). A 437-bp cDNA fragment of *Amhr2* was amplified using the primers CGTTTCTCCAGGTAATCCA and ATGGCGCATGACCTATCTTC and cloned into pCRII-TOPO with the TOPO TA Cloning kit (Invitrogen). The published plasmids containing fragments recognizing mouse *Wt1* (54) as well as *Lhx9* and *Sfl1* (58) mRNA were used.

RNA interference. Short interfering RNAs (siRNAs) directed against *Wt1* were designed using the online tool provided by the Whitehead Institute (www.whitehead.mit.edu) and synthesized by employing the Silencer siRNA Construction kit (Ambion) with the oligonucleotides AAGGATACAGCACGGTCACTTCCTGTCTC and AAAAGTGACCGTGTGTATCCCCTGTCTC (siRNA no. 1) as well as AATGACCTCCAGCTTGAATGCCTGTCTC and AACAT TCAAGCTGGGAGGTCACCTGTCTC (siRNA no. 2) as templates. M15 cells (3×10^5) were seeded in 6-cm dishes and transfected 24 h later by applying 15 nM siRNA and 10 µl siLentFect Lipid Reagent (Bio-Rad). Medium was changed 24 h posttransfection. Cells were harvested for RNA isolation about 48 h after transfection.

Western blotting analysis. For Western blotting, cellular lysates were extracted with 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% sodium dodecyl sulfate (SDS). Forty micrograms of cell lysate was electrophoretically separated by SDS-10% polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences). Proteins were detected with rabbit anti-*Wt1* antibody (C-19; 1:500 dilution; Santa Cruz Biotechnology) followed by horseradish peroxidase-coupled goat anti-rabbit antibody (1:2,500 dilution; DakoCytomation) or goat anti-lactate dehydrogenase antibody (1:10,000 dilution; Chemicon International) followed by horseradish peroxidase-coupled rabbit anti-goat antibody (1:2,500 dilution; DakoCytomation), respectively, and enhanced chemiluminescence analysis (ECL Plus Western Blotting Detection System; Amersham Biosciences).

EMSA. Electrophoretic mobility shift assay (EMSA) analysis using recombinant, bacterially expressed glutathione *S*-transferase (GST) fusion proteins of the zinc finger region of *WT1* with or without the KTS insertion (58), a Denys-Drash mutant harboring an exchange of Arg at position 394 to a Trp residue (41), and full-length *Sfl1* (51) was performed as described previously (58). *Amhr2* promoter fragment probes were amplified by PCR using a luciferase reporter construct containing 1.6 kb of the *Amhr2* promoter as template (53) and gel purified by employing a QIAEX II gel extraction kit (QIAGEN).

DNase footprinting assay. For footprinting analysis, a 362-bp fragment containing the *Sfl1* and *Wt1* binding sites and a 337-bp fragment containing the *Wt1* binding sites only were amplified using one ³²P-labeled 5' primer and one nonlabeled 3' primer (GGAACCATCTTGACAGAGC with CAGCCAAGGCTTCTACAAA and CCCTCTCCGAGGAGAAAAG with GLprimer2, CT TTATGTTTTGGCGTCTTCCA, respectively) and the 1.6-kb *Amhr2* promoter construct as template. DNase footprinting assays including RQ1-DNase I (Promega) treatment for 1 min were performed as described previously (58).

Luciferase reporter assay. Murine *Sfl1* cDNA was amplified using primers GGAAGCCTTGAATTCCTCTCCGTTCCAGCG and GCGCTCGAGAGGCAGTGGCATCCCTGCCTA, containing a HindIII and an XhoI restriction site, respectively, and ligated into pGEM-T (Promega). An *Sfl1* expression construct was obtained by subcloning this cDNA into pcDNA 3.1(+) (Invitrogen) via HindIII and XhoI restriction. *Sfl1* expression from this construct was confirmed by Western blot analysis. Construction of *Wt1*(+/-KTS) expression plasmids was described previously (58).

A luciferase reporter construct containing 1.6 kb of the *Amhr2* promoter sequence was kindly provided by Jose Teixeira (53). Mutagenesis constructs containing mutated *Wt1* consensus-binding sites were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). To generate the wild-type and mutated *pAmhr2* F4 constructs, fragments containing a 223-bp sequence from positions -148 to +75 of the *Amhr2* promoter were amplified from respective full-length constructs using the forward primer of F4 (GAAAAGAT TGATTCTCTGCTCTC) and GLprimer2 (see above), cloned into pCRII-TOPO (Invitrogen), verified by sequencing, and subcloned into the pGL3 basic reporter vector via SacI and NcoI restriction.

Transfection of murine cell lines was performed using SuperFect transfection reagent (QIAGEN) in a 24-well format. Cells were split at 5×10^4 to 8×10^4 TM4 cells or 2×10^4 to 3×10^4 M15 cells per well and transfected 24 h later with 750 ng expression construct, 200 ng promoter reporter construct, and 50 ng phRL-Tk control plasmid (Promega). Firefly and *Renilla* luciferase activities

were measured 48 h after transfection using the Dual-Luciferase Assay kit (Promega).

