Transcriptional regulation by the Wilms’ tumor protein, Wt1, suggests a role of the metalloproteinase Adams16 in murine genitourinary development

Charlotte L. J. Jacobi, Lucas J. Rudigier, Holger Scholz, and Karin M. Kirschner

From the Institut für Vegetative Physiologie, Charité-Universitätsmedizin Berlin, D-10117 Berlin, Germany

*Running title: *Wt1 regulates Adams16 in developing kidneys and gonads

To whom correspondence should be addressed: Holger Scholz, Institut für Vegetative Physiologie, Charité-Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany, Tel.: +49-30-450 528213, FAX: +49-30-450 528928, E-mail: holger.scholz@charite.de

**Keywords:** ADAMTS16; Wilms’ tumor protein; gene transcription

**Background:** ADAMTS16 is a mammalian metalloproteinase with unknown function. Results: Transcription of the Adams16 gene is regulated by Wilms’ tumor protein Wt1, and knockdown of Adams16 reduces branching morphogenesis in cultured embryonic kidneys. Conclusion: Adams16 is a Wt1 target gene during murine genitourinary development. Significance: The findings provide novel insights into gene regulatory networks controlling kidney and gonad development.

**SUMMARY**

ADAMTS16 (a disintegrin and metalloproteinase with thrombospondin motifs) is a secreted mammalian metalloproteinase with unknown function. We report here that murine Adams16 is co-expressed with the Wilms’ tumor protein, Wt1, in the developing glomeruli of embryonic kidneys. Adams16 mRNA levels were significantly reduced upon transfection of embryonic murine kidney explants with Wt1 antisense vivo-morpholinos. Antisense knockdown of Adams16 inhibited branching morphogenesis in kidney organ cultures. Adams16 was detected by in situ mRNA hybridization and/ or immunohistochemistry also in embryonic gonads, and in spermatids and granulosa cells of adult testes and ovaries, respectively. Silencing of Wt1 by transfection with antisense vivo-morpholinos significantly increased Adams16 mRNA in cultured embryonic XY gonads (11.5 and 12.5 d.p.c.), and reduced Adams16 transcripts in XX gonads (12.5 and 13.5 d.p.c.). Three predicted Wt1 consensus motifs could be identified in the promoter and the 5'-untranslated region of the murine Adams16 gene. Binding of Wt1 protein to these elements was verified by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). A firefly luciferase reporter gene under control of the Adams16 promoter was activated approximately 8-fold by transient co-transfection of human granulosa cells with a Wt1 expression construct. Gradual shortening of the 5’-flanking sequence successively reduced and eventually abrogated Adams16 promoter activation by Wt1. These findings demonstrate that Wt1 differentially regulates the Adams16 gene in XX and XY embryonic gonads. It is suggested that Adams16 acts immediately downstream of Wt1 during murine urogenital development. We propose that Adams16 is involved in branching morphogenesis of the kidneys in mice.
functional properties are related to, though distinct from the ADAM group of proteinases. ADAMTSs have several functional domains including a pro-domain, an N-terminal catalytic domain, a disintegrin domain, and a C-terminal ancillary domain. The latter one contains several thrombospondin-like repeats and is crucial for specific interactions with substrates (2,3). ADAMTS proteins lack the transmembrane domain present in ADAMs (4). To date, 20 ADAMTS family members have been identified whose multiple functions include proteolytic degradation of proteoglycans, collagen processing, inhibition of angiogenesis, and the control of blood coagulation (1,2,5-8). Additional roles of ADAMTS molecules may extend to embryonic development, inflammatory responses and fertility (9-13). Mutations in ADAMTS genes can give rise to human disorders. For example the Ehlers-Danlos syndrome (EDS) type VIIC (MIM 2254109) is a recessive connective tissue disease, which results from mutations in the gene for the procollagen N-proteinase, ADAMTS2 (14). Mutations in the gene encoding ADAMTS13, which cleaves the von Willebrand factor, can lead to inherited thrombotic thrombocytopenia (15).

ADAMTS16 is a recently cloned family member, whose physiological functions are currently unknown. A full-length recombinant protein was capable of cleaving the protease inhibitor α2-macroglobulin in vitro (16). ADAMTS16 mRNA was initially detected in human fetal lung and kidney, but also in adult brain and ovary (1). The cell types that express ADAMTS16 in these tissues have not been identified yet. Later studies demonstrated ADAMTS16 transcripts in human cartilage (17) and pancreas (16). Expression of ADAMTS16 in cartilage and synovium was increased in patients suffering from osteoarthritis compared to the tissues of healthy individuals (17). Variants of the ADAMTS16 gene were recently linked to inherited arterial hypertension (18) and high levels of ADAMTS16 could be detected in a significant portion of esophageal squamous cell carcinomas (19).

Whilst a role of ADAMTS16 in development and disease is beginning to emerge, very little is known about the molecular mechanisms regulating its tissue-specific expression. ADAMTS16 mRNA levels were increased in differentiated luteinizing human granulosa cells following treatment with follicle-stimulating hormone (FSH) and forskolin (16). These observations suggested that ADAMTS16 expression is activated via the cyclic AMP pathway (16). The promoter of the ADAMTS16 gene is GC-rich and contains several consensus motifs for Egr1 and Sp1 (20). Both transcription factors were found to activate ADAMTS16 promoter constructs in transient co-transfection experiments (20). However, it is neither known, whether Egr1 and Sp1 are physiological regulators of ADAMTS16 in vivo, nor have other trans-acting factors been ascertained so far.

In view of this background our study was aimed at providing novel insights into the gene regulatory mechanisms underlying the characteristic expression pattern of ADAMTS16. For this purpose we analyzed the cellular distribution of Adamts16 protein in various organs of mouse and rat. Furthermore, we sought to determine transcription factors that are involved in the tissue-specific control of Adams16. We report here that Adamts16 is a transcriptional downstream target gene of the Wilms’ tumor protein, Wt1. Importantly, knockdown of Adams16 by transfection with antisense vivo-morpholinos impaired branching morphogenesis in murine embryonic kidney cultures. These findings suggest a role for Adamts16 in the development of the genitourinary system in mice.

EXPERIMENTAL PROCEDURES

Plasmids - A 3250 base pair (bp) DNA sequence (from -3000 bp to +250 bp relative to the transcription start site) of the murine Adamts16 gene (NCBI accession no. NM_172053) was cloned by PCR utilizing a bacterial artificial chromosome (imaGenes, Berlin, Germany, clone RPCIB731I01114Q) as template. The amplified product was ligated into the SacI and HindIII restriction sites of the pGL3basic reporter plasmid (Promega, Mannheim, Germany). Adams16 promoter constructs with gradually shortened sequences were generated by PCR. The PCR primers that were used for DNA amplification are listed in Table 1. All constructs were analyzed by automated DNA sequencing (Eurofins MWG, Ebersberg, Germany). The expression construct for the murine Wt1(−KTS) protein was kindly provided by Dr. Daniel Haber (Massachusetts General Hospital, Boston, USA).

Animals – A heterozygous (Wt1+/−) mouse breeding pair [C57/B6 (B6) strain] was
obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and mated in the local animal facility in compliance with the current laws. The morning of vaginal plugs was considered as 0.5 d.p.c.

**Cell culture** - COV434 human granulosa cells (cat. no. 07071909, HPACC) were maintained in DMEM nutrient (PAA Laboratories, Pasching, Austria) supplemented with 10 % FCS (Biochrom KG, Berlin, Germany) and L-glutamine (PAA Laboratories). The UB27 and UD28 cell lines, which contain the murine Wt1(-KTS) and Wt1(+KTS) protein isoforms, respectively, regulated through a tetracycline-dependent promoter, were the gift of Dr. Christoph Englert. UB27 and UD28 cells, and the murine mesonephros-derived M15 cell line (21) were cultured as described elsewhere (22,23).

**Organ culture experiments** - Kidneys, gonads and hearts were excised from embryos of timed-pregnant mice (C57BL6 strain). The matched organs of each embryo were cultured separately on two polyethylene terephthalate Transwell® filters with 0.4 µm pore size (Corning, Berlin, Germany). This procedure allowed for a pairwise comparison between cultured explants from a single donor that had been transfected with either Wt1 antisense or mismatch vivo-morpholinos (see below). The Transwell® filters were kept in DMEM nutrient with stable L-glutamine (PAA Laboratories) supplemented with 10 % FCS (Biochrom KG), 100 IU/ml penicillin (PAA) and 100 µg/ml streptomycin (PAA) at 37 °C in a humidified atmosphere with 5 % CO₂. Embryonic hearts were kept in 96-well plates for 5 days to allow outgrowth of epicardium-derived cells. The epicardial origin of outgrown cells was proven by their characteristic cobblestone-like appearance and robust expression of Wt1 (data not shown). Prior to morpholino treatment of the cells the hearts were removed from the tissue culture plates. In vitro cultured organs and epicardial cell monolayers were incubated for 72 hours with either Wt1 antisense (CAGGTCCGCACGTGGAACCGCT) or mismatch (CAGCTCCGACACCTGCAAGCGATA) vivo-morpholinos at 10 µM each (Gene Tools, Philomath, USA) (24). Whole cell lysates and total RNA were prepared from cultured organs and cells as described below. Sex determination of the embryos was performed by PCR amplification of the Y-chromosomal gene Kdm5d from DNA using the following primers: mKdm5d-F, CTGAAGCCTTGGGCTTTTG; mKdm5d-R, CCACTGCCAATTCTTTTG (25).

