On the role of *Wt1*, *Dmrt8* and *Sox9* during murine gonad development and sex determination

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Zusammenfassung

Ist es ein Mädchen oder ein Junge? Das ist wohl immer die erste Frage, die jeden interessiert, wenn ein Kind geboren wird. Leider gibt es verschiedene menschliche Krankheiten, die dazu führen, dass diese Frage nicht immer ganz leicht zu beantworten ist. Hervorgerufen werden diese Krankheiten durch Mutationen in verschiedenen Genen, die eine Rolle bei der Entwicklung der primären Geschlechtsorgane (Gonaden) spielen. So können Patienten mit bestimmten Mutationen in den Genen *Wt1*, *Dmrt1* oder *Sox9* Merkmale beider Geschlechter besitzen. Alle diese Gene kodieren für Transkriptionsfaktoren.

Wt1 spielt eine fundamentale Rolle in der Entwicklung der Gonaden in beiden Geschlechtern. Mäuseembryonen ohne ein funktionsfähiges *Wt1* Gen beginnen zwar Gonaden zu entwickeln, verlieren diese aber im Laufe ihrer weiteren Entwicklung wieder. Welche Funktion das Wt1 Protein im Detail hat und welche Gene durch Wt1 reguliert werden ist noch immer weitgehend unverstanden. Im Rahmen dieser Arbeit wurde versucht, durch Vergleich der Genexpression in den Gonaden von $Wt1^{+/+}$ und $Wt1^{-/-}$ Mäuseembryonen neue Zielgene von Wt1 zu identifizieren. Dadurch konnte gezeigt werden, dass die Expression des Gens *AmhrII*, das eine wichtige Rolle in der männlichen Geschlechtsdifferenzierung spielt, von Wt1 abhängt. Weitere Untersuchungen haben ergeben, dass Wt1 den Promoter dieses Gens direkt bindet und aktiviert.

Die *Dmrt*-Gene gehören zur bisher einzigen bekannten Genfamilie, die für die Geschlechtsbestimmung verschiedener Tierstämme (z.B. Würmer, Fliegen und Wirbeltiere) wichtig ist. In mehreren Wirbeltieren scheint vor allem *Dmrt1* eine wichtige Rolle zu spielen. *Dmrt1*-Knockout-Mäuse zeigen allerdings nur einen schwachen Phänotyp. Deshalb wird vermutet, dass in Mäusen auch andere *Dmrt*-Gene für die Gonadenentwicklung wichtig sind. Im Rahmen dieser Arbeit wurde das Expressionsmuster eines bisher wenig untersuchten Gens – *Dmrt8* – analysiert, das in der Maus in drei Kopien vorkommt. Es stellte sich heraus, dass zwei dieser drei Kopien ein Expressionsmuster zeigen, das für eine Funktion in den männlichen Gonaden bzw. deren Entwicklung spricht.

Kollaborationspartnern ist es kürzlich gelungen, eine gonadenspezifische Knockout-Maus von *Sox9* zu erzeugen. Die in dieser Arbeit präsentierte Analyse der Genexpression in diesem Mausmodel zeigte, dass das Fehlen von *Sox9* zu einer

kompletten Geschlechtsumkehr in genetisch männlichen Embryonen führt. In sich entwickelnden Gonaden von *Sox9*-defizienten männlichen Embryonen ist das Gen *Foxl2*, das normalerweise nur in weiblichen Gonaden exprimiert wird, aktiviert. Das spezifisch männliche Gen *Amh* wird dagegen, trotz Expression des geschlechtsbestimmenden Gens *Sry*, nicht aktiviert. Somit scheint nicht *Sry*, sondern *Sox9* das zentrale Gen für die männliche Geschlechtsentwicklung zu sein. Weiter wurde Prostaglandin-D-synthase (*Ptgds*) als potentielles Zielgen von Sox9 identifiziert.

Abstract

Is it a girl or a boy? This is the first question everybody is interested in, when a baby is born. Unfortunately there are several human diseases, which complicate the answer to this question. These diseases are caused by mutations in genes, which play a role in the development of the primary sexual organs (gonads). Patients with certain mutations in the genes *Wt1*, *Dmrt1* or *Sox9,* for example, show sexual ambiguity. All these genes code for transcription factors.

Wt1 plays a fundamental role in the development of gonads of both sexes. Mouse embryos without an intact *Wt1* gene start to develop gonads but lose them again during further development. The detailed function of Wt1 protein and which genes are regulated by Wt1 is still quite unknown. Aim of this work was to identify new Wt1 target genes by comparison of gene expression in $Wt1^{+/+}$ and $Wt1^{-/-}$ embryos. It could be shown that *AmhrII*, a gene known to be important for male differentiation, depends on Wt1. Further experiments demonstrated that Wt1 directly binds and activates the promoter of this gene.

The *Dmrt*-genes belong to the only gene family known so far, which is important for sex determination in different metazoan phyla (e.g. worms, flies and vertebrates). Especially *Dmrt1* seems to play an important role in several vertebrates. However, the phenotype of *Dmrt1* knock-out mice is unexpectedly mild. Therefore it has been speculated that additional *Dmrt*-genes play a role in murine gonad development. In this work the expression pattern of *Dmrt8*, a relatively unexplored gene, of which three copies can be found in mice, was investigated. It was found, that two of these three copies show an expression pattern that suggests a role in male gonadal function and development.

Recently collaboration partners generated a gonad specific *Sox9* knock-out mouse. The analysis of gene expression in this mouse model, presented here, showed that the lack of *Sox9* leads to a complete male-to-female sex reversal. In developing *Sox9* mutant male gonads the female-specific gene *Foxl2* is upregulated. In contrast, the male-specific gene *Amh* is not activated although the sex determining gene *Sry* is expressed. Thus, not Sry, but Sox9 seems to be the master sex determining factor. In addition Prostaglandin D synthase (*Ptgds)* was identified as a potential target of Sox9.

Parts of this work have been published in:

Fischer A, **Klattig J**, Kneitz B, Diez H, Maier M, Holtmann B, Englert C, Gessler M. Hey basic helix-loop-helix transcription factors are repressors of GATA4 and GATA6 and restrict expression of the GATA target gene ANF in fetal hearts. Mol Cell Biol 2005; 25: 8960-8970.

Barrionuevo F, Bagheri-Fam S, **Klattig J**, Kist R, Taketo MM, Englert C, Scherer G. Homozygous Inactivation of Sox9 Causes Complete XY Sex Reversal in Mice. Biol Reprod 2006; 74: 195-201.

Veith AM*, **Klattig J***, Dettai A, Schmidt C, Englert C, Volff JN. Male-biased expression of X-chromosomal DM domain-less Dmrt8 genes in the mouse. Genomics 2006; in press. *These authors contributed equally to this work.

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1 Introduction

1.1 Gonad development

Nothing makes our life so wonderful and so complicated at the same time than the fact that there are two sexes – female and male. Thus, when a child is born, the first question, everybody is interested in, is: “Is it a boy or a girl?” In human beings and other mammals, the sex of a child is determined by the sex chromosomes X and Y. If an embryo possesses both – one X and one Y chromosome than it develops as a boy. When two X chromosomes, but no Y chromosome, are present, it develops as a girl. However, there are human diseases that cause problems in answering the simple question about the sex of a child. For example, mutations affecting the genes called *Sry*, *Dax1*, *Wt1*, *Sox9* and *Dmrt1* lead to partial or complete sex reversal. In these cases the chromosomal sex and the observed sex are not identical, or characteristics of both sexes are present[1].