ChIP. Chromatin immunoprecipitation (ChIP) using C-19 anti-Wt1 rabbit polyclonal antibody (Santa Cruz Biotechnology), Wt1 anti-Wt1 rabbit polyclonal antibody (17), A-14 anti-c-Myc rabbit polyclonal antibody (Santa Cruz Biotechnology), and anti-acetyl-histone H3 rabbit polyclonal antibody (Upstate Biotechnology) was performed essentially as described previously (48). The purified DNA was dissolved in 100 μ l water. One microliter was used for PCR analysis with 200 mM Tris, pH 8.4, 500 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.4 μ M primers, and 0.5 U *Taq* polymerase in a final volume of 25 μ l. Cycling conditions were 94°C for 1 min and then 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primer pairs used were GGTTCTGCTCTCCCTTT and GGTTCTGATCCCTCAGTCC (spanning bases -139 to -11 of the human *AMHR2* promoter), AGAGGCCACACCTCAGCA and TGGCAGTGATAAATCGGACA (binding ~2 kb downstream of the human promoter), CAGCTGGACAGCCAAGGTC and CAGCCAAGGCTTCTACAAA (spanning bases -209 to +24 of the murine *Amhr2* promoter), as well as AGATGTCTGCTCTGGGGTTG and CCTGTGGACTCTCTGGGAAA (binding ~10 kb downstream of the murine promoter).

Microarray data accession number. The microarray data of this work were deposited in the NCBI database GEO (accession number GSE6700).

RESULTS

***Amhr2* is not expressed in urogenital ridges of *Wt1*^{-/-} mouse embryos.** To identify targets of Wt1 in sexual development, we compared levels of gene expression in urogenital ridges of *Wt1*^{+/+} and *Wt1*^{-/-} mouse embryos using a microarray that covered 20,000 unique genes. The embryos had 11 to 12 tail somites (~E11.0), a stage immediately before the beginning of sex differentiation. Of the 12,043 genes that fulfilled the quality criteria, 142 showed differential expression at a level higher than 3-fold and 15 showed differential expression at a level higher than 10-fold. In most cases (e.g., 100 out of 142), expression of the respective gene was reduced in *Wt1*^{-/-} mutant urogenital ridges (for details, see http://www.fli-leibniz.de/www_molgen/supp-mat/MCB2007SupplTable.pdf). The gene which showed the greatest difference was the gene encoding the anti-Müllerian hormone receptor *Amhr2*, with 64-times-higher signal in wild-type versus knockout urogenital ridges. For comparison, the second and third most differentially expressed genes showed factors of 36 and 23, respectively.

To verify the expression difference and to obtain information about the localization of *Amhr2* mRNA in the urogenital ridge, RNA in situ hybridization was performed. We observed *Amhr2* expression in the gonadal part of the urogenital ridge of wild-type embryos starting from about E10.5, but not in knockout embryos (Fig. 1A, B, E, and F). In the latter, expression of *Lhx9*, a gene encoding another transcription factor involved in early gonad development (9), was diminished but could be clearly observed, as previously described (58) (Fig. 1C and D). The persistence of *Lhx9* expression demonstrates that the gonadal part of the urogenital ridge still exists in E11.5 knockout embryos. Differential expression of *Amhr2* was also confirmed by real-time PCR analysis (Fig. 1G). With this method we determined a 500-fold reduction in *Amhr2* expression in *Wt1*^{-/-} gonads from embryos with 12 tail somites compared to the respective wild-type tissue. Reduction of *Lhx9* was 2.8-fold.

***Amhr2* and *Wt1* show overlapping expression patterns.** Previous studies have identified the nuclear hormone receptor Sf1 as a potential regulator of *Amhr2* expression (6, 53). As *Sf1* itself is regulated by Wt1 during early gonad development (58), the loss of *Amhr2* expression in *Wt1*^{-/-} gonads could be indi-

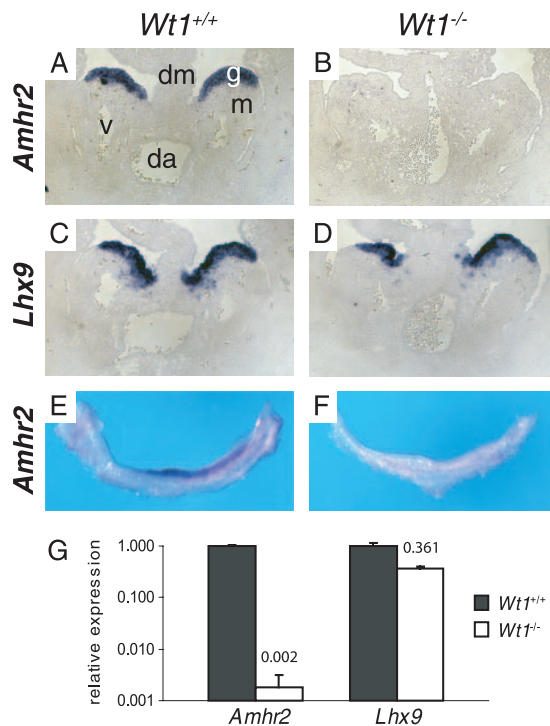


FIG. 1. *Amhr2* expression is lost in gonads of *Wt1*^{-/-} embryos. Expression of *Amhr2* (A, B, E, and F) and *Lhx9* (C and D) in urogenital ridges of *Wt1*^{+/+} (A, C, and E) and *Wt1*^{-/-} (B, D, and F) embryos with 12 (A to D), 15 (E), or 16 (F) tail somites (E11.0 to E11.5) was analyzed by RNA in situ hybridization on paraffin sections (A to D) and whole urogenital ridges (E and F). (G) Quantitative real-time RT-PCR analysis of *Amhr2* and *Lhx9* expression in *Wt1*^{-/-} urogenital ridges of the 12-tail-somite stage compared to wild-type tissue. Expression levels were normalized to *Actb*. For comparison, expression in the wild-type urogenital ridge was set as 1. In order to make the value for *Amhr2* expression in *Wt1*^{-/-} tissue visible, the scale of the y axis is logarithmic. Numbers above white bars indicate the ratio between mutant and wild-type expression levels. Data represent means and standard deviations of one representative experiment, measured in triplicate. da, dorsal aorta; dm, dorsal mesentery; g, gonad; m, mesonephros; v, subcardial vein.