In order to study branching morphogenesis the kidneys were isolated from mouse embryos at E11.5 (Taylor stage 20) and cultured in vitro as described above. The organ rudiments were transfected either with Adams16 antisense (5’-CGCAGCCAAACCTCGGGACTCCATG-3’) or mismatch vivo-morpholino (5’-CGGAGCGGACCTCAGCCTCCATG-3’) at 10 µM each (Gene Tools, Philomath, USA) (24). After 72 hours the explants were formalin-fixed and double-stained with FITC-conjugated anti-pan-cytokeratin (diluted 1:100, Sigma-Aldrich, Munich, Germany) and anti-Wt1 antibody (C19, diluted 1:50, Santa Cruz Biotechnology, Santa Cruz, USA). Cy3-coupled donkey anti-rabbit IgG (diluted 1:100, Jackson ImmunoResearch, Hamburg, Germany) was used for the detection of bound Wt1 antibody. Photographs were taken and branching of the pan-cytokeratin-stained ureter was analyzed using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

**Cell transfections and reporter gene assays** - COV434 cells were expanded to approximately 40 % confluence in 24-well tissue culture plates. Firefly luciferase constructs, 200 ng each, harboring gradually shortened sequences of the murine Adams16 promoter and the 5’UTR were transiently transfected along with a Wt1(-KTS) expression construct (200 ng) and a renilla luciferase plasmid (100 ng) using the Fugene6® reagent (4 µl per well) as described in the supplier’s manual (Roche Diagnostics, Mannheim, Germany). As negative controls, transfections were performed with the pGL3basic reporter plasmid (Promega) and the empty pCB6® expression vector, respectively. After 24 hours, the transfected cells were lysed in Reporter Lysis Buffer (Promega), and luciferase activities were measured in a luminometer (Microlite TLX1, MGM Instruments, Hamden, USA) as described (26,27). Data are presented as firefly luciferase activities normalized to renilla luciferase activities.

**Transfection of siRNA** - M15 cells were grown to approximately 60 % confluence in six well culture plates. A pool of four different siRNAs (50 pmol/well, Dharmacon, Thermo
Wt1 regulates Adamts16 in developing kidneys and gonads

Fisher Scientific, Bonn, Germany) targeting the murine Wt1 gene (ON-TARGETplus, SMARTpool siRNA, L-040686-01-0005, NM_144783) were transfected using the DharmaFECT 1 reagent (Dharmacon) according to the manufacturer’s protocol (22). Four different non-targeting siRNAs (siGENOME non-targeting siRNA pool #2, Dharmacon) were used as negative control. The cells were harvested for mRNA and protein analysis two days after siRNA transfection.

Reverse transcription (RT) real-time PCR - Total RNA was isolated from primary tissues and cells with the RNeasy Micro Kit (Qiagen, Hilden, Germany), and from permanent cell lines with the TRizol LS reagent (Invitrogen, Darmstadt, Germany). First-strand cDNA synthesis was carried out using oligo(dT) primers and SuperScript™ II Reverse Transcriptase (Invitrogen). Real-time PCR amplification was performed with the SYBR® Green PCR Master Mix and the StepOnePlus™ system (Life Technologies GmbH, Darmstadt, Germany) as described in detail elsewhere (28). The PCR primers for real-time RT-PCR are listed in Table 1. Relative transcript levels were obtained by subtracting the threshold cycle (Ct) value of the housekeeping gene (β-actin or Gapdh) from the corresponding Ct value of the gene of interest. Differences in mRNA levels were calculated according to the equation 2\(^{ΔΔCt}\).

SDS-PAGE and immunoblotting - Cells and tissues were lysed in Laemmli buffer (50 mM Tris-Cl, pH 6.8, 4 M urea, 1 % (w/v) sodium dodecyl sulfate (SDS), 0.001 % (w/v) bromophenol blue, 7.5 mM dithiothreitol, DTT), disrupted by ultrasonication (Labsonic U, B. Braun, Melsungen, Germany) and subsequently heated to 95 °C for 5 minutes. Protein concentrations were measured spectrophotometrically according to the method of Warburg and Christian (29). Twenty µg of protein were loaded per lane and separated on a 10 % denaturing polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Freiburg, Germany) with the use of a semidyrid blotting apparatus (Bio-Rad, München, Germany). Non-specific binding activity was reduced by incubating the membrane for 60 minutes at room temperature in 5 % non-fat milk (Roth, Karlsruhe, Germany) in TBS/ 0.1 % Tween 20. Incubation with Wt1 antibody (C19, cat. no. sc-192, diluted 1:400, Santa Cruz Biotechnology, Heidelberg, Germany) in 2.5 % non-fat milk (Roth) in TBS/Tween was performed overnight at 4 °C. A polyclonal antibody raised in rabbit (diluted 1:5,000, ab45048, Abcam, Cambridge, UK) was used for immunoblotting of Adamts16. After washing with TBS/Tween, the antibodies were detected with a peroxidase-coupled IgG (donkey anti-rabbit IgG-HRP, cat. no. sc-2313, diluted 1:20,000, Santa Cruz Biotechnology), and the reaction products were visualized with the Western Lightning™ Plus ECL reagents (PerkinElmer, Massachusetts, USA) following the user’s manual. Equal protein loading was assessed with antibodies against actin (anti-actin clone C4, cat. no. MAB1501R, diluted 1:6,000, Millipore, Darmstadt, Germany) and Gapdh (anti-Gapdh, cat. no. MAB374, diluted 1:300, Millipore), respectively, after stripping of the membranes with 1:5 diluted 1 N NaOH in distilled water for 10 minutes.

Electrophoretic mobility shift assay (EMSA) – Non-radioactive EMSAs were performed with purified GST-tagged recombinant Wt1 proteins (30) and double-stranded oligonucleotides (Table 2), which were selected on the basis of predicted Wt1 binding sites in the promoter of the murine Adamts16 gene. All oligonucleotides were 3’digoxigenin (DIG)-labeled using the DIG Oligonucleotide 3’-End Labeling Kit (Roche). Likewise, double-stranded oligonucleotides with mutations of the Wt1 binding motifs (Table 2) were DIG-labeled. For competition experiments we used an oligonucleotide with a previously identified Wt1 binding site in the promoter of the murine Adams16 gene. All oligonucleotides were 3’digoxigenin (DIG)-labeled using the DIG Oligonucleotide 3’-End Labeling Kit (Roche). Likewise, double-stranded oligonucleotides with mutations of the Wt1 binding motifs (Table 2) were DIG-labeled. For competition experiments we used an oligonucleotide with a previously identified Wt1 binding site in the promoter of the murine Adams16 gene. All oligonucleotides were 3’digoxigenin (DIG)-labeled using the DIG Oligonucleotide 3’-End Labeling Kit (Roche). Likewise, double-stranded oligonucleotides with mutations of the Wt1 binding motifs (Table 2) were DIG-labeled. For competition experiments we used an oligonucleotide with a previously identified Wt1 binding site in the promoter of the murine Adams16 gene. All oligonucleotides were 3’digoxigenin (DIG)-labeled using the DIG Oligonucleotide 3’-End Labeling Kit (Roche). Likewise, double-stranded oligonucleotides with mutations of the Wt1 binding motifs (Table 2) were DIG-labeled. For competition experiments we used an oligonucleotide with a previously identified Wt1 binding site in the promoter of the murine Adams16 gene.
Wt1 regulates *Adams16* in developing kidneys and gonads

USA) and treatment with blocking reagent (Roche) the membrane was incubated with anti-DIG-alkaline phosphatase antibody (cat. no. 11093274910, diluted 1:10,000, Roche). Proteins were detected by NBT/BCIP staining (Roche).

**Chromatin immunoprecipitation (ChIP) assays** - UB27 and UD28 cells were kept for 72 hours in the presence or absence of tetracycline (1 µg/ml) to either inhibit or stimulate expression of Wt1(-KTS) and Wt1(+KTS) proteins, respectively. ChIP assays were performed with approximately 4 x 10^6 cells. After fixation with 4% formaldehyde the cells were disrupted by ultrasonication (Labsonic U, B. Braun) and centrifuged. The supernatants were diluted in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1) and precleared for one hour at 4 °C with DNA-blocked protein G agarose (Millipore, Darmstadt, Germany). The following antibodies and control sera were used (0.6 µg each) for overnight incubation at 4 °C: normal rabbit IgG (cat. no. sc-2027, Santa Cruz Biotechnology), rabbit polyclonal anti-Wt1 antibody (cat. no. sc-192, Santa Cruz Biotechnology). Immunoprecipitates were bound to DNA-blocked protein G agarose (Millipore) during one hour incubation at 4 °C. Following several washing steps the DNA was eluted in 1% SDS, 0.1 M NaHCO₃, extracted in 25:24:1 phenol:chloroform:isoamyl alcohol according to the supplier’s instructions (Invitrogen) and subsequently precipitated in 100% ethanol. This protocol was also applied to M15 cells, which were used at approximately 1 x 10⁶ cells for ChIP assays.

Immunoprecipitation with an anti-histone H3 antibody (cat. no. 07-690, Millipore) was performed as a control. Immunoprecipitated DNA was amplified by quantitative PCR using primer pairs that flanked the identified Wt1 binding sites in the murine *Adams16* promoter (Table 1).

**Immunohistochemistry** - Immunofluorescent stainings were performed on mouse and rat tissues as described in detail elsewhere (26,31). Briefly, embryos between 13.5 and 16.5 days post conception (d.p.c.) and tissues from adult animals were frozen in tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, Netherlands) and sectioned using a cryostat. Frozen sections (8 µm) were treated with acetone:methanol (3:2) for 10 min at -20 °C and subsequently blocked for 5 min at room temperature in serum-free DakoCytomation protein block (cat. no. X0909, Dako, Hamburg, Germany). Single immunostainings for Wt1 and *Adams16* were performed on mouse tissue using the following primary antibodies diluted in ready-to-use antibody diluent (Zymed Laboratories Inc., Berlin, Germany): *Adams16* rabbit (diluted 1:500, ab45048, Abcam), *Adams16* rabbit (diluted 1:50, cat. no. sc-50490, Santa Cruz Biotechnology), Anti Wilms’ tumor protein Alexa Fluor® 488 (diluted 1:200, cat. no. ab140484, Abcam, Cambridge, UK). Double-immunostainings were performed on rat tissue using the above mentioned *Adams16* antibodies in combination with the Wt1 monoclonal mouse F6 antibody (diluted 1:600, cat. no. MAB4234, Millipore). For visualization of the bound primary antibodies Cy3-AffiniPure donkey anti-rabbit IgG (cat. no. 711-165-152, diluted 1:200, Jackson ImmunoResearch, Hamburg, Germany) and Alexa Fluor® 488-AffiniPure donkey anti-mouse IgG (diluted 1:50, cat. no. 715-545-151, Jackson ImmunoResearch) were used. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (dapi). Pictures of double stainings were taken with a digital camera connected to a confocal microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany) utilizing the LAS AF Lite software (Leica Microsystems). For pictures of single stainings an epifluorescence microscope (Axiovert100, Carl Zeiss, Berlin, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI) with the Spot software (Universal Imaging Corp., Marlow Buckinghamshire, UK) was used.