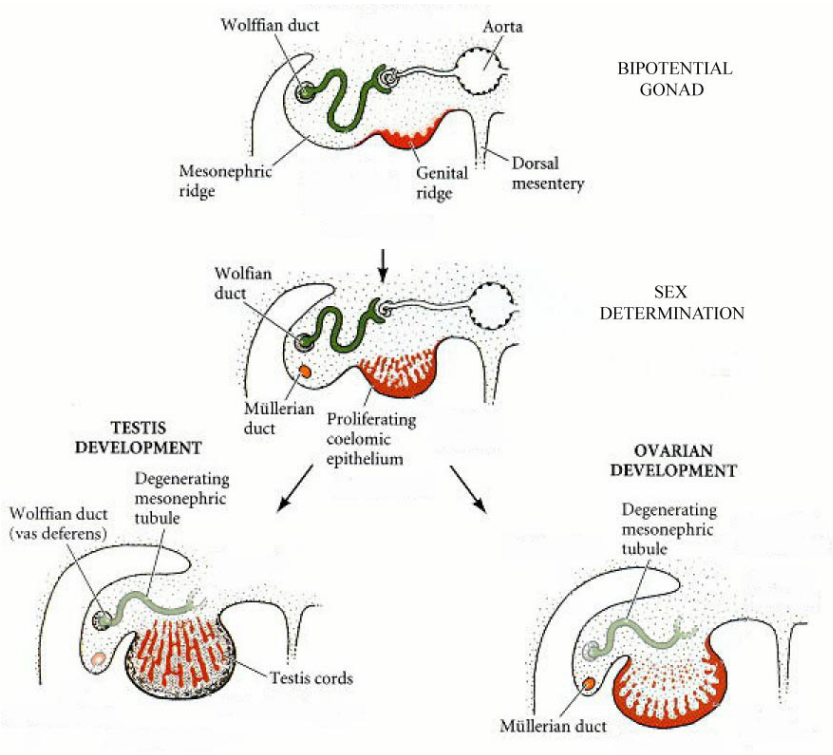

Fig. 1. Mammalian urogenital ridge development. Mammalian urogenital ridge development shown in transverse section. The future gonads (genital ridges) arise by proliferation of the coelomic epithelium adjacent to the mesonephric ridges on both sides of the dorsal aorta and the dorsal mesentery. No sexual differences can be observed at this developmental stage (bipotential gonad). At the time of sex determination two sexual ducts (Wolffian duct and Müllerian duct) are visible. After sex determination Müllerian ducts degenerate in male embryos while Wolffian ducts regress in female embryos. After sex determination male gonads can be distinguished from female gonads by the presence of testis cords. (Modified from Gilbert et al.[2])

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[2] Gilbert, S. F. (2003). "Developmental Biology." Sinauer Associates Inc., Sunderland, MA USA.

Before sex determination, the primary sexual organs of both sexes develop identically and are therefore called bipotential gonads (Fig. 1)[2]. The gonads arise as thickenings of the epithelium along the coelomic surface of the embryonic kidneys, the so called mesonephroi. This structure, consisting of developing gonad and mesonephros, is called urogenital ridge. Two pairs of sexual ducts, Müllerian ducts and Wolffian ducts, are formed in the mesonephroi of both sexes. Only one pair of sexual ducts is needed in each case later on, while the second pair regresses. Wolffian ducts develop into epididymes, vasa deferentia and seminal vesicles in males, while Müllerian ducts form Fallopian tubes, the uterus and the upper third of the vagina in females. In mice, gonad development starts about 10.0 days post coitum (E10.0). It takes almost two days before the first differences between male and female gonads are visible[3]. At day 11.5 of embryonic development (E11.5) sex determination occurs, male gonads grow faster than female gonads and structures called “testis cords” become visible, while ovaries remain relatively unstructured. Testis cords contain germ cells and supporting cells (Sertoli cells) and are surrounded by interstitial cells, like the steroid hormone producing Leydig cells.

1.2 Sex determination and *Sox9*

Male sex determination depends on expression of the Y-chromosomal gene *Sry* (sex-determining region on the Y chromosome). Gonads without a Y-chromosome and therefore without *Sry* develop as ovaries, while Sry protein induces testis differentiation in XY gonads. It has been shown, that transgenic expression of Sry is sufficient to induce testis differentiation in XX mice[4]. Murine *Sry* is expressed only during a short period of time from E10.5 to E12.5 with a peak at E11.5[5] and only in a certain cell type, the Pre-Sertoli cells[6,7]. The only function of Sry seems to be to induce Sertoli cell differentiation. Differentiating Sertoli cells then induce Leydig-cell

[3] Schmahl, J., Eicher, E. M., Washburn, L. L., and Capel, B. (2000). Sry induces cell proliferation in the mouse gonad. *Development* **127,** 65-73.
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differentiation, vascularization, proliferation of interstitial cells and migration of cells from the mesonephros into the gonad[8].

Whereas the testis-inducing function of SRY has been unambiguously demonstrated, its direct target gene or genes and molecular function still await identification. *Sry* contains a DNA-binding HMG (high mobility group) box and acts therefore most likely as transcription factor[9]. But also a function in mRNA splicing has been discussed[10]. As one likely direct and possibly only target of SRY, the *Sry*-related gene *Sox9* (*Sry* box containing 9) has emerged[11]. Sox9 is a transcription factor that contains an HMG-box which is very similar to that found in Sry. In humans, heterozygous *SOX9* mutations cause partial or complete male-to-female sex reversal in the context of the skeletal malformation syndrome campomelic dysplasia[12,13]. In turn, duplication of the chromosomal region 17q23.1-q24.3 encompassing the *SOX9* locus was present in an XX individual with female-to-male sex reversal[14]. During gonadogenesis of the mouse, *Sox9* is initially expressed in both sexes, but decreases in the developing ovary and strongly increases in the developing testis, concomitant with the peak of *Sry* expression at E11.5[15,16]. Furthermore, SOX9 has been shown to co-localize with SRY in the nucleus of Sertoli cell precursors as early as E11.5, consistent with the hypothesis that *Sox9* is a direct target of SRY[7]. *Sox9* can also substitute for *Sry* as a testis-determining factor, as ectopic expression of a *Sox9* transgene[17] and mutational

[8] Brennan, J., and Capel, B. (2004). One tissue, two fates: molecular genetic events that underlie testis versus ovary development. *Nat Rev Genet* **5,** 509-21.

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[17] Vidal, V. P., Chaboissier, M. C., de Rooij, D. G., and Schedl, A. (2001). Sox9 induces testis development in XX transgenic mice. *Nat Genet* **28,** 216-7.

upregulation of *Sox9* expression[18] cause testis development in XX mice. These latter data show that *Sox9* is sufficient for testis induction but do not prove that it is essential for this process. In contrast to the situation in humans, heterozygous *Sox9* mutations do not cause XY sex reversal in mice[19]. As mouse embryos homozygously mutant for *Sox9* die at E11.5 at the onset of testicular morphogenesis, the fate of the mutant XY gonad could only be studied *ex vivo* in organ culture, revealing no signs of testis cord formation after three days in culture[20].

To follow the development of *Sox9*$^{-/-}$ XY gonads *in vivo* and during the entire phase of gonadogenesis, a conditional, gonad-specific knock-out of *Sox9* is needed. The first attempt to create a gonad specific *Sox9* knock-out was not completely successful. A *Cre* transgene under control of an *Sf1* (steroidogenic factor 1, also known as *Nr5a1*) regulatory element has been used for this purpose, but due to inefficient and/or late Cre-mediated deletion of the *Sox9*^{flox} allele, mutant gonads always showed some testis cord formation[20]. Thus a different Cre line is needed to achieve early and efficient deletion of *Sox9*^{flox} alleles in gonads.