rectly caused by the loss of Sf1. We therefore analyzed the expression of all three genes in the urogenital ridge region from embryonic stage 13.5, a time point when *Amhr2* expression can predominantly be observed in the cells of the coelomic epithelium in proximity to the Müllerian duct (59). Our analysis showed that at this time point *Wt1* was expressed in testis cords of male embryos, the mesonephros, and throughout the coelomic epithelium (Fig. 2A), conforming to its published expression pattern (2, 44). *Amhr2* was expressed in testis cords of male embryos and in the coelomic epithelium next to the Müllerian duct, thus overlapping with the *Wt1* expression domain (Fig. 2B and insets in A and B). In contrast to *Wt1*, however, *Sf1* was not expressed in all regions of *Amhr2* expression in the developing embryo. We did not detect any expression of *Sf1* in cells surrounding the Müllerian duct and only weak expression within testis cords at E13.5 (Fig. 2C). As published by others, we found strong *Sf1* expression in interstitial cells of the gonad (27).

When we examined *Amhr2* and *Wt1* expression in female urogenital ridges of later stages, we found that both genes were

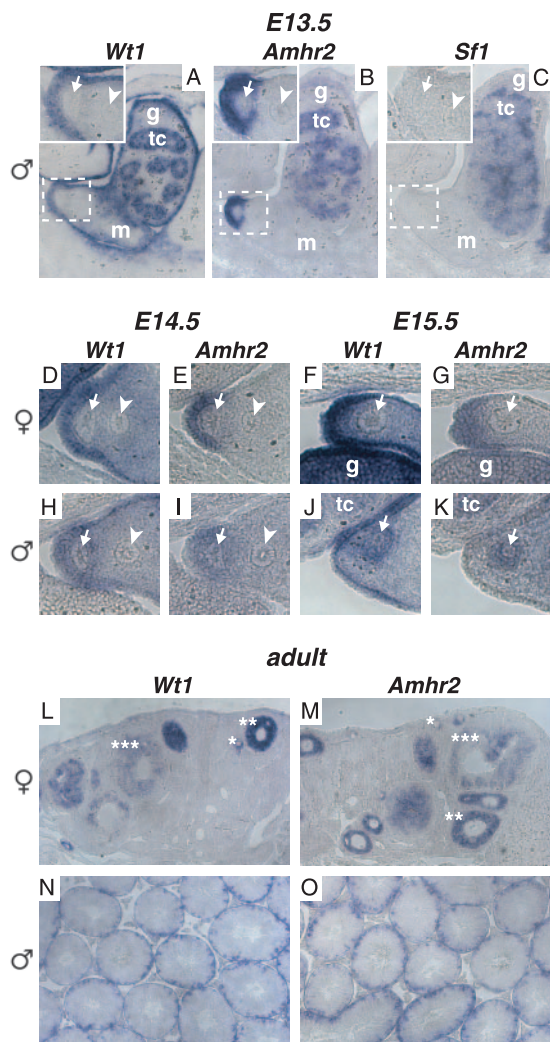


FIG. 2. *Wt1* and *Amhr2* show overlapping expression patterns in the region of the developing Müllerian duct as well as in embryonic and adult gonads. Expression of *Wt1* (A, D, F, H, and J), *Amhr2* (B, E, G, I, and K), and *Sfl* (C) in the urogenital region of E13.5 (A to C), E14.5 (D, E, H, and I), and E15.5 (F, G, J, and K) wild-type male (A to C and H to K) and female (D to G) embryos was investigated by RNA in situ hybridization on paraffin sections. Insets in A, B, and C show areas marked by dashed boxes in higher magnification. Expression of *Wt1* (L and N) and *Amhr2* (M and O) was also analyzed in adult ovary (L and M) and testis (N and O). tc, testis cord; g, gonad; m, mesonephros; arrowhead, Wolffian duct; arrow, Müllerian duct; *, primary follicle; **, secondary follicle; ***, tertiary follicle.

expressed in the coelomic epithelium but not around the Müllerian duct (Fig. 2D to G). In contrast, in male urogenital ridges both *Amhr2* and *Wt1* expression became weaker in the coelomic epithelium at E14.5 than at E13.5, and *Amhr2*- as well as *Wt1*-expressing cells became visible between the Müllerian and the Wolffian duct (Fig. 2H and I). One day later both genes were expressed around the degenerating Müllerian duct (Fig. 2J and K). Thus, the expression of *Wt1* mirrors the dynamic and sexually dimorphic expression pattern of *Amhr2* in the urogenital ridge. Of note, *Wt1* and *Amhr2* were both expressed in developing female gonads as well as in testis cords at all time points analyzed (Fig. 2F, G, J, and K and data not shown).