**In situ mRNA hybridization** - In situ hybridization for *Adams16* mRNA was performed according to the protocol of Stricker *et al.* (32). Briefly, tissues were cryosectioned and fixed in freshly prepared paraformaldehyde (4% in PBS). Following acetylation with acetaldehyde the sections were pre-hybridized for 4 hours at room temperature in hybridization buffer (5x SSC/50% formamide). Hybridization was carried out overnight at 68 °C with digoxigenin-labeled murine *Adams16* sense and *antisense* riboprobes, respectively. After washing with formamide (1x SSC/50% formamide) the
slides were treated with RNase for 30 min at 37 °C and additional washing steps with SSC buffers (2x SSC/ 0.2x SSC) were performed. The tissue sections were blocked for 1.5 hours at room temperature (1 % blocking solution in maleic acid buffer) and then incubated overnight at 4 °C with an alkaline Phosphatase-conjugated anti-digoxigenin antibody. Bound antibody was visualized with NBT/ BCIP reagent.

Statistics - Student's paired t-test and ANOVA with Tukey as post-hoc test were performed as indicated to reveal statistical significances. P-values less than 0.05 were considered significant.

RESULTS

Adams16 and Wt1 proteins are expressed in gonads and kidneys. While Adams16 mRNA has been detected by RT-PCR in several organs including kidneys and gonads (1), very little is known about the protein localization in these tissues. We used specific polyclonal rabbit antibodies (ab45048, Abcam and sc-50490, Santa Cruz Biotechnology) to visualize Adams16 expressing cells in the genitourinary system of mouse and rat. Adams16 and Wt1 proteins were readily visible in XY and XX embryonic gonads (Fig. 1A a-c, B a-c) though more distinct in embryonic testes (Fig. 1A a-c). According to previous reports (33,34), Wt1 protein was present in developing and mature Sertoli cells (Fig. 1A a, d-f), whereas immunohistochemistry (Fig. 1A b-f) and in situ hybridization (Fig. 1C a, c, d) detected Adams16 mainly in germ cells of embryonic and adult testes. Expression of Adams16 was predominant in elongated spermatids of adult testes (Fig. 1A d-f), which was confirmed by in situ hybridization (Fig. 1C e, d). Both proteins were also detected in differentiated follicular granulosa cells and in mesothelial cells covering the surface of mature ovaries (Fig. 1B d–f). In situ hybridization confirmed Adams16 transcripts in follicular granulosa cells of the ovaries (Fig. 1C e, f). Adams16 was demonstrated by immunostaining also in the developing glomeruli of embryonic kidneys (Fig. 2 a), which are known sites of Wt1 expression (33,34, Fig. 2 b). Co-expression of both proteins in glomerular progenitors was revealed by double immunolabeling with two different Adams16 antibodies (Fig. 2 c, d, with corresponding single stainings in Fig. 2 e-h). No fluorescence signal was obtained when normal sera instead of primary antibodies were used (Fig. 2 i, k). Adams16 in murine embryonic kidneys (14 d.p.c.) was confirmed by in situ mRNA hybridization with a specific antisense riboprobe (Fig. 2 l). No hybridization signal was obtained with the use of an Adams16 sense probe (Fig. 2 m).

Expression of Adams16 in developing gonads and kidneys is Wt1-dependent. Co-expression of Adams16 and Wt1 suggests a regulatory link between both proteins in different cell-types of the genitourinary system. We therefore addressed the question of whether Wt1 is indeed necessary for normal expression of Adams16 in embryonic gonads and kidneys. To this end Adams16 transcripts were measured in isolated gonadal ridges of wild-type (Wt1+/+), heterozygous (Wt1+/−) and Wt1-deficient (Wt1−/−) murine embryos at 13 d.p.c. Compared to the gonads of normal (Wt1+/+) embryos Adams16 mRNA levels were significantly lower (P<0.05, ANOVA) in the developing gonadal ridges of Wt1−/− mice (Fig. 3A). Later developmental stages could not be studied due to the embryonic lethality of Wt1-deficient mice (35). These observations suggest that Adams16 mRNA levels in the developing gonads depend, at least to some extent, on Wt1. However, it is also conceivable that reduced Adams16 transcripts were simply due to an arrest of gonad formation with subsequent apoptosis of the undifferentiated tissue in the absence of Wt1.

To distinguish between these possibilities we established in vitro cultures of embryonic gonads. For this purpose the gonads were excised from normal murine embryos at 11.5, 12.5 and 13.5 d.p.c., respectively, and grown in a culture medium supplemented with either Wt1 antisense or mismatch vivo-morpholinos. After 72hours total RNA was isolated from the explants and Adams16 transcripts were measured by quantitative RT-PCR (Fig. 3B-D). Down-regulation of Wt1 in response to Wt1 antisense morpholino treatment, which was assessed by immunoblot analysis, had differential effects on Adams16 mRNA levels in female and male gonads (Fig. 3C, D). Thus, Adams16 transcripts were significantly (P<0.05, Student's paired t-test) increased by knockdown of Wt1 in XY gonads obtained from mouse embryos at 11.5 and 12.5 d.p.c., but not at 13.5 d.p.c. (Fig. 3C). In contrast, Wt1 silencing significantly (P<0.05, Student's
paired t-test) reduced Adams16 mRNA levels in XX gonads at 12.5 and 13.5 d.p.c., but had no significant effect on embryonic day 11.5 (Fig. 3D). These findings suggest, that Wt1 inhibits Adams16 expression in XY gonads at early developmental stages (11.5 and 12.5 d.p.c.), and activates Adams16 expression in XX gonads at later points in time (12.5 and 13.5 d.p.c.). Consistently, Adams16 mRNA levels were increased in both, XX and XY gonads treated with mismatch morpholinos, at 13.5 d.p.c. compared to earlier developmental stages ($P<0.05$, ANOVA) (Fig. 3B). Based on differences in Ct-values Adams16 mRNA levels were at least 10-fold higher in male and female gonads from 13.5 d.p.c. vs. 11.5 d.p.c. embryos (Fig. 3B). At all developmental stages analyzed Adams16 transcripts were 2- to 3-fold higher in XX compared to XY gonads ($P<0.05$, Student’s paired t-test) (Fig. 3B). Similar results were obtained when β-actin mRNA was used as a housekeeping gene for normalization (data not shown).

Since Wt1−/− mouse embryos lack kidneys (35), they are not useful for analyzing Wt1 downstream signaling pathways during nephrogenesis. To overcome this problem we established in vitro organ cultures of normal embryonic kidneys, in which we knocked-down Wt1 expression by antisense treatment (24). Densitometric analysis of the immunoblots using the ImageJ software (National Institutes of Health) revealed a reduction of band intensities in response to Wt1 antisense treatment by 57±10 % ($P<0.05$, Student’s paired t-test, n=6). Embryonic kidney explants treated with Wt1 antisense vivo-morpholinos had significantly ($P<0.05$, Student’s paired t-test) lower Adams16 mRNA levels than the corresponding mismatch controls (Fig. 4A). These findings indicate that normal expression of Adams16 in embryonic kidneys and gonads requires Wt1.

Knockdown of Adams16 inhibits branching morphogenesis in kidney organ culture. To explore the potential role of Adams16 in kidney development, we transfected embryonic renal organ cultures (Fig. 4B) with either Adams16 antisense or mismatch vivo-morpholinos. Knockdown efficiency was determined by immunoblot analysis of Adams16 using the same antibody (ab45048, Abcam) as for the immunostainings. This antibody produced a signal of the predicted molecular mass (approx. 135 kDa) of the Adams16 protein (Fig. 4C). Importantly, the protein band was clearly diminished in kidney organ cultures that had been treated with antisense vivo-morpholinos against Adams16, indicating specificity of the antibody used (Fig. 4C). In vitro knockdown of Adams16 resulted in an abnormal kidney morphology which was assessed by phase contrast microscopy (Fig. 4B a, b) and by immunostaining of the ureteric bud and the metanephric mesenchyme, respectively (Fig. 4B, c-f). Microscopic analysis of the immunolabeled whole-mount preparations revealed a significant ($P<0.05$, Student’s paired t-test) reduction in the number of ureteric bud branching points upon Adams16 knockdown (Fig. 4B g, h; Fig. 4C). These observations suggest a functional role of Adams16 in branching morphogenesis of murine kidneys.

Wt1 and Adams16 expression levels are correlated in permanent cell lines and primary epicardial cells. Next we examined whether Wt1 and Adams16 levels are associated in permanent cells derived from embryonic kidneys. M15 cells were originally established from the mesonephros of mice transgenically expressing the large T protein of polyoma virus under control of the early viral enhancer (21). The cells were transfected with a pool of 4 different siRNAs to inhibit Wt1 expression. Control experiments were performed by treatment with pooled non-targeting siRNAs. Silencing of Wt1 significantly ($P<0.001$, Student’s paired t-test) reduced Adams16 mRNA levels in M15 cells indicating that Adams16 expression in these cells depends on Wt1 (Fig. 5A). To analyze the expression of Adams16 and Wt1 in primary cells, we established cultures from epicardial progenitor cells. The epicardium is a mesothelial tissue on the outer heart surface, which expresses Wt1 from the early developmental stages on throughout adulthood (34). We also detected Adams16 protein by immunohistochemistry in the developing epicardium of murine embryos (data not shown). Transfection of primary epicardial cells with Wt1 antisense vivo-morpholinos strongly reduced Wt1 protein levels, and this effect was associated with a significant ($P<0.05$, Student’s paired t-test) reduction of Adams16 mRNA (Fig. 5B).