Only two gonadal targets of Sox9 are known so far, namely the genes encoding the anti-Müllerian hormone (Amh)[21,22] and Vanin-1[23]. Especially the function and regulation of Amh, also called Müllerian inhibiting substance (Mis), has been intensively studied[24]. Amh, a member of the *transforming growth factor β* superfamily, is secreted by Sertoli cells and binds to a heterodimeric serine/threonine kinase receptor (Amh-receptor type I and type II)[25]. The ligand binding part of the receptor, AmhrII, is well known and characterized, while several candidates are discussed as

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possible type I receptors. The receptor complex is located in mesenchymal cells surrounding the Müllerian duct and mediates Müllerian duct regression[26,27]. Expression of *Amh* is regulated by Sox9 in cooperation with several other transcription factors, known to be involved in gonad development, namely Sf1, Gata4

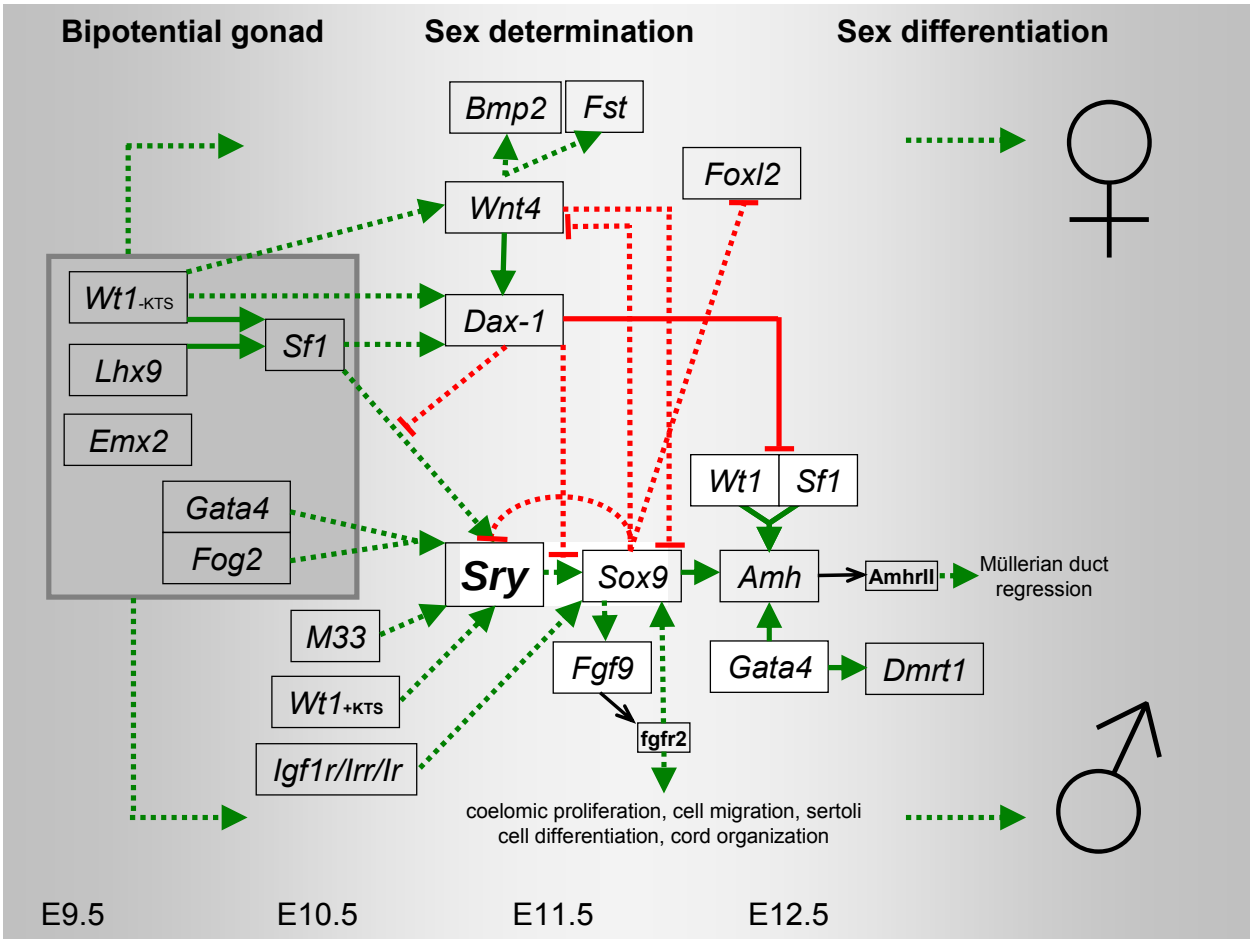

Fig. 2. Regulation of gonad development.
Gonads develop in three stages: bipotential stage, sex determination and sex differentiation. In mice this development starts at about E10.0. Sex determination occurs at E11.5. Gonad development is regulated by a complicated network of factors, which activate (green arrows) or inhibit (red lines) each others expression. Clearly shown direct interactions are indicated by solid lines. Supposed or indirect interactions are indicated by dashed lines. The factors can be roughly divided into three groups: First, factors important for development of the bipotential gonad in both sexes (boxed in dark grey); second, factors specifically expressed in female gonads after sex determination (Wnt4, Bmp2, Fst, Foxl2); finally, factors, which are important for male sex determination and differentiation upstream or downstream of the sex determining gene *Sry*. Some factors involved in early gonad development are also important for sex differentiation (Wt1, Sf1, Gata4). Action of Fgf9 and Amh is mediated by binding to their specific receptors Fgfr2 and AmhrII. Modified from Clarkson and Harley[28].

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(GATA binding protein 4), Dax1 (also known as Nr0b1) and Wt1 (Wilms tumour protein 1) (Fig. 2). Besides activation and action of Amh the processes involved in gonad development, upstream and downstream of Sry and Sox9, are still poorly understood (Fig. 2). Even less understood than male sex differentiation is the development of ovaries. Only a few female-specific genes, like *Wnt4* (wingless-related MMTV integration site 4), *Bmp2* (bone morphogenetic protein 2), *Fst* (follistatin) and *Foxl2* (forkhead box L2), have been identified so far (Fig. 2)[8]. It has become clear, that gonadogenesis, sex determination and sex differentiation are regulated by a complicated network of interacting factors. Many aspects of this network, including genes and modes of interaction, still await discovery.

1.3 Wt1

One gene that plays a fundamental role in gonad development of both sexes is the Wilms tumour suppressor gene *Wt1*. *Wt1* was initially identified as a gene inactivated in 10-15% of Wilms tumours (WT), a form of pediatric kidney cancer found in 1 of 10,000 children[29,30]. The *Wt1* gene covers 50 kb with 10 exons and encodes a protein family of at least 24 different proteins arising from usage of alternative translation initiation sites, RNA editing, and two alternative splicing events (Fig. 3)[31]. Wt1 proteins show typical characteristics of transcription factors with a glutamine/proline-rich N-terminus, activation and repression domains and four zinc fingers of the Cys_2-His_2 type at the C-terminus. One of the two alternative splicing events includes the mammalian specific exon 5, which encodes 17 amino acids, in the central part of the protein. The function of this alternative exon has not yet been defined. In contrast, the second alternative splicing event seems to be essential for the function of this gene and leads to two proteins with distinct functions[32]. These two variants of Wt1 are formed by alternative splicing at the end of exon nine, leading to the inclusion or exclusion of three amino acids, lysine, threonine, and serine (KTS), between zinc fingers three and four. The resulting variants are called Wt1(-KTS) and

[29] Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., and et al. (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60,** 509-20.

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Wt1(+KTS). These isoforms are expressed in a constant ratio of about +KTS/-KTS = 2:1[33]. It has been proven, that Wt1(-KTS) binds to DNA and acts as a transcription factor, while the DNA binding capacity of Wt1(+KTS) is much lower and Wt1(+KTS) function is still a matter of speculation[34]. Due to its colocalisation and association with splicing factors and small nuclear ribonucleoproteins (snRNPs) a role in RNA processing is discussed.

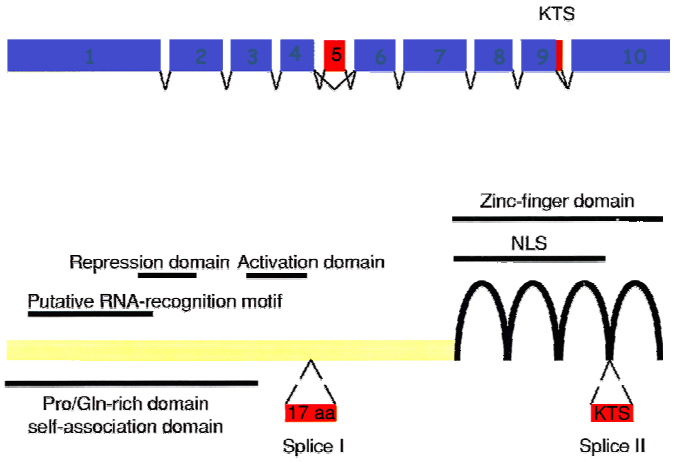

Fig. 3. Structure of *Wt1* mRNA and Wt1 protein[31].
Structure of the *Wt1* mRNA (top) and Wt1 protein (bottom). Wt1 is encoded by 10 exons. Alternative splicing events affect Exon 5, encoding 17 amino acids, and 9 bp at the end of exon 9, leading to inclusion or exclusion of the three amino acids lysine (K), threonine (T) and serine (S) between zinc fingers three and four. The protein contains four zinc fingers at the C-terminus, several interaction domains and a nuclear-localization signal (NLS).