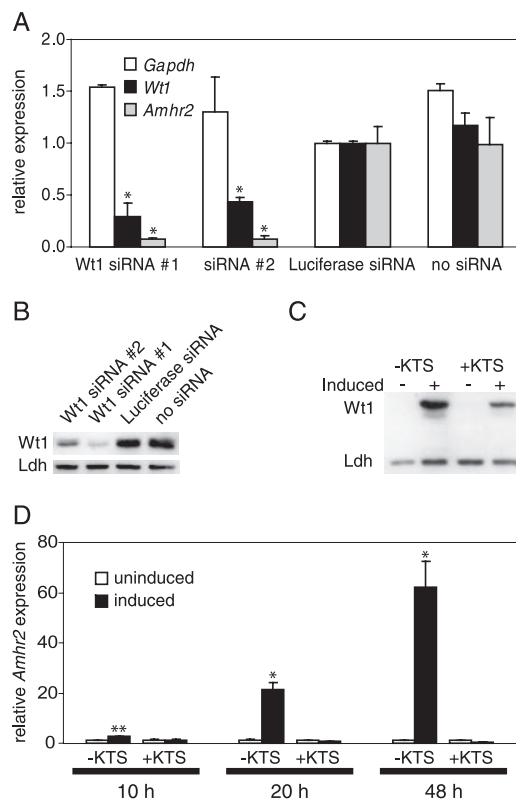


FIG. 3. *Amhr2* expression is altered by *Wt1* knockdown and *Wt1* induction. (A) M15 cells were treated with two different siRNAs directed against *Wt1*, an siRNA directed against luciferase, or transfection reagent only. Expression of *Amhr2*, *Wt1*, and *Gapdh* was measured 48 h after transfection by real-time RT-PCR and normalized to *Actb* expression. Expression levels in luciferase siRNA-treated cells were set as 1. Data represent the means and standard deviations of two independent experiments. Asterisks indicate statistically significant differences ($P < 0.05$; Student's *t* test) from expression in both controls (luciferase siRNA and cells not treated with siRNA). (B) Western blot analysis of *Wt1* protein levels in siRNA-treated cells 48 h after transfection. Lactate dehydrogenase (Ldh) served as a loading control. (C) Western blot analysis of cells harboring an inducible allele of *Wt1* ($-KTS$) or *Wt1* ($+KTS$) 24 h after induction. (D) *AMHR2* expression in uninduced and induced cells was measured by real-time RT-PCR 10 h, 20 h, and 48 h after induction of *Wt1* ($-KTS$) or *Wt1* ($+KTS$) in UB27 and UD28 cells. Expression levels were normalized to *ACTB*, and expression levels in uninduced cells were set as 1. Data represent the means and standard deviations of two independent experiments. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test) from expression in uninduced cells.

As *Wt1* and *Amhr2* have also been reported to be expressed in adult gonads, we examined the expression patterns of both genes in the gonads of adult mice. In ovaries, both genes showed expression in granulosa cells during follicle maturation, with the highest expression in secondary follicles (Fig. 2L and M). This is in line with previous analyses of rat ovaries (3, 26). In testis, expression of both genes was detected in Sertoli cells (Fig. 2N and O), as described by others (4, 42). However, with the method used here, we were not able to detect the reported weak *Amhr2* expression in Leydig cells (43).

These results show a significant coexpression of *Wt1* and *Amhr2* in developing as well as in adult tissue and are compatible with a direct regulation of *Amhr2* by *Wt1*. The data also

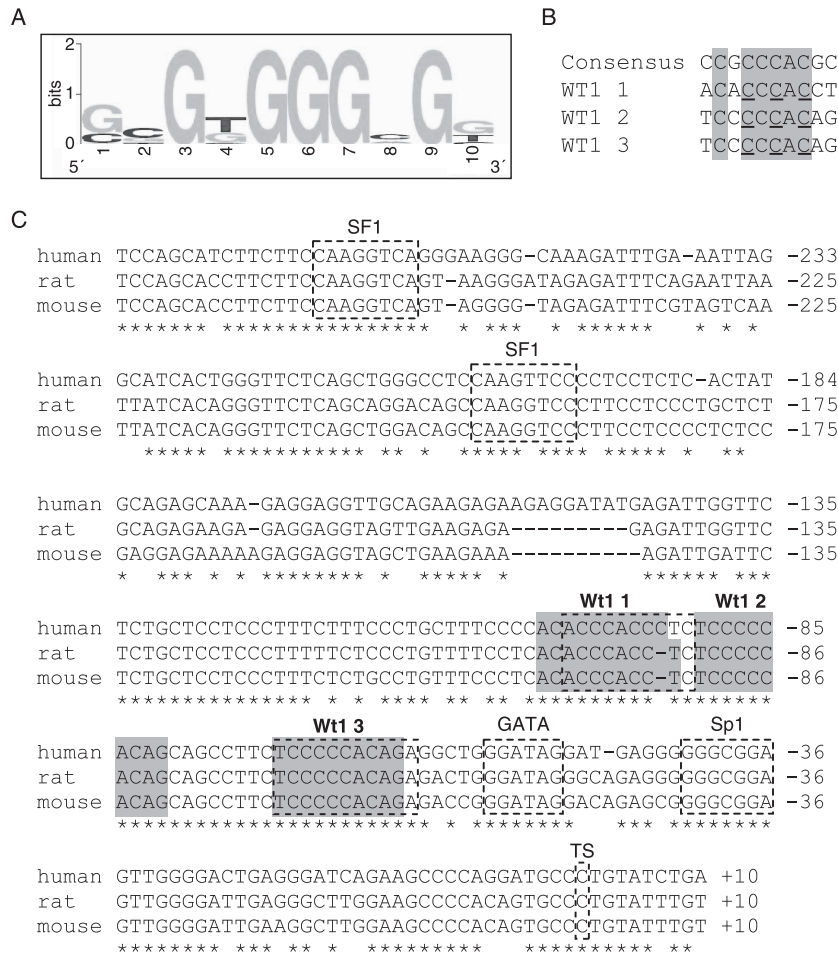


FIG. 4. *Amhr2* promoter contains three conserved Wt1(-KTS) consensus binding sites. (A) Graphical representation of the more G-rich strand of the Wt1(-KTS) consensus binding site designed with <http://weblogo.berkeley.edu>. (B) Alignment of the consensus sequence with the three Wt1(-KTS) consensus binding sites found in the murine *Amhr2* promoter. In all cases the more C-rich strand is shown in the 5' to 3' direction. Conserved positions are boxed in gray. Positions mutated for EMSA and reporter gene assays (C to A) are underlined. (C) The mouse *Amhr2* promoter sequence (accession number AF092445) was aligned with homologous human and rat sequences acquired from <http://genome.ucsc.edu/index.html> (human assembly, May 2004; rat assembly, November 2004) using the T-coffee program (<http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee.cgi/index.cgi>). Conserved transcription factor binding sites for Sf1, Gata, Sp1, and two unknown factors as well as the transcriptional start site (TS) (53) are indicated by dashed boxes. All sequences contain three conserved Wt1(-KTS) consensus binding sites (gray shaded boxes) overlapping with the two described binding sites for unknown factors (53).

rule out regulation of *Amhr2* by Sf1 during Müllerian duct regression.