Importantly, the Wt1 mRNA is subject to alternative splicing. The usage of an alternative splice donor site at the end of exon 9 leads to
Wt1 regulates Adamts16 in developing kidneys and gonads

the insertion of 3 additional amino acids - lysine, threonine, serine (KTS) - between zinc finger 3 and 4 of the Wt1 protein (36). Structural and functional data demonstrate that Wt1(-KTS) proteins, which lack the 3 amino acids, act as transcription factors (37,38). Compared to the Wt1(+KTS) isoforms Wt1 proteins with the KTS insertion exhibit increased RNA binding affinity and play a presumed role in mRNA processing (39,40). The human osteosarcoma-derived cell lines UB27 and UD28 express the Wt1(-KTS) and Wt1(+KTS) proteins, respectively, under control of a tetracycline-dependent promoter (23). A consistent rise of Wt1 proteins in UB27 and UD28 cells was achieved by removal of tetracycline from the tissue culture medium (Fig. 5C, D). Induction of Wt1(-KTS) protein in UB27 cells resulted in a time-dependent increase of ADAMTS16 mRNA levels (Fig. 5C), whereas accumulation of Wt1(+KTS) protein in UD28 cells had no significant effect on ADAMTS16 transcripts (Fig. 5D). These results indicate that Adamts16 is co-regulated with Wt1 in primary epicardial cells and permanent cell lines. Restriction of the stimulatory effect on Adamts16 expression to the Wt1(-KTS) protein suggests that the underlying regulatory mechanism acts on the level of gene transcription.

Wt1 protein binds to the promoter of the Adamts16 gene in vivo. Chromatin immunoprecipitation (ChIP) was performed to examine whether Wt1 interacts with the promoter of the Adamts16 gene in M15 cells. The PCR primers for amplification of immunoprecipitated DNA bound to the proximal promoter and the 5'-flanking region of the Adamts16 gene, respectively (Fig. 6A). Compared to the use of normal rabbit serum, Adamts16 promoter DNA was enriched more than 2.5-fold with anti-Wt1 antibody (P<0.01, Student’s t-test) (Fig. 6B). An approximately 2-fold enrichment of amplicons within the promoter of the Amhr2 gene (P<0.05, Student’s t-test), a known downstream target of Wt1 (41), was obtained by immunoprecipitation with anti-Wt1 antibody (Fig. 6B). In contrast, amplified DNA fragments containing an actin promoter sequence were not enriched by immunoprecipitation of Wt1 protein (Fig. 6B). These results could be validated using UB27 cells with inducible expression of the Wt1(-KTS) protein. Thus, Adamts16 promoter DNA accumulated approximately 3-fold in UB27 cells with Wt1(-KTS) expression compared to uninduced cells (P<0.05, Student’s t-test). No differences in Wt1-bound actin promoter DNA were observed between unstimulated and Wt1(-KTS) expressing UB27 cells (Fig. 6C). Immunoprecipitation with anti-Wt1 antibody failed to enrich Adamts16 promoter DNA and actin DNA in UD28 cells that had been stimulated to express the Wt1(+KTS) protein (Fig. 6D).

The Wt1(-KTS) isoform stimulates the Adamts16 promoter. To study the molecular interaction of Wt1 protein with the Adamts16 promoter in detail, we co-transfected a firefly luciferase reporter construct, carrying approximately 3 kb of the upstream sequence of the murine Adamts16 gene, and in addition a Wt1(-KTS) expression plasmid into human granulosa cells (COV434). A nearly 8-fold stimulation of the Adamts16 promoter was measured in response to Wt1(-KTS) expression compared to co-transfection of the empty vector (P<0.01, Student’s t-test) (Fig. 7A). Sequence analysis revealed the presence of three putative high-affinity Wt1 binding sites in the promoter and 5'-flanking region of the murine Adamts16 gene. All three elements were necessary for maximal activation of the Adamts16 promoter by the Wt1(-KTS) protein (Fig. 7A). Truncated constructs with gradual deletion of the predicted binding motifs were successively less activated by co-transfection of the Wt1(-KTS) expression vector. Stimulation by the Wt1(-KTS) protein was completely lost in the absence of all three binding motifs (Fig. 7A). Electrophoretic mobility shift assays (EMSAs) testified that the Wt1(-KTS) protein indeed bound to each of these elements (Fig. 7B-D). Interaction of recombinant Wt1(-KTS) protein could be competed with excess amounts of unlabeled oligonucleotide carrying the previously identified Wt1 binding site of the Ntrk2 gene promoter (Fig. 7B-D) (31). Likewise, binding of Wt1 protein was abrogated by introducing single base pair mutations into the three oligonucleotides (Fig. 7B-D). These results demonstrate that Wt1(-KTS) protein interacts with several cis-elements in the 5'-upstream sequence and stimulates the promoter of the Adamts16 gene. In contrast, recombinant Wt1(+KTS) protein did not interact with any of the identified binding sites (Fig. 7E).
DISCUSSION

ADAMTS16 is a mammalian metalloproteinase whose function is largely unknown. Our initial microarray gene expression analysis showed that Adamts16 transcripts were strongly reduced upon RNAi silencing of the Wilms’ tumor protein Wt1 in mesonephros-derived M15 cells. The following chain of evidence suggest that Adamts16 is indeed a molecular downstream target gene of the Wt1 transcription factor: Firstly, Wt1 and Adamts16 mRNA levels were closely correlated in various tissues and cells (Figs. 3-5). Secondly, a reporter construct containing the murine 5’-upstream sequence of the Adamts16 gene was stimulated approximately 8-fold by co-transfection of ovarian granulosa cells with a Wt1 expression vector (Fig. 7A). Moreover, Wt1(-KTS) protein bound to three presumed cis-elements in the promoter and the 5’-flanking region of the murine Adamts16 gene (Figs. 6, 7B-E). Importantly, knockdown of Wt1 in cultured embryonic murine kidneys and XX gonads (12.5 and 13.5 d.p.c.) by treatment with Wt1 antisense morpholinos significantly reduced Adamts16 transcripts suggesting that Wt1 stimulates Adamts16 expression in these tissues (Figs. 3, 4A).

Transcriptional regulation of Adamts16 by Wt1 may become relevant during various biological processes in the embryonic and adult organism. Thus, mammalian kidney formation depends on the reciprocal interaction between the metanephric mesenchyme and the invading ureteric bud (reviewed in 42). Signals emanating from the branching ureteric bud tips induce the surrounding mesenchymal cells to condensate and differentiate into comma- and s-shaped bodies, which will eventually give rise to the glomeruli and build-up parts of the tubular system of the kidneys (42). Epithelial transition of the metanephric mesenchyme in turn is a prerequisite for sustained branching of the ureteric bud tips during nephrogenesis (42). Metalloproteinases are considered as major candidates for the modulation of mesenchymal-epithelial interactions in embryonic kidneys and other tissues. Several matrix metalloproteinases (MMPs) and their inhibitors, i.e. MMP-2, MT1-MMP and TIMP-2, are spatially and temporally regulated in the developing kidney (42). Moreover, targeted inactivation of the gene encoding ADAMTS1, which cleaves the major cartilage proteoglycan aggrecan produced kidney abnormalities in mice, i.e. shrinkage of the renal parenchyma and enlargement of the caliceal space (44). Our observation that vivo-morpholino knockdown of Adamts16 impaired branching morphogenesis in cultured murine embryonic kidneys suggests an involvement of this metalloproteinase in nephrogenesis (Fig. 4B, C). Consistently, silencing of the nephrogenic Wt1 protein significantly reduced Adamts16 expression in kidney rudiments (Fig. 4A). Wt1 belongs to the Cys2-His2 class of zinc finger proteins and promotes mesenchymal-to-epithelial transition (MET) during the early stages of kidney development (35-45). Kidney formation in Wt1-deficient mouse embryos, which are commonly lethal by 13.5 d.p.c. was disrupted due to apoptosis of the metanephric mesenchyme and a failure of ureteric bud branching (35). Subsequent in vitro organ culture experiments and transgenic animal studies proved a requirement of Wt1 not only for the early inductive events, but also during the later phases of kidney formation, i.e. the differentiation and maturation of the glomeruli (46), and the integrity of mature kidneys (47-49). Interestingly, recent disruption of the Adamts16 gene in Dahl Salt-sensitive rats caused splitting and thickening of glomerular capillaries in addition to a significant reduction of arterial blood pressure (50). Renal damage was associated with increased proteinuria suggesting that Adamts16 is indeed necessary for the structural and functional preservation of the kidneys in this model of genetic hypertension (50).