Since the cloning of *Wt1*, several human diseases have been shown to be associated with *Wt1* mutations[31]. Among them are three syndromes associated with sex reversal or genitourinary malformations. Urogenital abnormalities in patients with WAGR syndrome (*Wilms Tumour*, *Aniridia*, *genitourinary malformations*, *mental retardation*), Denys-Drash syndrome (DDS) or Frasier syndrome range from undescended testes (cryptorchidism) and abnormal location of the penis opening (hypospadias) in male WAGR patients to streak gonads and sex reversal in extreme DDS or Frasier cases[35,36,37,38,39,40]. The reasons for these diseases are heterozygous

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mutations in the Wt1 locus. WAGR is caused by deletions in the chromosomal region 11p13, where *Wt1* and *Pax6*, a master gene for eye development, are located. According to the two-hit model, a Wilms' tumour occurs in these patients, if the second *Wt1* allele is mutated in renal precursor cells[41]. Like WAGR, also DDS leads to predisposition to Wilms' tumour and sexual ambiguity, but with higher incidence. DDS is caused by missense mutations in the zinc finger region of the Wt1 gene. The most common mutation consists of an arginine to tryptophan change at residue 394 of the Wt1 zinc finger III, which abrogates DNA binding of Wt1[42]. The more severe phenotype of DDS compared with WAGR may be explained by a dominant-negative effect of the DDS mutant form of Wt1 due to interaction with wild-type Wt1[43,44,45,46]. Frasier syndrome is associated with a point mutation in the intron 9 donor splice site of one *Wt1* allele, resulting in the loss of Wt1(+KTS) expression from that allele and an altered –KTS/+KTS ratio[38,40]. That both forms of Wt1 and the correct ratio of these forms are important for gonad and kidney development has also been shown in a mouse model[32]. Thus, it has become clear by analysis of human patients and different mouse models, that Wt1 plays an essential role in the development of gonads, kidneys and other organs[47].

In contrast to human patients, mice with heterozygous inactivation of *Wt1* appear normal and do not develop tumours. *Wt1*$^{-/-}$ embryos however show failure of gonad

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[46] Holmes, G., Boterashvili, S., English, M., Wainwright, B., Licht, J., and Little, M. (1997). Two N-terminal self-association domains are required for the dominant negative transcriptional activity of WT1 Denys-Drash mutant proteins. *Biochem Biophys Res Commun* **233,** 723-8.

[47] Discenza, M. T., and Pelletier, J. (2004). Insights into the physiological role of WT1 from studies of genetically modified mice. *Physiol Genomics* **16,** 287-300.

and kidney development[48]. In these embryos urogenital ridge development is initiated, but after E11.5 the cells of the urogenital ridge undergo apoptosis. *Wt1*$^{-/-}$ embryos die before birth, most likely due to cardiac failure[49].

In mice, *Wt1* is expressed in the urogenital ridge region as soon as E9.5 and continues to be expressed throughout gonad and kidney development[50,51,52]. In adult gonads *Wt1* mRNA can be detected in Sertoli cells of the testis and the epithelial and granulosa cells (female supporting cells) of the ovary[50].

The search for Wt1 target genes is still ongoing. Although several genes have been shown to be activated or inhibited by Wt1(-KTS) in transactivation assays, the identification of physiologically relevant targets is still underway. Most of the targets identified so far are activated by Wt1(-KTS)[34]. Some of the genes induced by Wt1 are important for gonad development. For example, the human *Sry* is activated by Wt1(-KTS) in cell culture experiments[53]. Furthermore, *Sry* expression is highly reduced in embryos lacking *Wt1(+KTS)*[32]. However, the exact relationship of *Sry* and *Wt1(+KTS)* is not known. Another target of Wt1 is *Sf1* (also called *Nr5a1*). Gonadal expression of *Sf1* is activated by Wt1(-KTS) in cooperation with a second transcription factor, called Lhx9 (LIM homeobox protein 9)[54]. Like *Sf1*, also *Dax1* expression is highly reduced in *Wt1* and *Wt1(-KTS)* knock-out mice[32,54]. This could be due to direct or indirect activation, as Wt1 and Sf1 have been shown to activate *Dax1* in transactivation assays[55,56]. Also the female-specific gene *Wnt4* seems to be regulated by Wt1[57,58]. Due to the drastic knock-out phenotype and the broad

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[54] Wilhelm, D., and Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16,** 1839-51.

[55] Kim, J., Prawitt, D., Bardeesy, N., Torban, E., Vicaner, C., Goodyer, P., Zabel, B., and Pelletier, J. (1999). The Wilms' tumor suppressor gene (wt1) product regulates Dax-1 gene expression during gonadal differentiation. *Mol Cell Biol* **19,** 2289-99.

[56] Kawabe (1999)

[57] 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,** 235-46.

temporal and local expression of *Wt1* in the urogenital ridge, it is most likely that many genes regulated by Wt1 during urogenital ridge development are still unknown. Identification of more Wt1 target genes should help to gain insites into the mechanisms underlying the phenotypes of human patients and mouse models.

1.4 Dmrt8

The mechanism of sex determination is poorly conserved throughout the animal kingdom. Very different mechanisms, like environmental sex determination by factors such as temperature and various ways of genetic sex determination are found. Also the genes involved in sex determination are weakly conserved. However, there is one gene family that seems to be an exception. The *Caenorhabditis elegans* gene *MAB-3*, the *Drosophila melanogaster* gene *doublesex* (*dsx*) and the vertebrate *Dmrt1* (Dmrt = DSX and MAB-3 related transcription factor) share a DNA binding motif termed DM domain and play a role in sex determination of the respective organisms[59]. In human, *DMRT1* is physically clustered with two other genes, *DMRT2* and *DMRT3,* of the same gene family on chromosome 9p24. Mutations and deletions in this region are associated with male-to-female sex reversal[60,61,62]. *Dmrt1* has been suggested to play a role in sexual development in a variety of vertebrate species, including fish, reptiles, birds and mammals[59]. In contrast to the human patients mentioned above, *Dmrt1* knock-out mice show a relatively mild phenotype, with degeneration of seminiferous tubules after birth[63]. This observation led to the idea that, in addition to *Dmrt1,* other *Dmrt* genes might be involved in mouse gonad development. Of six genes (*Dmrt2 - 7*), investigated so far, three (*Dmrt3*, *Dmrt4* and

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[62] Ottolenghi, C., Veitia, R., Quintana-Murci, L., Torchard, D., Scapoli, L., Souleyreau-Therville, N., Beckmann, J., Fellous, M., and McElreavey, K. Ibid.The region on 9p associated with 46,XY sex reversal contains several transcripts expressed in the urogenital system and a novel doublesex-related domain. **64,** 170-8.

[63] Raymond, C. S., Murphy, M. W., O'Sullivan, M. G., Bardwell, V. J., and Zarkower, D. (2000). Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* **14,** 2587-95.

Dmrt7) show an expression pattern consistent with a role in gonadal development. *Dmrt3* is expressed in interstitial cells. *Dmrt4* is expressed in gonads of both sexes and *Dmrt7* in somatic and germ cells in the ovary, but solely in germ cells in testis[64]. Another putative *Dmrt* gene, related to *Dmrt7*, called *DMRTC1/Dmrt8* is present on the X chromosome in human and mouse[65]. The expression pattern of this gene has not been investigated so far, as, in contrast to all other known *Dmrt* genes, the DM domain-encoding sequence of *DMRTC1*/*Dmrt8* is not conserved in human and mouse and it has been doubted if *Dmrt8* genes have the potential to encode functional DM domain-containing proteins in any species[54,55].