Endogenous *Amhr2* expression is altered after Wt1 knock-down or Wt1 induction in mammalian cell lines. To analyze the short-term effect of Wt1 reduction on *Amhr2* expression, we chose RNA interference and established two different *Wt1*-directed siRNAs. Both siRNAs led to a specific knockdown of Wt1 mRNA and protein with slightly different efficiencies (Fig. 3A and B). M15 mesonephric mouse cells treated with siRNA directed against *Wt1* mRNA showed a drastic reduction of *Amhr2* expression (Fig. 3A). The fact that *Amhr2* expression is more drastically reduced upon *Wt1* siRNA treatment than *Wt1* itself suggests that a threshold level of Wt1 is necessary to achieve proper *Amhr2* activation.

After having shown that *Amhr2* expression is decreased by inactivation of *Wt1* in gonads and M15 cells, we studied the effect of *Wt1* overexpression on *Amhr2* expression. We em-

ployed the human osteosarcoma cell lines UB27 and UD28, which express Wt1(-KTS) and Wt1(+KTS), respectively, under the control of a tetracycline-regulatable promoter (17) (Fig. 3C). *AMHR2* was upregulated in a time-dependent manner after induction of *Wt1(-KTS)* but not *Wt1(+KTS)*. Ten hours after Wt1 induction, *AMHR2* expression was about three times higher than that in uninduced cells. *AMHR2* mRNA levels were increased more than 20-fold after 20 h and approximately 60-fold 48 h after *Wt1(-KTS)* induction (Fig. 3D). Thus, expression of endogenous murine and human *Amhr2* showed an immediate response upon changes in Wt1 concentration in the respective cell lines, suggesting a direct regulation of *Amhr2* by Wt1(-KTS).

The *Amhr2* promoter contains potential Wt1 binding sites. On the basis of an optimized binding site for Wt1(-KTS), called WTE (38), and considering several naturally occurring Wt1 binding sites that have recently been characterized, we