Interaction of the Wt1 transcription factor with the Adamts16 gene may also play a role in the development and function of the gonads. The mammalian gonads are formed by tissue which is derived from the intermediate mesoderm on either side of the embryonic midline axis (51). Establishment of the bipotential gonadal ridge occurs in a non-sex-specific manner in XX and XY individuals and is completed by approximately 12 d.p.c. in rats and mice (51). Wt1 acts on multiple steps during gonad development. Gonadal ridge formation in Wt1 null mice is initiated in both males and females, but then degenerates due to apoptosis leading to gonadal agenesis (35). The sex determining factor Sry and the anti-Müllerian hormone receptor (Amhr) 2, which is critical for the regression of the female urogenital ducts, are among the genes that are regulated by Wt1 (41,52,53). Consistently,
Wt1 regulates Adamts16 in developing kidneys and gonads

male-to-female sex reversal or genital tract abnormalities were observed in XY patients with heterozygous Wt1 germline mutations (54) underscoring the role of Wt1 in testis determination. Our results indicate that Wt1 controls Adamts16 expression in the developing gonads in a time-dependent and sex-specific fashion. Thus, silencing of Wt1 significantly increased Adamts16 transcripts in in vitro cultured XY gonads of 11.5 d.p.c. and 12.5 d.p.c. embryos (Fig. 3C). In contrast, Adamts16 mRNA levels in female but not in male gonads (12.5 and 13.5 d.p.c.) were significantly reduced by Wt1 antisense inhibition (Fig. 3C, D). These results suggest that Wt1 inhibits Adamts16 expression in XY gonads during the early developmental stages, and stimulates Adamts16 expression in XX gonads at later points in time. Repression of Adamts16 by Wt1 in developing XY gonads is in line with our failure to clearly co-localize both proteins in the same cells in this tissue (Fig. 1A, B). Further on, Wt1 may directly contribute to the developmental increase of Adamts16 mRNA levels in female gonads (Fig. 3B). Reporter gene assays and protein-DNA binding studies testified that Wt1(-KTS) protein indeed stimulates transcription of the Adamts16 gene in ovarian granulosa cells (Figs. 6, 7). The molecular mechanisms underlying the variable stage-specific effects of Wt1 on Adamts16 expression in male and female gonads are currently unknown, but may involve the physical interaction of Wt1 with other proteins in the control of gene transcription (55). Detection of Adamts16 in spermatids of mature testes (Fig. 1A, C) points to a role in male germ cell maturation and/or function throughout adulthood. Generation of mouse mutants with conditional inactivation of the Adamts16 gene in the gonads will be helpful in clarifying this issue.

It has been shown previously that follicle-stimulating hormone (FSH) induced the expression of ADAMTS16 in parietal granulosa cells of pre-ovulatory human follicles (16). In contrast, FSH attenuated basal and nerve growth factor-stimulated Wt1 protein expression in isolated granulosa cells of 21-day-old mice (56). Recently, Wt1 has been reported to inhibit expression of the FSH receptor in immature rat granulosa cells through repression of the FSH receptor promoter (57). These combined observations suggest that Wt1 is an important element in the hormonally-regulated transcriptional network in pre-ovulatory follicles. Stimulation of Adamts16 expression by Wt1 in parietal granulosa cells may have a role in extracellular matrix (ECM) remodeling, which is a prerequisite for oocyte liberation during ovulation. Notably, various members of the ADAMTS protease family, in particular ADAMTS1, have been implicated in ECM transformation in the ovary (44,58-60). The involvement of other proteases and substrates in this process adds some functional redundancy which may facilitate ovulation even in the absence of one or more constituents (61).

Another interesting aspect of our study pertains to the presumed origin of the follicular granulosa cells which is still a matter of controversy. Indeed, three major sources for pregranulosa cells have been under discussion for many decades: 1) mesothelial cells on the surface of the developing ovary (62), 2) cells originating from a centrally located blastema (63) and 3) mesonephric cells derived from the rete ovarii system (64). The results of a more recent histological study in sheep favored the idea that >95 % of the granulosa cells in newly formed primordial follicles arise from the ovarian surface epithelium (65). During this process mesothelial cells on the outside of the ovary are expected to undergo epithelial-to-mesenchymal (EMT) transition and migrate into the ovarian cortex where they differentiate to follicular granulosa cells. Remarkably, Wt1 protein has been identified to act as a key molecule for EMT by down-regulating E-cadherin expression in the epicardium of mouse embryos and other mesothelial tissues (66). Thus, co-localization with Wt1 protein in the ovarian surface epithelium suggests that Adamts16 promotes a mesenchymal commitment of these cells, possibly through ECM remodeling.

In summary, our findings demonstrate that transcription of the gene encoding the Adamts16 metalloproteinase is regulated by the Wilms’ tumor protein Wt1 in a sex- and tissue-specific fashion. This regulatory link has a presumed role in the development of murine kidneys and gonads.
Wt1 regulates Adamts16 in developing kidneys and gonads

REFERENCES

1. Cal, S., Obaya, A. J., Llamazares, M., Garabaya, C., Quesada, V., and Lopez-Otin, C. (2002) Cloning, expression analysis, and structural characterization of seven novel human ADAMTSs, a family of metalloproteinases with disintegrin and thrombospondin-1 domains. *Gene* **283**, 49-62

2. Apte, S. S. (2009) A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. *J. Biol. Chem.* **284**, 31493-31497

3. Kuno, K., and Matsushima, K. (1998) ADAMTS-1 protein anchors at the extracellular matrix through the thrombospondin type I motifs and its spacing region. *J. Biol. Chem.* **273**, 13912-13917

4. Kuno, K., Kanada, N., Nakashima, E., Fuji, F., Ichimura, F., and Matsushima, K. (1997) Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. *J. Biol. Chem.* **272**, 556-562

5. Colige, A., Beschin, A., Samyn, B., Goebels, Y., Van, B. J., Nusgens, B. V., and Lapière, C. M. (1995) Characterization and partial amino acid sequencing of a 107-kDa procollagen I N-proteinase purified by affinity chromatography on immobilized type XIV collagen. *J. Biol. Chem.* **270**, 16724-16730

6. Fernandes, R. J., Hirohata, S., Engle, J. M., Colige, A., Cohn, D. H., Eyre, D. R., and Apte, S. S. (2001) Procollagen II amino propeptide processing by ADAMTS-3. Insights on derm harshasis. *J. Biol. Chem.* **276**, 31502-31509

7. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., Rockwell, A., Yang, F., Duke, J. L., Solomon, K., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, H., Wiswall, B. H., Murphy, K., Hillman, M. C., Hollis, G. F., Newton, R. C., Magolda, R. L., Trzaskos, J. M., and Arner, E. C. (1999) Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. *Science* **284**, 1664-1666

8. Vazquez, F., Hastings, G., Ortega, M. A., Lane, T. F., Oikemus, S., Lombardo, M., and Iruela-Arispe, M. L. (1999) METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a novel family of proteins with angio-inhibitory activity. *J. Biol. Chem.* **274**, 23349-23357

9. Clark, M. E., Kelner, G. S., Turbeville, L. A., Boyer, A., Arden, K. C., and Maki, R. A. (2000) ADAMTS9, a novel member of the ADAM-TS/ metallospindin gene family. *Genomics* **67**, 343-350

10. Hurskainen, T. L., Hirohata, S., Seldin, M. F., and Apte, S. S. (1999) ADAM-TS5, ADAM-TS6, and ADAM-TS7, novel members of a new family of zinc metalloproteinases. General features and genomic distribution of the ADAM-TS family. *J. Biol. Chem.* **274**, 25555-25563

11. Kim, J., Kim, H., Lee, S. J., Choi, Y. M., Lee, S. J., and Lee, J. Y. (2005) Abundance of ADAM-8, -9, -10, -12, -15 and -17 and ADAMTS-1 in mouse uterus during the oestrous cycle. *Reprod. Fertil. Dev.* **17**, 543-555

12. Sylvester, J., Liacini, A., Li, W. Q., and Zafarullah, M. (2004) Interleukin-17 signal transduction pathway implicated in inducing matrix metalloproteinase-3, -13 and aggrecanase-1 genes in articular chondrocytes. *Cell Signal.* **16**, 469-476
Wt1 regulates Adamts16 in developing kidneys and gonads

13. Worley, J. R., Baugh, M. D., Hughes, D. A., Edwards, D. R., Hogan, A., Sampson, M. J., and Gavrilovic, J. (2003) Metalloproteinase expression in PMA-stimulated THP-1 cells. Effects of peroxisome proliferator-activated receptor-gamma (PPR gamma) agonists and 9-cis-retinoic acid. J. Biol. Chem. 278, 51340-51346

14. Colige, A., Nuytinck, L., Haussier, I., van Essen, A. J., Thiry, M., Herens, C., Ades, L. C., Malfait, F., Paepe, A. D., Franck, P., Wolff, G., Oosterwijk, J. C., Smitt, J. H., Lapière, C. M., and Nusgens, B. V. (2004) Novel types of mutation responsible for the dermatosparactic type of Ehlers-Danlos syndrome (Type VIIC) and common polymorphisms in the ADAMTS2 gene. J. Invest. Dermatol. 123, 656-663

15. Levy, G. G., Nichols, W. C., Lian, E. C., Foroud, T., McClintick, J. N., McGee, B. M., Yang, A. Y., Siemieniak, D. R., Stark, K. R., Gruppo, R., Sarode, R., Shurin, S. B., Chandrasekaran, V., Stabler, S. P., Sabio, H., Bouhassira, E. E., Upshaw, J. D., Jr., Ginsburg, D., and Tsai, H. M. (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 413, 488-494

16. Gao, S., De Geyter, G. C., Kossowska, K., and Zhang, H. (2007) FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles. Mol. Hum. Reprod. 13, 465-471

17. Kevorkian, L., Young, D. A., Darrah, C., Donell, S. T., Shepstone, L., Porter, S., Brockbank, S. M., Edwards, D. R., Parker, A. E., and Clark, I. M. (2004) Expression profiling of metalloproteinases and their inhibitors in cartilage. Arthritis Rheum. 50, 131-141

18. Joe, B., Saad, Y., Lee, N. H., Frank, B. C., Achinike, O. H., Luu, T. V., Gopalakrishnan, K., Toland, E. J., Farms, P., Yerga-Woolwine, S., Manickavasagam, E., Rapp, J. P., Garrett, M. R., Coe, D., Apte, S. S., Rankinen, T., Perusse, L., Ehret, G. B., Ganesh, S. K., Cooper, R. S., O’Connor, A., Rice, T., Weder, A. B., Chakravarti, A., Rao, D. C., and Bouchard, C. (2009) Positional identification of variants of Adamts16 linked to inherited hypertension. Hum. Mol. Genet. 18, 2825-2838

19. Sakamoto, N., Oue, N., Noguchi, T., Sentani, K., Anami, K., Sanada, Y., Yoshida, K., and Yasui, W. (2010) Serial analysis of gene expression of esophageal squamous cell carcinoma: ADAMTS16 is upregulated in esophageal squamous cell carcinoma. Cancer Sci. 101, 1038-1044