1.5 Aim of this work

Aim of this work was to investigate the roles of *Wt1*, *Sox9* and *Dmrt8* in murine gonad development. The obtained results are presented in the three parts of this thesis. In the first part, gene expression in urogenital ridges of $Wt1^{-/-}$ and $Wt1^{+/+}$ was compared, to identify new Wt1 target genes. Further analysis showed that *AmhrII*, one of the identified potential targets, is indeed regulated by Wt1. The second part describes the expression pattern of two newly identified murine *Dmrt8* genes, *Dmrt8.1* and *Dmrt8.2*. The intention was to get an idea whether these genes could be involved in gonad development or gonadal function, in addition to other *Dmrt* genes, in the mouse. In the third part a conditional *Sox9* knock-out mouse, generated by collaboration partners, was used, to investigate the role of Sox9 during sex determination.

[64] Kim, S., Kettlewell, J. R., Anderson, R. C., Bardwell, V. J., and Zarkower, D. (2003). Sexually dimorphic expression of multiple doublesex-related genes in the embryonic mouse gonad. *Gene Expr Patterns* **3,** 77-82.

[65] Ottolenghi, C., Fellous, M., Barbieri, M., and McElreavey, K. (2002). Novel paralogy relations among human chromosomes support a link between the phylogeny of doublesex-related genes and the evolution of sex determination. *Genomics* **79,** 333-43.

2 Materials and Methods

2.1 Materials

2.1.1 Oligonucleotides

PCR-primers were designed using the Primer3 program (http://fokker.wi.mit.edu/cgibin/primer3/primer3_www.cgi) in most cases and all oligonucleotides were synthesized by Metabion (Martinsried) or MWG (Ebersberg). The oligonucleotides used are listed in the appendix.

2.1.2 Plasmids

Already available plasmids used are listed in table 1. Construction of new plasmids is described 2.2.

Table 1. Plasmids.

Name	Description	Source	Reference
pGl3-basic	Promoter-less reporter-vector, containing a multiple cloning site (MCS) 5' of *Photinus pyralis* (Firefly) luciferase cDNA; Ampicillin resistance	Promega, USA	
pGl3-amhrII	Promoter of the anti-Müllerian hormone type II receptor cloned into the MCS of pGl3-basic	Jose Teixeira	Teixeira et al.[66]
phRL-Tk	Expression vector containing *Renilla reniformis*-Luciferase coding sequence under the control of *HSV-TK*-promoter; Ampicillin resistance	Promega, USA	
phRL-CMV	Expression vector containing *Renilla reniformis*-Luciferase coding sequence under the control of *CMV*-promoter, Ampicillin resistance	Promega, USA	
pSEAP2-Control	Expression vector containing *SEAP (secreted alkaline phosphatase)* coding sequence under the control of *SV40* early promoter; Ampicillin resistance	BD Bio-sciences Clontech	
pGEX-KG	Expression plasmid containing gluthathione-S-transferase (GST) coding sequence under the control of an IPTG inducable bacterial promoter and a 3' polycloning site for heterologous expression of		Guan & Dixon[67]

[66] Teixeira, J., Kehas, D. J., Antun, R., and Donahoe, P. K. (1999). Transcriptional regulation of the rat Mullerian inhibiting substance type II receptor in rodent Leydig cells. *Proc Natl Acad Sci U S A* **96,** 13831-8.

[67] Guan, K. L., and Dixon, J. E. (1991). Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* **192,** 262-7.

	GST fusion proteins; Ampicillin resistance		
Gex1	Zinc finger region of *WT1* without the KTS insertion cloned into pGEX-KG to express a GST fusion protein		Wilhelm & Englert[68]
Gex2	Zinc finger region of *WT1* with the KTS insertion cloned into pGEX-KG to express a GST fusion protein		Wilhelm & Englert[68]
Gex3	Like Gex1, harboring an exchange of Arg at position 394 to Trp; Denys-Drash mutant of the zinc finger region of WT1		Wilhelm & Englert[68]
pGex-Sf1	Full length *Sf1* cloned into pGEX-4T-3 (Amersham Pharmacia) to express a GST fusion protein	Dagmar Wilhelm	Schepers et al.[69]
pCRII-TOPO	TA-cloning vector	Invitrogen	
pBstZic1	Fragment of mouse *Zic1* cDNA (1678-2011, d32167) subcloned into *Bam*HI/*Eco*RI-digested pBluescript II KS (-) (Stratagene); suitable for making antisense and sense riboprobes with T3/T7- RNA polymerase after linearization with *Bam*HI/*Eco*RI	Jun Aruga	Aruga et al.[70]
pBS(SK)-G14a	cDNA representing the 5' half of the mouse *Gata4* mRNA subcloned into pBluescript II KS (Stratagene); suitable for making an antisense riboprobe with T7 RNA-polymerase after linearization with *Bam*HI	David B. Wilson	Arceci et al.[71]
Cres	Mouse *Cres* (*Cst8*) cDNA cloned into pBSK II; suitable for making antisense and sense riboprobes with T7/T3- RNA polymerase after linearization with *Eco*RI /*Xho*I	Gail A. Cornwall	Cornwall et al.[72]
Wt1-670	Mouse *Wt1* cDNA (pos. 758-1841) cloned into pBSKII; suitable for making antisense and sense riboprobes with T7/T3- RNA polymerase after linearization with *Bam*HI/*Hind*III		Herzer et al.[73]

[68] Wilhelm, D., and Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16,** 1839-51.

[69] Schepers, G., Wilson, M., Wilhelm, D., and Koopman, P. (2003). SOX8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the Amh promoter in vitro. *J Biol Chem* **278,** 28101-8.

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[71] Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* **13,** 2235-46.

[72] Cornwall, G. A., Orgebin-Crist, M. C., and Hann, S. R. (1992). The CRES gene: a unique testis-regulated gene related to the cystatin family is highly restricted in its expression to the proximal region of the mouse epididymis. *Mol Endocrinol* **6,** 1653-64.

[73] Herzer, U., Crocoll, A., Barton, D., Howells, N., and Englert, C. (1999). The Wilms tumor suppressor gene wt1 is required for development of the spleen. *Curr Biol* **9,** 837-40.

pGTe-Lhx9	Mouse *Lhx9* cDNA (pos. 4-1137) cloned into pGEM-T easy (Promega); suitable for making antisense and sense riboprobes with T7/Sp6- RNA polymerase after linearization with *Sal*I/*Nco*I		Wilhelm & Englert[68]
pcSF1c	Expression vector pcDNA 3.1(+) containing mouse SF1 coding sequence under the control of *CMV*-promoter	Dagmar Wilhelm	
pRc/CMV	Plasmid containing a *CMV*-promoter and a 3' MCS for the construction of expression vectors; Ampicillin resistance	Invitrogen	
pCA	Expression vector pRc/CMV containing mouse Wt1-A (-exon 5, -KTS) coding sequence		Wilhelm & Englert[68]
pCC	Expression vector pRc/CMV containing mouse Wt1-C (-exon 5, +KTS) coding sequence		Wilhelm & Englert[68]
rCHlhx9	Expression vector pRc/CMV containing mouse Lhx9 and HA-epitope coding sequence for expression of a HA-Lhx9 fusion protein		Wilhelm & Englert[68]
pEGFP-C3	Expression vector containing EGFP coding sequence under the control of a *CMV*-promoter and a 3' MCS	BD Bio-sciences Clontech	

2.1.3 Antibodies

Anti WT1 (human, mouse, rat) (C-19), rabbit polyclonal (Santa Cruz Biotechnology)
Anti c-Myc (human) (A-14), rabbit polyclonal (Santa Cruz Biotechnology)
Anti Sf1 (mouse, human, bovine), rabbit polyclonal (Upstate Biotechnology)
Anti Digoxigenin, alkaline phosphatase linked, sheep polyclonal (Roche)

2.1.4 Bacterial strains

The following strains of *Escherichia coli* were used for cloning, plasmid propagation and heterologous protein expression:

DH5α — Recombination defective, suppressive strain for cloning, Genotype: *sup*E44 ΔlacU169 ø80lacZΔM15 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