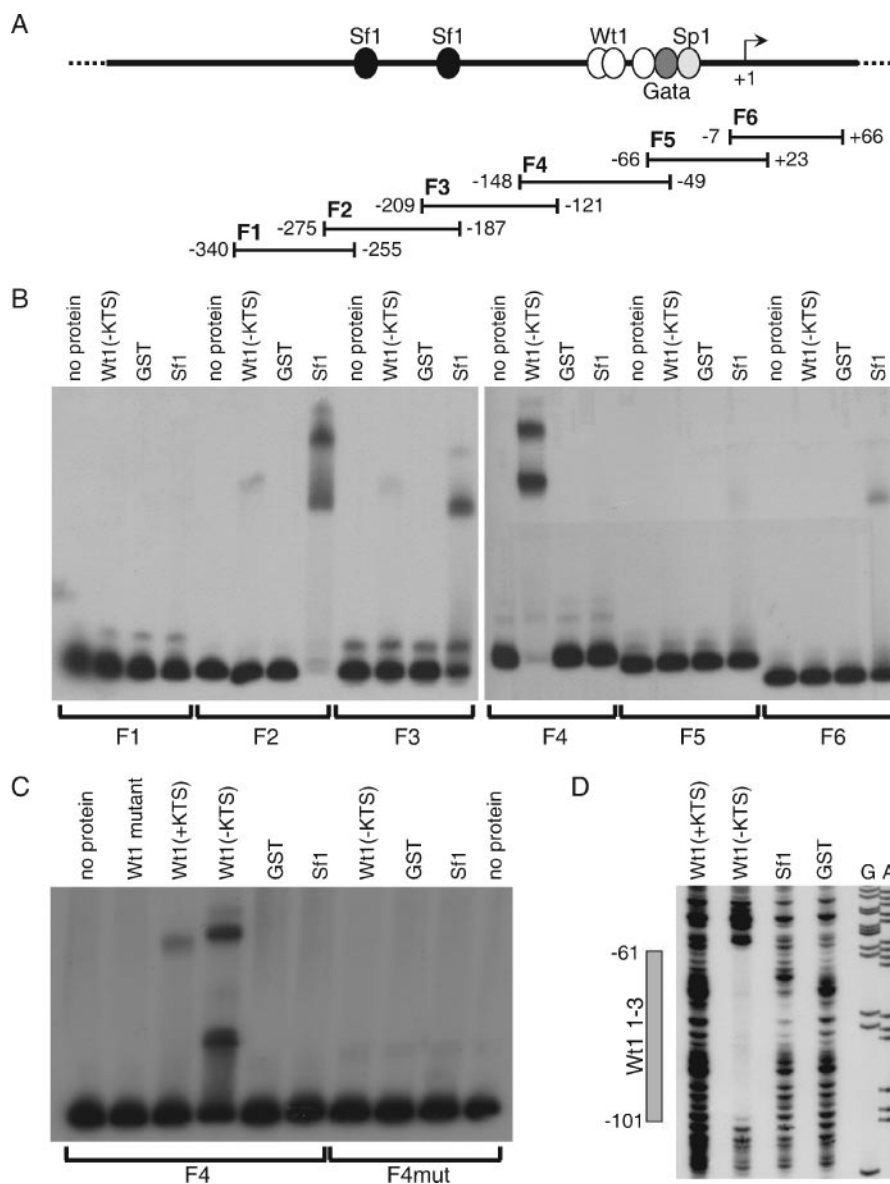


FIG. 5. Wt1(-KTS) and Sf1 bind to the murine *Amhr2* promoter in vitro. (A) Schematic representation of the *Amhr2* promoter, containing potential transcription factor binding sites, and the fragments used for EMSA. (B) For EMSA analysis, labeled fragments F1 to F6 were incubated without protein and with recombinant GST, GST-Wt1(-KTS), and GST-Sf1. (C) Wild-type and mutated F4 were additionally analyzed by adding GST-Wt1(+KTS) and a mutant form of GST-Wt1(-KTS). F4 was mutated by replacing three of the five conserved cytosines with adenines in all three Wt1 binding sites. (D) Binding of Wt1 to the consensus binding sites was also shown by DNase footprinting analysis using the same recombinant proteins and a 5'-labeled PCR fragment. Sequencing reactions for A and G bases of the same fragment are shown. The gray box indicates the region protected by Wt1(-KTS) spanning all three consensus binding sites. Numbers in panels A and D refer to the murine *Amhr2* promoter sequence (53).

have generated a refined Wt1 consensus binding motif (Fig. 4A and B; see also http://www.fli-leibniz.de/www_molgen/supp-mat/MCB2007SupplFig.pdf). Using this motif we have found three potential Wt1 binding sites in the published 1.6-kb murine *Amhr2* promoter sequence (53). This promoter fragment had been shown to activate a reporter gene in the rat Leydig cell tumor line R2C but not in other cell lines without endogenous *Amhr2* expression, and it harbors Gata, Sp1, and Sf1 consensus binding sites (Fig. 4C). The latter were protected in a DNase footprinting assay using extracts from R2C cells (53). In addition to these sites, two more sites were protected, for which the

binding factor was not identified. These two sites overlap with the first and third Wt1 binding motifs (Fig. 4C). The three Wt1 binding motifs are highly conserved between mouse, human, and rat (Fig. 4C). The same is true for the transcriptional start site, the Gata binding site, the Sp1 binding site, and the first of the two Sf1 binding sites.

Wt1(-KTS) binds to the *Amhr2* promoter in vitro. We used electrophoretic mobility shift assays (EMSA) to investigate whether Wt1 actually binds to the identified consensus binding motives in the *Amhr2* promoter. For EMSA, the *Amhr2* promoter was divided into six overlapping fragments (F1 to F6)

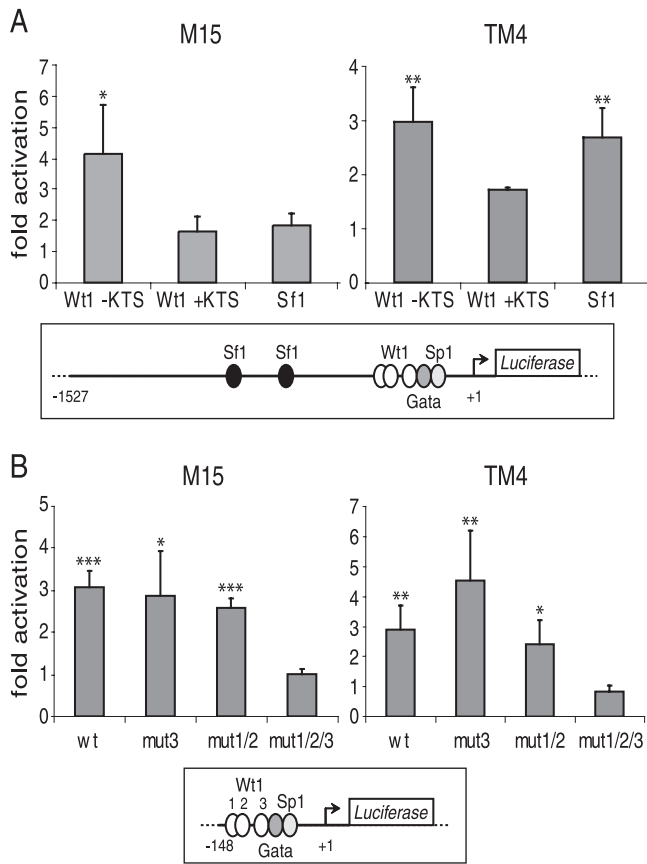


FIG. 6. Wt1(-KTS) transactivates the *Amhr2* promoter. (A) M15 and TM4 cells were transfected with the 1.6-kb *Amhr2* promoter construct together with expression constructs for Wt1(-KTS), Wt1(+KTS), and Sf1. Subsequently, luciferase reporter assays were performed. (B) Constructs harboring a shorter fragment and mutated forms of this fragment with mutations in binding site 3 (mut3), 1 and 2 (mut1/2), or all three sites together (mut1/2/3) were cotransfected with Wt1(-KTS). The respective *Amhr2* promoter fragments are indicated schematically below the diagrams. Results are given as relative activation of the reporter by the expression constructs compared to the empty vector. Data represent the means and standard deviations of three (A) and four (B) independent experiments. Asterisks indicate statistically significant differences of activation compared to cells transfected with an empty expression construct (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; analysis of variance).

(Fig. 5A). As expected, the most efficient binding by Wt1(-KTS) was observed for F4, which contained the three Wt1 consensus binding sequences (Fig. 5B). Sf1 bound most efficiently to F2 and F3, the fragments containing the published Sf1 binding sites. Additionally, much weaker binding of Wt1(-KTS) to F2 and F3 as well as of Sf1 to F5 and F6 was observed. Subsequently, Wt1 binding of F4 was analyzed in more detail. In contrast to wild-type Wt1(-KTS), a mutated form, which contained the most common Denys-Drash syndrome-causing mutation in zinc finger III (R394W), did not bind to this fragment at all, while weak binding of Wt1(+KTS) could be observed (Fig. 5C, left). This is in line with previously published weak binding of Wt1(+KTS) to Wt1(-KTS) binding sites (46). When all potential Wt1 binding sites were mutated (see Fig. 4B), Wt1 binding to F4 was completely lost (Fig. 5C, right).