20. Surridge, A. K., Rodgers, U. R., Swingler, T. E., Davidson, R. K., Kevorkian, L., Norton, R., Waters, J. G., Goldring, M. B., Parker, A. E., and Clark, I. M. (2009) Characterization and regulation of ADAMTS-16. Matrix Biol. 28, 416-424

21. Larsson, S. H., Charliu, J. P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V., and Hastie, N. D. (1995) Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell 81, 391-401

22. Sciesielski, L. K., Kirschner, K. M., Scholz, H., and Persson, A. B. (2010) Wilms' tumor protein Wt1 regulates the Interleukin-10 (IL-10) gene. FEBS Lett. 584, 4665-4671

23. Englert, C., Hou, X., Maheswaran, S., Bennett, P., Ngwu, C., Re, G. G., Garvin, A. J., Rosner, M. R., and Haber, D. A. (1995) WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. EMBO J. 14, 4662-4675

24. Hartwig, S., Ho, J., Pandey, P., Macisaac, K., Taglienti, M., Xiang, M., Alterovitz, G., Ramoni, M., Fraenkel, E., and Kreidberg, J. A. (2010) Genomic characterization of Wilms' tumor suppressor 1 targets in nephron progenitor cells during kidney development. Development 137, 1189-1203
Wt1 regulates Adamts16 in developing kidneys and gonads

25. Clapcote, S. J. and Roder, J. C. (2005) Simplex PCR assay for sex determination in mice. *Biotechniques* **38**, 702, 704, 706

26. Dame, C., Kirschner, K. M., Bartz, K. V., Wallach, T., Hussels, C. S., and Scholz, H. (2006) Wilms' tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. *Blood* **107**, 4282-4290

27. Kirschner, K. M., Hagen, P., Hussels, C. S., Ballmaier, M., Scholz, H., and Dame, C. (2008) The Wilms' tumor suppressor Wt1 activates transcription of the erythropoietin receptor in hematopoietic progenitor cells. *FASEB J.* **22**, 2690-2701

28. Martens, L. K., Kirschner, K. M., Warnecke, C., and Scholz, H. (2007) Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator of the TrkB neurotrophin receptor gene. *J. Biol. Chem.* **282**, 14379-14388

29. Warburg, O., and Christian, W. (1942) Isolation and crystallization of enolase. *Biochem. Z.* **310**, 384-421

30. Wagner, K. D., Wagner, N., Sukhatme, V. P., and Scholz, H. (2001) Activation of vitamin D receptor by the Wilms' tumor gene product mediates apoptosis of renal cells. *J. Am. Soc. Nephrol.* **12**, 1188-1196

31. Wagner, N., Wagner, K. D., Theres, H., Englert, C., Schedl, A., and Scholz, H. (2005) Coronary vessel development requires activation of the TrkB neurotrophin receptor by the Wilms' tumor transcription factor Wt1. *Genes Dev.* **19**, 2631-2642

32. Stricker, S., Verhey van Wijk, N., Witte, F., Brieske, N., Seidel, K., and Mundlos, S. (2006) Cloning and expression pattern of chicken Ror2 and functional characterization of truncating mutations in Brachydactyly type B and Robinow syndrome. *Dev. Dyn.* **235**, 3456-3465

33. Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., van Heyningen, V., and Hastie, N.D. (1990) The candidate Wilms' tumour gene is involved in genitourinary development. *Nature* **346**: 194-197

34. Armstrong, J.F., Pritchard-Jones, K., Bickmore, W.A., Hastie, N.D., and Bard, J.B. (1993). The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mech. Dev.* **40**: 85-97

35. Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., and Jaenisch, R. (1993) WT-1 is required for early kidney development. *Cell* **74**, 679-691

36. Haber, D. A., Sohn, R. L., Buckler, A. J., Pelletier, J., Call, K. M., and Housman, D. E. (1991) Alternative splicing and genomic structure of the Wilms' tumor gene WT1. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9618-9622

37. Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P., and Rauscher F. J. 3rd (1991) Transcriptional repression mediated by the WT1 Wilms tumor gene product. *Science* **253**, 1550-1553

38. Hamilton, T. B., Barilla, K. C., and Romaniuk, P. J. (1995) High affinity binding sites for the Wilms’ tumour suppressor protein WT1. *Nucleic Acids Res.* **23**, 277-284

39. Laity, J. H., Dyson, H. J., and Wright, P. E. (2000) Molecular basis for modulation of biological function by alternate splicing of the Wilms’ tumor suppressor protein. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11932-11935
Wt1 regulates Adams16 in developing kidneys and gonads

40. Bor, Y. C., Swartz, J., Morrison, A., Rekosh, D., Ladomery, M., and Hammarskjöld, M. L. (2006) The Wilms’ tumor 1 (WT1) gene (+KTS isoform) functions with a CTE to enhance translation from an unspliced RNA with a retained intron. *Genes Dev.* 20, 1597-1608

41. Klattig, J., Sierig, R., Kruspe, D., Besenbeck, B., and Englert, C. (2007) The Wilms’ tumor protein Wt1 is an activator of the anti-Müllerian hormone receptor gene Amhr2. *Mol. Cell. Biol.* 27(12), 4355-4364

42. Michos, O. (2009) Kidney development: from ureteric bud formation to branching morphogenesis. *Curr. Opin. Genet. Dev.* 19(5), 484-490

43. Pohl, M., Sakurai, H., Bush, K. T., and Nigam, S. K. (2000) Matrix metalloproteinases and their inhibitors regulate in vitro ureteric bud branching morphogenesis. *Am. J. Physiol. Renal Physiol.* 279, F891-F900

44. Mittaz, L., Russell, D. L., Wilson, T., Brasted, M., Tkalcevic, J., Salamonsen, L. A., Hertzog, P. J., and Pritchard, M. A. (2004) Adams-1 is essential for the development and function of the urogenital system. *Biol. Reprod.* 70, 1096-1105

45. Miller-Hodges, E., and Hohenstein, P. (2012) WT1 in disease: shifting the epithelial-mesenchymal balance. *J. Pathol.* 226(2), 229-240

46. Davies, J. A., Ladomery, M., Hohenstein, P., Michael, L., Shafe, A., Spraggon, L., and Hastie, N. (2004) Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation. *Hum. Mol. Genet.* 13, 235-246

47. Guo, J. K., Menke, A. L., Gubler, M. C., Clarke, A. R., Harrison, D., Hammes, A., Hastie, N. D., and Schedl A. (2002) WT1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. *Hum. Mol. Genet.* 11(6), 651-659

48. Schumacher, V., Scharer, K., Wuhl, E., Altrogge, H., Bonzel, K. E., Guschmann, M., Neuhaus, T. J., Pollastro, R. M., Kuwertz-Broking, E., Bulla, M., Tondera, A. M., Mundel, P., Helmchen, U., Waldherr, R., Weirich, A., and Royer-Pokora, B. (1998) Spectrum of early onset nephrotic syndrome associated with WT1 missense mutations. *Kidney Int.* 53, 1594-1600

49. Niaudet, P., and Gubler, M. C. (2006) WT1 and glomerular diseases. *Pediatr. Nephrol.* 21(11), 1653-1660

50. Gopalakrishnan, K., Kumarasamy, S., Abdul-Majeed, S., Kalinoski, A. L., Morgan, E. E., Gohara, A.F., Nauli, S. M., Filipiak, W. E., Saunders, T. L., and Joe, B. (2012) Targeted disruption of *Adams16* gene in a rat genetic model of hypertension. *Proc. Natl. Acad. Sci. U. S. A.* 109(50), 20555-20559

51. Swain, A., and Lovell-Badge, R. (1999) Mammalian sex differentiation: a molecular drama. *Genes Dev.* 13, 755-767

52. Shimamura, R., Fraizer, G. C., Trapman, J., Lau. Y. f. C., and Saunders, G.F. (1997) The Wilms’ tumor gene WT1 can regulate genes involved in sex determination and differentiation SRY, Müllerian-inhibiting substance, and the androgen receptor. *Clin. Cancer Res.* 3, 2571-2580
Hossain, A., and Saunders, G. F. (2001) The human sex-determining gene SRY is a direct target of WT1. *J. Biol. Chem.* **276**, 16817-16823

Pelletier, J., Bruening, W., Kashtan, C. E., Mauer, S. M., Manivel, J. C., Striegel, J. E., Houghton, D. C., Junien, C., Habib, R., Fouser, L., Fine, R. N., Silverman, B. L., Haber, D. A., and Housman, D. (1991) Germline mutation in the Wilms' tumor suppressor gene are associated with abnormal urogenital development and Denys-Drash syndrome. *Cell* **67**, 437-447

Roberts, S. G. (2006) The modulation of WT1 transcription function by cofactors. *Biochem. Soc. Symp.* **73**, 191-201

Roh, J., Bae, J., Lee, K., Mayo, K., Shea, L., and Woodruff, T. K. (2009) Regulation of Wilms' tumor gene expression by nerve growth factor and follicle-stimulating hormone in the immature mouse ovary. *Fertil. Steril.* **91**, 1451-1454

Yoon, O., and Roh, J. (2012) Regulation of FSH receptor expression by the Wilms' tumor 1 gene product (WT1) in immature rat granulosa cells. *Mol. Reprod. Dev.* **79**, 368

Espey, L. L., Yoshioka, S., Russell, D. L., Robker, R. L., Fujii, S., and Richards, J. S. (2000) Ovarian expression of a disintegrin and metalloproteinase with thrombospondin motifs during ovulation in the gonadotropin-primed immature rat. *Biol. Reprod.* **62**, 1090-1095

Madan, P., Bridges, P. J., Komar, C. M., Beristain, A. G., Rajamahendran, R., Fortune, J. E., and MacCalman, C. D. (2003) Expression of messenger RNA for ADAMTS subtypes changes in the periovulatory follicle after the gonadotropin surge and during luteal development and regression in cattle. *Biol. Reprod.* **69**, 1506-1514

Robker, R. L., Russell, D. L., Espey, L. L., Lydon, J. P., O'Malley, B. W., and Richards, J. S. (2000) Progesteron-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc. Natl. Acad. Sci. U. S. A* **97**, 4689-4694