TOP10F' — Chemically competent cells for high efficiency cloning and plasmid propagation, provided with TOPO TA Cloning Kit,

Genotype: F' {*lac*I^q, Tn*10*(Tet^R)} *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) ø80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen, USA)

ElectroMAX Stbl4 — Electro competent cells for transformation of large and unstable plasmids, Genotype: mcrA Δ(mcrBC-hsdRMS-mrr) recA1 endA1 gyrA96 gal$^-$ thi-1 supE44 λ$^-$ relA1 Δ(lac-proAB)/F' proAB$^+$, (Invitrogen, USA)

BL21 — Strain for expression of fusion proteins, Genotype: F- ompT hsdSB (rB- mB-) dcm gal (DE3) pLysS (Cmr)

2.1.5 Eukaryotic cell lines

Table 2. Cell lines

Name	Description	Reference
M15	mesonephric mouse cell line, high endogenous level of Wt1	Larsson et al.[74]
TM4	Mouse sertoli cell line	Mather et al.[75] Beverdam et al.[76]
UB27	Derivative of the human osteosarcoma cell line U2OS, harboring a tetracycline inducible *Wt1-KTS* allele	Englert et al.[77]
UD28	Derivative of the human osteosarcoma cell line U2OS, harboring a tetracycline inducible *Wt1+KTS* allele	Englert et al.[77]

Cells were kept in 10 cm dishes at 6% CO2, 95% humidity and 37°C and handled under a clean bench. All cell lines were grown in Dulbecco's MEM (DMEM, Invitrogen) with 10% fetal calf serum (FCS).

UB27 and UD28 cells harbor *Wt1* alleles that can be controlled by a *tet* system[78]. As long as these cells are growing in tetracycline containing medium Wt1 expression is

[74] Larsson, S. H., Charlieu, 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.

[75] Mather, J. P. (1980). Establishment and characterization of two distinct mouse testicular epithelial cell lines. *Biol Reprod* **23,** 243-52.

[76] Beverdam, A., Wilhelm, D., and Koopman, P. (2003). Molecular characterization of three gonad cell lines. *Cytogenet Genome Res* **101,** 242-9.

[77] Englert, C., Hou, X., Maheswaran, S., Bennett, P., Ngwu, C., Re, G. G., Garvin, A. J., Rosner, M. R., and Haber, D. A. (1995a). WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. *Embo J* **14,** 4662-75.

suppressed, while upon tetracycline removal Wt1 expression is induced. The cells were propagated 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 with PBS and twice with DMEM containing 10% FCS.

To detach cells, medium was removed and the cells were incubated in 2 ml trypsin/EDTA (Invitrogen) for several minutes at 37°C. Trypsinization was stopped by addition of 8 ml medium. After resuspension cells were pipetted into fresh dishes in a desired dilution. In case that an exact cell number was needed, cells were counted in a Neubauer chamber.

To store cell lines, logarithmically growing cells were trypsinized, centrifugated (300g, 3min), resuspended in freezing medium (DMEM, 20% FCS, 10% DMSO), cooled down slowly to –80°C and placed into liquid nitrogen.

Cells were thawn quickly at 37°C in a waterbath, diluted in medium, centrifugated, resuspended in fresh medium and distributed into fresh cell culture dishes.

PBS: 8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4; per liter in H_2O

2.1.6 Mice

For *Sox9* mutant analysis dissected gonads provided by Francisco Barrionuevo and Gerd Scherer (Institute of Human Genetics and Anthropology, University of Freiburg) were used. To obtain these gonads *Sox9*$^{flox/+}$ mice, originally on a 129P2/OlaHsd x C57BL/6 mixed genetic background[79], had been backcrossed to C57BL/6 for three generations. These N3 mice had been made *Sox9*$^{flox/flox}$ by sister-brother matings and crossed with the *Ck19:Cre* transgenic mouse line[80]. Resultant *Cre/+*; *Sox9*$^{flox/+}$ offspring had been backcrossed to *Sox9*$^{flox/flox}$ mice to obtain *Cre/+*; *Sox9*$^{flox/flox}$ mice. Genotyping for the *Ck19:Cre* allele[81], for the *Sox9* and *Sox9*^{flox} alleles[79] and for *Sry*[82] was also carried out in Freiburg.

[78] Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89,** 5547-51.

[79] Kist, R., Schrewe, H., Balling, R., and Scherer, G. (2002). Conditional inactivation of Sox9: a mouse model for campomelic dysplasia. *Genesis* **32,** 121-3.

[80] Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *Embo J* **18,** 5931-42.

[81] Lecureuil, C., Fontaine, I., Crepieux, P., and Guillou, F. (2002). Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice. *Genesis* **33,** 114-8.

[82] Daneau, I., Pilon, N., Boyer, A., Behdjani, R., Overbeek, P. A., Viger, R., Lussier, J., and Silversides, D. W. Ibid.The porcine SRY promoter is transactivated within a male genital ridge environment. 170-80.

For all other experiments Mf1 wild-type or mice carrying a *Wt1* knock-out allele on an Mf1 background were used[83]. Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated as E0.5. Embryos from E10.5 and E12.5 were staged by counting the number of tail somites (ts) posterior to the hindlimb bud (Fig. 4). 8 ts is approximately E10.5, 18 ts correlates with E11.5 and 30 ts is approximately E12.5[84]. The embryos were genotyped by PCR on proteinase K digested tissue, like tail tips or amnions, using primers Wt1-pgk, Wt1-shared and Wt1-wt for *Wt1*-genotyping and Zfy 5' and Zfy 3' for testing for presence or absence of the Y chromosome (for primers see appendix).

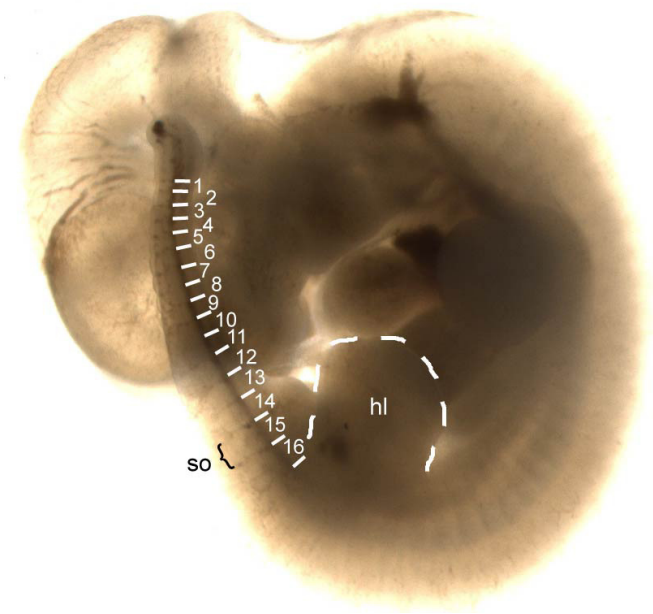

Fig. 4. Tail somites.
From E10.5 to E12.5 embryos can be staged by the number of tail somites. Tail somites are all somites (so) between the tip of the tail and the hindlimb bud (hl).

[83] Herzer, U., Crocoll, A., Barton, D., Howells, N., and Englert, C. (1999). The Wilms tumor suppressor gene wt1 is required for development of the spleen. *Curr Biol* **9,** 837-40.
[84] Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development* **121,** 1603-14.

2.2 Methods

2.2.1 Standard techniques

Molecular biology standard techniques, including plasmid propagation, preparation and analysis of DNA and RNA, enzymatic manipulation of DNA and RNA, analysis of proteins, polymerase chain reaction and protein expression, were performed according to Sambrook et al.[85] or Ausubel et al.[86]

2.2.2 RNA isolation

Total RNA from embryonic tissues was isolated using Absolutely RNA Microprep Kit (Stratagene), including a *DNase* treatment and eluted in 30 µl prewarmed elution buffer.

Total RNA from up to 100 mg of adult tissues was isolated using PeqGold TriFast (PeqLab) and dissolved in 100 µl. RNA concentration was determined with a spectrophotometer and set to 100 ng/µl before reverse transcription.