To confirm the EMSA results and to determine the position

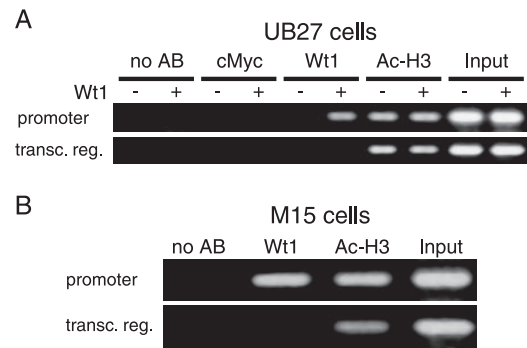


FIG. 7. Wt1(-KTS) binds the *Amhr2* promoter in living cells. PCR analysis of DNA purified after chromatin immunoprecipitation with (A) uninduced and induced UB27 cells and (B) M15 cells. Two different antibodies against Wt1 were used, Wt1 C-19 (A) and WTe8 (B). Immunoprecipitation using an antibody against c-Myc or without antibody served as negative controls, while antibody against acetylated histone H3 and input DNA served as positive controls. Primer pairs binding to the transcribed region (transc. reg.) of human and murine *Amhr2*, 2 and 10 kb downstream of the transcriptional start site, respectively, were used as controls for PCR.

of Wt1 binding in more detail, we performed a DNase footprinting assay. This assay corroborated specific binding of Wt1(-KTS) to the identified consensus binding sites (Fig. 5D). A region spanning all three consensus binding sites was protected by Wt1(-KTS) but not by Wt1(+KTS), Sf1, and GST. As shown in Fig. 5B, the bandshift assay showed weak binding of Wt1 to F2 and F3, two fragments without perfect Wt1 consensus binding sites. However, these fragments contain several G- or C-rich sites with some similarity to the Wt1(-KTS) consensus. DNase footprinting assays revealed less pronounced protection of sequences within F2 and F3 by Wt1(-KTS) (data not shown).

Wt1 transactivates the *Amhr2* promoter in a reporter gene assay. After having shown that Wt1 binds the *Amhr2* promoter in vitro, we wanted to examine whether Wt1 was also able to transactivate this promoter. Using the 1.6-kb *Amhr2* promoter fragment in the context of a luciferase reporter plasmid, we observed transactivation by Wt1(-KTS) but not Wt1(+KTS) in the mesonephric cell line M15 and the Sertoli-like cell line TM4, both from mouse (Fig. 6A). Transactivation by Sf1 was only observed in TM4 cells. There was no synergism between the effects of Wt1 and Sf1 on the *Amhr2* promoter (data not shown). To address the specificity of the interaction, we generated a shorter *Amhr2* promoter construct by deleting sequences 5' of F4 (see Fig. 5A). This construct, which contains no Sf1 binding site, still showed transactivation by Wt1 in both cell lines (Fig. 6B). When all three potential Wt1 binding sites were mutated (see Fig. 4B), however, the resulting promoter fragment could not be activated by cotransfection with a Wt1(-KTS) expression construct (Fig. 6B). Mutation of one or two binding sites was not enough to abolish transactivation by Wt1.

Wt1(-KTS) binds to the *Amhr2* promoter in living cells. To examine whether Wt1(-KTS) binds the *Amhr2* promoter also in living cells, ChIP was employed. We used human UB27 cells, harboring an inducible Wt1(-KTS) allele (Fig. 7A), and murine M15 cells, expressing significant levels of endogenous Wt1

(Fig. 7B). After the ChIP procedure the DNA was tested for the presence of an *Amhr2* promoter fragment by PCR with specific primer pairs spanning the identified Wt1 consensus binding sites. As controls, primer pairs binding within the transcribed region of the human and murine *Amhr2* gene were used. DNA isolated directly from the formaldehyde-treated cells served as positive controls for PCR (Fig. 7, input). As a positive control for ChIP we used an antibody against acetylated histone H3, while an antibody directed against the transcription factor c-Myc and samples with no antibody added served as negative controls. Significant enrichment of *Amhr2* promoter fragments could be observed when an anti-Wt1 antibody was combined with material from cells containing Wt1, either expressed from the transgene (Fig. 7A) or expressed endogenously (Fig. 7B). However, when uninduced UB27 cells were used, no PCR product was obtained. No or background signals were observed for genomic fragments located within the transcribed region of *Amhr2*. Thus, when Wt1 is present in UB27 or M15 cells, it specifically binds to the human and murine *Amhr2* promoters.

DISCUSSION

The different splice forms of the Wilms' tumor protein Wt1 have been implicated in several aspects of mammalian gonad development and sex determination. Wt1, particularly the -KTS splice form, is required for development and survival of the gonad primordium (21). However, Wt1 also has a role in the process of sex determination, a role that is demonstrated by reduction of *Sry* expression in Wt1(+KTS) knockout mice (21). Recently, a role of Wt1 after the initial phase of sex differentiation, namely, in the maintenance of testis architecture, has been reported (18).