Richards, J. S., Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Teuling, E., Lo, Y., Boerboom, D., Falender, A. E., Doyle, K. H., LeBaron, R. G., Thompson, V., and Sandy, J. D. (2005) Regulated expression of ADAMTS family members in follicles and cumulus oocyte complexes: evidence for specific and redundant patterns during ovulation. *Biol. Reprod.* **72**, 1241-1255

Gondos, B. (1975) Surface epithelium of the developing ovary. Possible correlation with ovarian neoplasia. *Am. J. Pathol.* **81**, 303-321

Peters, H., and Pedersen, T. (1967) Origin of follicle cells in the infant mouse ovary. *Fertil. Steril.* **18**, 309-313

Byskov, A. G. (1978) The anatomy and ultrastructure of the rete system in the fetal mouse ovary. *Biol. Reprod.* **19**, 720-735

Sawyer, H. R., Smith, P., Heath, D. A., Juengel, J. L., Wakefield, S. J., and McNatty, K. P. (2002) formation of ovarian follicles during fetal development in sheep. *Biol. Reprod.* **66**, 1134-1150

Martinez-Estrada, O. M., Lettice, L. A., Essafi, A., Guadix, J. A., Slight, J., Veleceta, V., Hall, E., Reichmann, J., Devenney, P. S., Hosten, N., Hill, R. E., Muñoz-Chápuli, R., and Hastie, N. D. (2010). Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. *Nat. Genet.* **42**(1), 89-93

15
Wt1 regulates Adamts16 in developing kidneys and gonads

67. Morris, J. F., Madden, S. L., Tournay, O. E., Cook, D. M., Sukhatme, V. P., and Rauscher, F. J. (1991). Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. Oncogene 6, 2339-2348

Acknowledgments - We thank Mrs. Ulrike Neumann for her expert technical assistance.

FOOTNOTES
*This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Scho634/8-1). C.J. received fellowships from the Sonnenfeld-Stiftung and the FAZIT-STIFTUNG.

1To whom correspondence may be addressed: Holger Scholz, Institut für Vegetative Physiologie, Charité-Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany, Tel.: +49-30-450 528213, FAX: +49-30-450 528928, E-mail: holger.scholz@charite.de

3The abbreviations used are: ADAMTS16, a disintegrin and metalloproteinase with thrombospondin motifs; Wt1, Wilms’ tumor gene 1; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

FIGURE LEGENDS

FIGURE 1. Expression of Adamts16 and Wt1 in embryonic and adult gonads.

Panel A: Specific antibodies against Wt1 (green) and Adamts16 (red) were used to detect both proteins in the XY gonads of embryonic (14 d.p.c.) mice (a-c) and adult rats (d-f). Please, note that two different antibodies were applied for the detection of Adamts16: the results shown in b, d, e were obtained with antibody ab45048 (Abcam), and antibody sc-50490 (Santa Cruz Biotechnology) was used for the stainings presented in c and f. Double-immunolabeling identified Sertoli cells as the sites of Wt1 expression in adult testes (arrowheads in d and f). Antibody ab45048 detected Adamts16 protein mainly in the spermatids (asterisks in e), whereas antibody sc-50490 produced a signal in Sertoli cells (arrowheads in f) in addition to spermatids (asterisks in f). Cell nuclei were counterstained with dapi (blue).

Panel B: Wt1 (green) and Adamts16 (red) proteins in XX gonads of embryonic (13 d.p.c.) mice (a, b) and adult rats (d-f). Data shown in b were obtained with antibody ab45048 (Abcam), whereas antibody sc-50490 (Santa Cruz Biotechnology) was used for the stainings presented in d-f. Co-expression of Wt1 and Adamts16 was observed in granulosa cells of preovulatory tertiary (d) and secondary (asterisks in e) follicles, and in mesothelial cells on the surface of the ovaries (arrowheads in e and f). Incubation of the tissue sections with normal sera produced no staining signal (c). O, oocyte; A, antrum folliculi. Cell nuclei were counterstained with dapi (blue).

Panel C: Expression of Adamts16 in developing (a) and adult (c, d) testis was confirmed by in situ hybridization using a specific antisense riboprobe. Note, that Adamts16 transcripts were also present in granulosa cells of the ovaries (e, f). No hybridization signal was seen with the use of an Adamts16 sense probe (b). The data shown are representative for the 5 tissue samples from 3 different animals each.

FIGURE 2. Expression of Adamts16 and Wt1 in embryonic kidneys.

Specific antibodies against Wt1 (green) and Adamts16 (red) were used to detect both proteins in the kidneys of embryonic (16 d.p.c.) rats. Double immunofluorescent labeling indicates co-expression of Adamts16 and Wt1 in the developing glomeruli (c, d). The corresponding single stainings are shown in e, g, and f, h, respectively. Two different antibodies were applied for the detection of Adamts16: the results shown in a, c, e were obtained with antibody ab45048 (Abcam), and antibody sc-50490 (Santa Cruz Biotechnology) was used for the staining in d and f. Tissue sections were incubated with normal sera instead of primary antibodies as negative control (i, k). Cell nuclei were visualized with dapi (blue). Please, note that the single immunostainings shown in figures a and b were performed on two different tissue sections. Adamts16 mRNA was detected in the kidneys of murine embryos (14.5 d.p.c.) by in situ hybridization using a specific antisense riboprobe (l). No hybridization signal was obtained with the use of an Adamts16 sense probe (m). a.g., adrenal gland.
FIGURE 3. Adams16 mRNA in embryonic gonads.
Panel A: Adams16 transcripts in freshly isolated gonadal ridges of wild-type (Wt1+/+)
and Wt1-deficient (Wt1−/−) murine embryos at 13 d.p.c. Adams16 mRNA levels were measured
by quantitative RT-PCR and normalized to Gapdh transcripts. Values are means±SEM, n=9
(Wt1+/+), n=12 (Wt1+/−), n=14 (Wt1−/−). *P<0.05 vs. wild-type, ANOVA.
Panel B: Adams16 transcript levels in mismatch morpholino-treated (72 hours) gonads presented as
differences in Ct-values (Ct_{Adams16} - C_{Gapdh}). Assuming a doubling of amplicons during each cycle of
the PCR, Adams16 mRNA levels were approximately 10-times higher in both XX and XY gonads at
13.5 d.p.c. vs. 11.5 d.p.c.
Panels C and D: Adams16 mRNA in cultured gonads of wild-type male (C) and female (D) embryos
at 11.5, 12.5 and 13.5 d.p.c. The isolated gonads were incubated for 72 hours in the presence of either
mismatch or Wt1 antisense vivo-morpholinos. Results are shown as x-fold differences between
cultures treated with mismatch and Adams16 antisense morpholinos, respectively. Wt1 and Gapdh
proteins in the morpholino-treated tissues were determined by immunoblotting, and representative
blots are shown. The expected molecular masses of the different Wt1 isoforms vary between 50 and
55 kDa (67). Adams16 and Gapdh transcripts were measured by RT-PCR. Data are presented as
means±SEM, n=12. *P<0.05, Student’s t-test (C, D), ANOVA (B).

FIGURE 4. Regulation and function of Adamts16 in embryonic murine kidneys.
Panel A: Adamts16 mRNA is reduced in kidney organ culture upon antisense inhibition of Wt1.
Kidneys were removed from 13 d.p.c. murine embryos and incubated in vitro for 72 hours with
mismatch and Wt1 antisense morpholinos, respectively. Knockdown efficiency was assessed by
immunoblotting of Wt1 and β-actin proteins in the morpholino-treated cultures. A representative
Western blot is shown in the lower panel in A. Adams16 and Gapdh transcripts were measured by
RT-PCR. Data are presented as means±SEM, n=7, *P<0.05, Student’s t-test.
Panel B: In vitro differentiation of embryonic kidneys is impaired by antisense inhibition of Adamts16.
Kidneys were isolated from murine embryos at E11.5 (Taylor stage 20) and cultured for
72 hours in the presence of either mismatch or Adamts16 antisense morpholinos. The tissue explants
were analyzed by phase contrast microscopy (a, b) and immunofluorescent staining of the ureteric bud
and the metanephric mesenchyme using antibodies against pan-cytokeratin (green) and Wt1 (red),
respectively (c-f). Reconstructions of typical branching trees of the ureter are depicted in g and h.
Panel C: The number of branching points of the ureteric bud was significantly reduced in response to
Adamts16 knockdown. Adams16 knockdown in the kidney explants was assessed by immunoblot
analysis yielding a band of approximately 135 kDa (1,19). A representative Western blot is shown in
the lower panel in C. Data are presented as means±SEM, n=7, *P<0.02, Student’s paired t-test.

FIGURE 5. Adams16 transcripts and Wt1 protein in cell lines and primary epicardial cells.
Panels A: Adams16 mRNA levels in murine mesonephros-derived M15 cells transfected with a pool of
four different siRNAs targeting the Wt1 gene. Control experiments were performed by incubation with
non-targeting siRNAs.
Panel B: Adams16 transcripts in primary epicardial cells obtained from murine embryos. The cells
were incubated either with Wt1 antisense or mismatch vivo-morpholinos.
Panels C and D: Adams16 mRNA levels in UB27 (C) and UD28 (D) cells, which express the
Wt1(-KTS) and Wt1(+KTS) isoforms, respectively, under control of a tetracycline-sensitive promoter.
Adams16 and Gapdh transcripts were quantified by real-time RT-PCR. Values shown as
means±SEM, n=4, M15 cells; n=4, epicardial cells; n=3, UB27/UD28 cells. Statistical significance is
indicated by asterisks (**P<0.001, *P<0.01, *P<0.05, Student’s t-test). Wt1 protein was detected
by immunoblotting (lower panels) and compared to β-actin and Gapdh proteins, respectively.
Representative Western blots are shown.