Total RNA from eukaryotic cell lines was isolated using RNeasy Mini Kit (Qiagen) after homogenization with QIAshredder spin columns (Qiagen) and eluted in a total volume of 80 µl RNase free water. 17µl of RNA were treated with RQ1 RNase-Free DNase (Promega) before reverse transcription.

2.2.3 Microarray analysis

For microarray analysis urogenital ridges (mesonephros + gonad) of five (four female, one male) *Wt1*$^{+/+}$ (wild-type, wt) and *Wt1*$^{-/-}$ (knock-out, ko) embryos with 11 to 12 tail somites were pooled before RNA isolation. To estimate the average amount of RNA isolated by this method, total RNA from 17 pooled pairs of E11.5 urogenital ridges was isolated. The obtained amount was approximately 0.6 µg RNA per embryo. Thus, the wt and ko samples contained approximately 3 µg total RNA. The RNA was sent on dry ice to Amersham Biosciences (now part of GE Healthcare), where the it was used for a proof of performance experiment employing CodeLink

[85] Sambrook, J., and Russell, D. W. (2001). "Molecular Cloning: a laboratory manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

[86] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (2002). "Short protocols in molecular biology: a compendium of methods from Current protocols in molecular biology." John Wiley & Sons, Inc.,

UniSet Mouse 20k microarray slides. Both samples were measured three times. The results provided by Amersham Biosciences included Microsoft Excel worksheets for each of these six measurements named T00232140-T00232142 for the *Wt1*$^{-/-}$ sample and T00232137- T00232139 for the *Wt1*$^{+/+}$ sample. The normalized intensities of all measurements were copied into one Excel worksheet, to calculate mean ko intensities, mean wt intensities and wt/ko ratios. The data provided by Amersham Biosciences also included quality information, like “good”, “near background”, “background contaminated”, “irregular shape” or “saturated”, for the signal of each spot on the microarray slide. To take this quality information into account, the results were preselect according to the quality information for measurements T00232141 and T00232137. Only microarray probes with a “good” signal for at least ko or wt were used for further analysis. Probes with a “good” signal for only one of the two samples were only included, if the second sample showed a “near background” signal.

EASE analysis (http://david.niaid.nih.gov/david/ease.htm) was done to compare all mRNAs with more than three times higher expression in ko and/or wt to a group of 3070 mRNAs with no differential expression (ko/wt = 1±0.1). The mRNA sequences were identified by the accession numbers provided by Amersham Biosciences with the microarray data.

2.2.4 RT-PCR analysis

2.2.4.1 cDNA synthesis

An aliquot of 10 µl RNA was reverse transcribed with SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) and oligo dT primers (Roche) in a total volume of 20 µl. To verify the absence of genomic DNA contamination, a parallel reaction without reverse transcriptase was performed in each case. 1 µl of cDNA was used for subsequent PCR analysis.

2.2.4.2 Polymerase Chain Reaction (PCR)

For a standard PCR an appropriate amount of DNA-template was used with 2.5 µl 10x PCR-Puffer (200mM Tris pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM Primers and 0.5 U *Taq*-Polymerase in a final volume of 25 µl. The Cycling program was started with denaturation at 94°C for 1 min followed by 35 cycles (40

cycles for real-time RT-PCR) including denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30s.

2.2.4.3 Quantitative Real-Time RT-PCR (qRT-PCR)

Whenever possible, primer pairs for RT-PCR covered at least one intron.
qRT-PCR analysis was performed in a 96-well format employing the QuantiTect SYBR green real-time PCR kit (Qiagen) on a Biorad iCycler.
For comparison of gene expression in *Wt1*$^{+/+}$ and *Wt1*$^{-/-}$ urogenital ridges, the standard PCR mix plus 0.75 µl of a 1/2000 dilution of SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications) and 0.25 µl of 1 µM Fluorescein Calibation Dye (BioRad) was used as an alternative to the QuantiTect real-time PCR Mix.
qRT-PCR allows quantification of DNA templates by determination of the so called threshold cycle (C_T). Therefore the intensity of fluorescent light, which is emitted by the DNA staining fluorescent dye SYBR Green I, is measured. The number of amplification cycles which is needed to reach a fluorescent signal significantly higher than background is the C_T. The more templates are present at the beginning of the reaction, the fewer is the number of cycles it takes to reach the C_T. This method can be used to measure gene expression by using cDNA pools as template. Gene expression levels in different samples can be compared by different methods: When same amounts of cDNA are used, expression levels of a certain gene in different samples can be compared by calculating differences of the C_T values (ΔC_T). The factor difference in expression is then $2^{-\Delta CT}$. If different amounts of cDNA are used, the ΔC_T values of a gene of interest can be normalized to a housekeeping gene by subtracting the ΔC_T values ($\Delta\Delta C_T$) for the two genes. The normalized factor difference in expression is then $2^{-\Delta\Delta CT}$ (double delta method) [87]. All these calculations are done under the assumption that the PCR efficiency is 100%. As this is only the case under optimal conditions, a more accurate measurement can be achieved by employing a standard curve: For analysis of *Sox9*, *Amh*, *Foxl2* and *Sry* expression in *Sox9* mutants, a standard curve for each gene was generated, using serial dilutions of a cDNA pool from 16 male embryos with 13-15 tail somites. A serial dilution of adult testis cDNA was used for determination of *Dmrt8*A expression patterns and *L-*

[87] Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25,** 402-8.

Ptgds expression in *Sox9* mutants. Expression levels were determined in one plate for all samples simultaneously and normalized to the corresponding amounts of *Tbp* (TATA box binding protein) cDNA measured within the same plate.

2.2.5 RNA *in situ* hybridization

2.2.5.1 Generation of digoxigenin-labeled riboprobes

For generation of digoxygenin labeled riboprobes plasmids containing cDNA fragments of the respective gene were linearized by restriction and purified employing QiaExII (Qiagen) or phenol-chloroform extraction. 1-4 µg purified plasmid were used for transcription with T3, T7 or Sp6 RNA-Polymerase (Roche) in presence of DIG RNA Labeling Mix (Roche) and rRNasin (Promega).

Plasmids containing fragments recognizing mouse *Wt1* and *Lhx9* mRNA existed in the lab (see table 1). cDNA fragments of *Dbx1*, *Gdf8*, *Dmrt2*, *Cbln1*, *Muc16*, *Ptprv*, *Neo* and *AmhrII* were amplified by PCR (Primers see Appendix) and cloned into pCRII-TOPO with the TOPO TA Cloning Kit (Invitrogen). A plasmid containing Aldh1a1 cDNA sequence was provided by Shahidul Makki, a member of our lab. For *Dmrt8.1* a 610 bp cDNA fragment and for *Dmrt8.2* a 609bp cDNA fragment from testis, subcloned into pBluescript II KS^+ by Anne-Marie Veith (Department of Physiological Chemistry I, University of Würzburg) were used. Plasmids suitable for the generation of *Zic1*, *Gata4* and *Cst8* riboprobes were kind gifts from other labs (see table 1).

2.2.5.2 RNA *in situ* hybridization on paraffin sections

In situ hybridizations on paraffin sections were performed using digoxigenin-labeled (Roche) antisense and sense riboprobes according to Leimeister et al.[88] with some modifications. Briefly, the respective embryos or tissue samples were dissected in PBS and fixed in PFA/PBS. Tissues were washed in PBS and 0.9% NaCl, dehydrated in a graded isopropanol or ethanol series, infiltrated with chloroform or xylol and embedded in paraffin. Sections of 10 µM were mounted on poly-lysine coated slides (Menzel), dried on a heating plate at 40°C and stored at 4°C.

Sections were dewaxed in chloroform, rehydrated in a graded ethanol series, washed in PBS, refixed for 30 min in 4% PFA/PBS, digested with 10 µg/ ml proteinase K for

[88] Leimeister, C., Bach, A., and Gessler, M. (1998). Developmental expression patterns of mouse sFRP genes encoding members of the secreted frizzled related protein family. *Mech Dev* **75,** 29-42.