In this report we provide evidence for another late role for Wt1 in the process of sex development, namely, in regression of the Müllerian duct, a process that is essential for normal male development to occur. This process is initiated by binding of the anti-Müllerian hormone Amh to its cognate receptor, *Amhr2*. Our data suggest that Wt1 is required for cells to gain the competence for *Amhr2* expression. As is the case for *Amhr2* (59), *Wt1* is initially expressed in the coelomic epithelium. Subsequently, in male urogenital ridges the expression domains for both genes migrate towards the Müllerian duct and eventually surround the regressing ductal epithelium. This dynamic expression pattern involves cell migration as well as epithelial-to-mesenchymal transition, a process in which Wt1 has been implicated (23). However, not all *Wt1*-expressing cells show *Amhr2* activity. This becomes obvious especially in the coelomic epithelium and suggests the involvement of additional factors in *Amhr2* regulation. Initially, only cells of the coelomic epithelium that directly neighbor the Müllerian duct express *Amhr2*, while *Wt1* is expressed throughout the coelomic epithelium. Thus, a second determinant is necessary to define those cells that express *Amhr2*. One possible candidate is *Wnt7a*, a secreted signaling molecule originating from the Müllerian duct (40). It has been shown that *Wnt7a* knockout mice of both sexes lack *Amhr2* expression in the Müllerian duct region, leading to persistent Müllerian ducts in males. In support of these data, *Amhr2* has been shown to be a target of β -catenin, a downstream component of the canonical Wnt

signaling pathway (25). Sf1, another factor that had been implicated in *Amhr2* activation (6, 25, 53), was not expressed in the cells that mediate Müllerian duct regression. This excludes Sf1 as a decisive factor during Müllerian duct regression but is still compatible with Sf1-mediated *Amhr2* activation in cells that do express *Sf1* and *Amhr2* but not *Wt1*, for example, in Leydig cells, as previously suggested (53).

In addition to the region surrounding the Müllerian duct, we have observed coexpression of *Wt1* and *Amhr2* in developing gonads of both sexes and in Sertoli cells of adult males as well as throughout development of the ovarian follicle. Although our knowledge about the role of *Wt1* in adult tissues is still limited, there is evidence to suggest that Wt1 and Amh signaling affect the same processes in adult gonad biology. Regarding the testis, *Amhr2* knockout mice show Leydig cell hyperplasia and vacuolization of Sertoli cells (36). The latter phenotype has also been reported for mice with adult Sertoli cell-specific *Wt1* inactivation (45). In ovaries, both Wt1 and Amh signaling have been suggested to function as inhibitors of early follicular growth (12, 15).

One prediction from our finding that Wt1 is an activator of *Amhr2* is that males with *Wt1* mutations should show signs of persisting derivatives of the Müllerian ducts. Because of the degeneration of urogenital ridges in *Wt1* mutant embryos after E11.5, we could not analyze Müllerian duct regression in those mice. There are, however, cases where remnants of the Müllerian ducts have been reported to occur in human Denys-Drash syndrome patients. This syndrome comprises the triad of partial gonadal dysgenesis, congenital or infantile nephropathy, and Wilms' tumor. All components of the syndrome appear to be caused by dominant loss-of-function mutations of the *WT1* gene. In most cases, DDS mutations are point mutations that alter DNA contact sites within the Wt1 zinc fingers (41). We have used the most frequent DDS mutant protein (R394W) and shown that it is not able to bind to the *Amhr2* promoter. One of the initial publications about Denys-Drash syndrome described a patient who possessed internal genitalia that were very reminiscent of persistent Müllerian duct syndrome (13). In addition to a normally developed vagina and uterus, two pairs of ducts as well as one histologically normal testis and a vas deferens were present. In a similar case, a 4-month-old patient with a 46 XY karyotype was presented who developed a Wilms' tumor and had a vagina, uterus, testes, epididymis, and vas deferens (5). Further cases have been reported since then (35).

The existence of male internal genitalia in these patients demonstrates normal testis development, including the generation of Sertoli and Leydig cells as well as the respective hormones without which structures like the epididymis and vas deferens would not form. Thus, the persistence of Müllerian duct structures cannot be explained by the activation of a female default pathway caused by the failure of male sex determination to occur but rather by missing execution of a genetic program that normally leads to regression of the Müllerian duct. While we provide evidence for *Amhr2* as a target of Wt1, the latter has also been implicated in the activation of the *Amh* gene encoding the Amhr2 ligand (24, 37). In our microarray analysis, which was performed using RNA from mouse embryos of stage E11.0, a time point when *Amh* starts to be expressed, *Amh* levels were low but detectable and were not

different between wild-type and *Wt1* knockout embryos. It is still possible that XY Denys-Drash syndrome patients like the ones indicated above show reduced *Amh* expression, although testis differentiation seems to proceed normally, and that these reduced *Amh* levels might also contribute to the Denys-Drash phenotype. The regulation of two components within one pathway, namely, the *Amh* ligand and its receptor, by *Wt1* could explain why Müllerian duct regression seems to be more drastically affected in the mentioned Denys-Drash syndrome patients than other processes depending on *Wt1*, namely, early gonad development and testis differentiation.

In summary, we have identified the *Amhr2* gene as a physiologically relevant target of the Wilms' tumor protein *Wt1*. This provides new insights into the role of *Wt1* in sexual development and indicates a novel function for *Wt1* in sex differentiation, in addition to its known function in early gonad development and sex determination.

ACKNOWLEDGMENTS

We thank Helen Morrison, Frank Bollig, and Falk Weih for discussions and for critically reading and improving the manuscript. We also thank Susanne Spahr and Debra Weih as well as the students Nicole Borth and Christian Linke for technical support, Dagmar Wilhelm for fruitful discussions and the pGex-Sf1 construct, and Jose Teixeira for providing the *Amhr2* promoter constructs and his readiness to reannotate the corresponding sequence. The help from Richard Münch in generating a *Wt1* consensus binding sequence is gratefully appreciated.

This work was supported by grants En280/6-1 and En280/6-2 from the Deutsche Forschungsgemeinschaft.

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