FIGURE 6. Binding of Wt1 protein to the 5’-flanking region of the Adams16 gene.
Chromatin immunoprecipitation (ChIP) was performed to detect Wt1 protein bound to the 5’-flanking
region of the Adams16 gene in its native chromosomal configuration. The drawing (A) delineates the
three predicted Wt1 binding sites (Wt1-A, Wt1-B, Wt1-C) in the promoter and 5’UTR of the
Adams16 gene, and allocates the PCR primers used for DNA amplification. Specific antibodies
against Wt1 and histone proteins were chosen for immunoprecipitation of M15 whole cell lysates.
Amplicons encompassing the 5'-flanking region of the Adamts16 gene were enriched approximately 2.5-fold with the use of anti-Wt1 antibody compared to normal rabbit IgG (NRb-IgG). No differences in actin DNA were observed between anti-Wt1 antibody and normal rabbit IgG (B). The gene encoding anti-Müllerian hormone receptor 2 (Amhr2), a previously identified Wt1 target (41), served as a positive control (B). Binding of Wt1(-KTS) protein to the Adamts16 promoter was confirmed in stimulated UB27 cells (C). Wt1(+KTS) protein failed to interact with the promoter of the Adamts16 gene in UD28 cells (D). Data shown are means±SEM. Statistical significances are indicated by asterisks (*P<0.05, **P<0.01, Student’s t-test, n=4, M15 cells; n=3, UB27/ UD28 cells).

**FIGURE 7.** Functional interaction of Wt1(-KTS) protein with the promoter of the Adamts16 gene.
Panel A: COV434 ovarian granulosa cells were transiently co-transfected with the indicated Adamts16 promoter luciferase reporter plasmids and a Wt1(-KTS) expression construct. A Renilla luciferase plasmid was utilized for normalization of transfection efficiencies. Data shown are means±SEM, n=4. Statistical significance vs. transfection with empty expression vector is indicated by asterisks (*P<0.05, ANOVA).
Panels B-D: Binding of Wt1(-KTS) to each one of the predicted binding sites (lanes 1 and 2) was proven by electrophoretic mobility shift assay. Mutation of the Wt1 consensus motifs abrogated Wt1(-KTS) binding (lanes 3 and 4). Interaction of Wt1(-KTS) protein with the wild-type oligonucleotides could be competed with unlabeled competitor DNA (lanes 5-7) corresponding to the previously identified Wt1 binding element in the Ntrk2 promoter (31).
Panel E: The Wt1(+KTS) isoform, which lacks tree amino acids in its zinc finger domain (36), did not bind to the oligonucleotides (lanes 3, 6 and 9).
TABLE 1

List of PCR primers

| Cloning primers       | qPCR-primers         | ChIP-primers      |
|-----------------------|----------------------|------------------|
| mAdams16-Prom-3000    | mAdams16-F           | mAdams16-Prom-BS-F2 |
| mAdams16-Prom-250     | mAdams16-R           | mAdams16-PromBS-R2 |
| mAdams16-Prom-I       | hADAMTS16-F          | mActin-ChIP-F1    |
| mAdams16-Prom-45      | hADAMTS16-R          | mActin-ChIP-R1    |
| mAdams16-Prom-80      | mGapdh-F             | mAImhr2-PromBS-F1 |
|                       | mGapdh-R             | mAImhr2-PromBS-R1 |
|                       | hADAMTS16-Prom-BS-F5 | hADAMTS16-PromBS-R-F1 |
|                       | hADAMTS16-Prom-BS-R1 | hACTIN-ChIP-F1    |
|                       | hACTIN-ChIP-R1       | hACTIN-ChIP-R1    |

Cloning primers

- mAdams16-Prom-3000: CGTGAGCTCAATTCAGACCTTTGCATA
- mAdams16-Prom-250: CGTAAGCTTCCCCGGGGCTCACAGGACT
- mAdams16-Prom-I: GTAGAGCTCCCTTGGCCTCCCTCAGTCC
- mAdams16-Prom-45: ATAGAGCTCCACTGCCACCTACCTCC
- mAdams16-Prom-80: ATAGAGCTCCCTGCCCCGGTTGCTCCTTTG

qPCR-primers

- mAdams16-F: TTTCCACCAGAAGAGAACTG
- mAdams16-R: GGTGATCATCAAGACTTCC
- hADAMTS16-F: TGGCTTTATTGTGCAAGACG
- hADAMTS16-R: GGCCTTCTCGTCACTATCAT
- mGapdh-F: ACAGACCCTTCATGACCTCA
- mGapdh-R: TTTGGCTTACCCCTTCAAGTG
- hGAPDH-F: ACAGTCAGCCGACATCTTCTT
- hGAPDH-R: GACAGTCCCAGTTCTCAG

ChIP-primers

- mAdams16-PromBS-F2: GTCATAGGCCGTGAAGGATGC
- mAdams16-PromBS-R2: CCATGGCAGCTACGAGTTATC
- mActin-ChIP-F1: ATAGAAGCTCCCTTTCTATGACG
- mActin-ChIP-R1: TGGCTTTATTGTGCAAGACG
- mAImhr2-PromBS-F1: CAGGCTGACAGCCACAGGTC
- mAImhr2-PromBS-R1: CAGCCAAGGCTTCCATACAAA
- hADAMTS16-Prom-BS-F5: TGCTCTTGTCCCTGCACTTCTC
- hADAMTS16-Prom-BS-R1: CGCTCCAGGGGCTAGGGGC
- hACTIN-ChIP-F1: GTGAGTGGCCCGCTAACT
- hACTIN-ChIP-R1: CTTGGTACACCGAGCCAG
TABLE 2

| List of oligonucleotides used for gel shift experiments (mutated nucleotides are underlined) |
|--------------------------------------------------|
| mAdamts16-wtA-sense | TCCTCTATCCCTCCCCCTCTCTCTCTTT |
| mAdamts16-wtA-antisense | AAAGGAGAGGAGAGGGAGAGGGATAGAGGA |
| mAdamts16-wtB-sense | GTGCCCTTCGCCCCCCCCCACCACCTGCA |
| mAdamts16-wtB-antisense | TGGCAGTGTGGCAGGCCCCGGGCAAGAC |
| mAdamts16-wtC-sense | CTGTTCGGACCAGGGAGGTGGAGTGTCAG |
| mAdamts16-wtC-antisense | TCCCTCTATGAAATCCACTCTCTCTCTTT |
| mAdamts16-wtAmut-sense | AAAGGAGAGTAGAGTAGTTCGATAGAGGA |
| mAdamts16-wtBmut-sense | GTGCCGTTCAGTTCACAGTCACTCAGTCA |
| mAdamts16-wtBmut-antisense | TGGCAGTGTGGCAGGCCCCGGGCAAGAC |
| mAdamts16-wtCmut-sense | CTGTTCGGACCAGGGAGGTGGAGTGTCAG |
| mAdamts16-wtCmut-antisense | TCCCTCTATGAAATCCACTCTCTCTCTTT |
| mNtrk2-wt-F | TGTGAACTCCCACATGCTGCTG |
| mNtrk2-wt-R | CAGCAGCATGTGGGAGTTCCA |
Figure 1

Wt1 regulates Adamts16 in developing kidneys and gonads
Figure 2
Figure 3

Wt1 regulates Adamts16 in developing kidneys and gonads

A

B

C

D

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

Adams16 mRNA/Gapdh mRNA

Wt1+/+
Wt1+/−
Wt1−/−

Adams16 mRNA/Gapdh mRNA

11.5 d.p.c. 12.5 d.p.c. 13.5 d.p.c.

Adams16 mRNA/Gapdh mRNA

XY gonads

XX gonads

Wt1 antisense mismatch

Wt1 antisense mismatch

Wt1 Gapdh

Wt1 Gapdh

kDa

35 49 56

kDa

35 49 56
Wt1 regulates Adamts16 in developing kidneys and gonads

Figure 4

A

B

C

| mismatch | Wt1 antisense | Adamts16 mRNA / Gapdh mRNA |
|----------|---------------|-----------------------------|
| 1.0      | 1.0           | *                           |

Wt1 antisense mismatch

Adamts16 mRNA / Gapdh mRNA

ß-actin

- 56 - 49 - 43 kDa

ureteric bud branching points

| mismatch | Wt1 antisense | ureteric bud branching points |
|----------|---------------|------------------------------|
| 10       | 10            | *                           |

Adams16 mismatch

ß-actin

- 135 - 43 kDa
Figure 5

A. M15 cells

B. Primary epicardial cells

C. UB27 cells

D. UD28 cells

Wt1 regulates Adamts16 in developing kidneys and gonads.
Figure 6

Wt1 regulates Adamts16 in developing kidneys and gonads

A

ChIP primer

Exon1

ChIP primer

Wt1-A

Wt1-B

Wt1-C

promoter

5'UTR

B

M15 cells

C

UB27 cells

D

UD28 cells

NRb-IgG

anti-Wt1

anti-histone

Amhr2

Adamts16

Actin

ACTIN

ADAMTS16

uninduced

Wt1(-KTS)

uninduced

Wt1(+KTS)
Wt1 regulates Adamts16 in developing kidneys and gonads

Figure 7

A

![Diagram](image1)

![Diagram](image2)

![Diagram](image3)

![Diagram](image4)

![Diagram](image5)
Transcriptional regulation by the Wilms tumor protein, Wt1, suggests a role of the metalloproteinase Adamts16 in murine genitourinary development
Charlotte L.J. Jacobi, Lucas J. Rudigier, Holger Scholz and Karin M. Kirschner

J. Biol. Chem. published online May 9, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.464644

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts
Transcriptional regulation by the Wilms tumor protein, Wt1, suggests a role of the metalloproteinase Adamts16 in murine genitourinary development.

Charlotte L. J. Jacobi, Lucas J. Rudigier, Holger Scholz, and Karin M. Kirschner

PAGE 18817:

Lanes 3–6 of the original Western blot image in Fig. 3C contained the same bands as lanes 3–6 of the Western blot image in Fig. 3D, and both panels failed to indicate that separate lanes from the original blots had been spliced together. The correct Western blot image for Fig. 3C is now provided, and lines have been added to indicate the area where the blot images were joined. These changes do not affect the interpretation or conclusions of this work.