10 min, washed and fixed again. After washing in PBS, 2x SSC and Tris/glycine buffer, sections were hybridized with 0.1 µg/ml digoxygenin labeled probes in hybridization buffer for 16-20 h at 70°C under plastic foil in a 5x SSC humified chamber. Following hybridization, sections were washed three times for 20 min in 5x SSC at room temperature, 1 h in 0.5x SSC/20% formamide at 60°C, 15 min at 37°C in RNase buffer and treated with 10 µg/ml *RNase A* for 30 min. After washes in RNase buffer for 15 min at 37°C, in 0.5x SSC/20% formamide for 30 min at 60°C and in 2x SSC for 30 min at room temperature, sections were blocked with 1% blocking reagent (Roche) in MABT and incubated with anti-digoxygenin antibodies (Roche) at a dilution of 1:5000 for 16-20 h at 4°C. Sections were washed five times for 10 min in TBST, once for 10 min in NTMT, 10 min in NTMT containing 2 mM levamisol and then developed for 1-5 days in BM-purple substrate (Roche) containing 2 mM levamisol and 0.1% Tween 10. After staining sections were washed twice in NTMT for 15 min and 10 min in PBS. Slides were mounted in Kaisers glycerin gelatine (Merck).

PBS:	8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4; per liter in H_2O
PFA/PBS:	4% (w/v) paraformaldehyde in PBS
Proteinase K buffer:	20 mM Tris pH 7.5; 1 mM EDTA
1x SSC:	0.15 M NaCl; 15 mM tri-sodium citrate; pH 5
Tris/glycine buffer:	0.1 M Tris-HCl; 0.1 M glycine
Hybridization buffer:	50% formamide; 1.3x SSC pH 5; 5 mM EDTA; 0.5% CHAPS; 100 µg/ml heparin; 0.2% Tween 20; 100 µg/ml torula yeast RNA
RNase buffer:	0.5 M sodium chloride; 10 mM Tris-HCl pH7.5; 5 mM EDTA
MABT:	0.1 M maleic acid; 0.15 M NaCl; 0.1% Tween 20; pH 7.5
TBST:	140 mM sodium chloride; 2.7 mM potassium chloride; 25 mM Tris-HCl pH 7.5; 0.1% Tween 20
NTMT:	10 mM sodium chloride; 50 mM magnesium chloride; 100 mM Tris-HCl pH 9.5; 0.1% Tween 20

2.2.5.3 Whole mount *in situ* hybridization (WISH)

PFA-fixed embryos were washed in PBS, dehydrated in a graded methanol series and stored at -20°C. Before hybridization embryos were rehydrated in a graded methanol series, washed in PBT, bleached with 6% H_2O_2 in PBT for 1 h, washed again, treated with 20 µg/µl proteinase K in PBT for 3 min, washed with PBT, RIPA buffer and again PBT and refixed for exactly 20 min in 4% PFA/PBT with 0.2% glutaraldehyde. After washing in PBT, 1:1 PBT/hybridization buffer and hybridization

buffer alone, embryos were prehybridized in hybridization buffer containing 100 µg/ml torula yeast RNA for 1-3 h at 70°C and hybridized with 0.1 µg/ml digoxygenin labeled probes in hybridization buffer for 16-20 h at 70°C in glas vials. Following hybridization, embryos were washed in hybridization buffer at 65°C for 30 min three times, followed by 30 min RNase solution at 37°C and incubated in 100 µg/ml RNaseA at 37°C for 30 min two times. After washes in RNase solution, in hybridization buffer at 65°C and in 1:1 hybridization buffer/TBST at 65°C for 30 min, in TBST for 10 min at room temperature two times and in MABT for 10 min at room temperature two times, embryos were blocked with 10% blocking reagent (Roche) in MABT for 1 h and incubated with anti-digoxygenin antibodies (Roche) at a dilution of 1:5000 in MABT containing 1% blocking reagent for 16-20 h at 4°C. Embryos were washed several times in MABT for 1-2 days, followed by washing in NTMT two times 10 min, incubation for 10 min in NTMT containing 2 mM levamisol and stained for 1-14 days in BM-purple substrate (Roche) containing 2 mM levamisol and 1% Tween 20. After staining embryos were washed twice in NTMT for 10 min, fixed and stored in 4% PFA/PBS.

PBT:	PBS containing 0.1% Tween 20
RIPA buffer:	0.1% SDS, 150 mM NaCl; 1% NP40, 0.5% Deoxycholate; 1 mM EDTA; 50 mM Tris pH 8
RNase solution:	0.5 M sodium chloride; 10 mM Tris-HCl pH7.5; 0.1% Tween 20

For additional buffers see 2.2.5.2.

2.2.6 siRNA

The protocol used for knock-down of *Wt1* by RNA interference was established by Ralph Sierig within his diploma work[89].

Briefly, siRNAs were synthesized employing the Silencer siRNA Construction Kit (Ambion) including control templates for synthesis of a control siRNA against *Gapdh*. The oligonucleotides Wt1si5 and Wt1si6 were used as templates for synthesis of a siRNA directed against *Wt1*.

$3x10^5$ M15 cells were split into 6 cm dishes and transfected 24h later applying 15 nM siRNA and 10 µl siLentFect Lipid Reagent (Bio-Rad) according to the manufacturer. Medium was changed 4 h post transfection. Cells were harvested for RNA isolation about 55 h after transfection.

[89] Sierig, R. (2005). Die Rolle von Wt1 in der Regulation der AmhrII-Expression, Vol. Diploma Thesis. University of Würzburg.

2.2.7 Luciferase reporter assay

2.2.7.1 Generation of *Gata4* promoter constructs

To obtain a *Gata4* promoter fragment, colony macroarrays containing a genomic mouse cosmid library (RZPD number 121, strain 129/ola) were screened with a cDNA probe containing exon 1 and part of exon 2 of murine *Gata4.* Five clones were identified and further characterized by restriction and Southern analysis. Of these five clones one (MPMGc121F03498Q) contained approximately 34 kb *Gata4* 5' upstream sequence. This clone was used to obtain a 10.6 kb *KspI-KspI* fragment that harbored half of exon1 and additional 5' flanking region of *Gata4* (-10,534 to +72 from transcriptional start site). This fragment was treated with Klenow enzyme and ligated into the *SmaI* site of the pGl3-basic vector (Promega) and propagated using Stbl4 cells. Constructs containing 5 kb, 2.5 kb, 2.2 kb and 0.5 kb of the *Gata4* 5' region were obtained by religation of the 10.6 kbp construct after digestion with *NruI* and *PacI*, *MluI*, *KpnI* or *SacI*.

2.2.7.2 Generation of a *Gata4* expression construct

Gata4 coding sequence was amplified using primers *gata4-EcoRI* and *gata4-ApaI* with adult murine heart cDNA as a template. The PCR product was gel purified employing QIAEX II gel extraction kit (Qiagen) and cloned into pGemT easy vector (Promega). Digestion of this construct with *EcoRI* and ligation into pcDNA3.1 containing an N-terminal hemaglutinin (HA) tag lead to the HA-Gata4 expression construct. Integrity and expression of this construct was verified by sequencing and immunofluorescence. All other expression constructs used for cotransfection experiments were existing in the lab (Table 1).

2.2.7.3 Luciferase reporter assay

Transfection of murine cell lines was performed using Superfect transfection reagent (Qiagen) in a 24 well format. Cells were split at 2-10x10^4 TM4 cells or 2x10^4 M15 cells per well and transfected 24 h later with 750-800 ng expression construct, 150-200 ng promoter reporter construct and 10 ng control plasmid, according to the instructions of the manufacturer.

Firefly and *Renilla* luciferase activity were measured 48h after transfection using the Dual-Luciferase Assay Kit (Promega) as described by the manufacturer on a Mithras LB 940 plate reader (Berthold).

2.2.8 Electrophoretic mobility shift assay (EMSA)

EMSA analysis was performed as described by Wilhelm and Englert[90] using recombinant, bacterially expressed GST fusion proteins of the zinc finger region of WT1 with (Plasmid: Gex2, see table 1) or without (Gex1) the KTS insertion, a Denys-Drash mutant harboring an exchange of Arg at position 394 to a Trp residue[91] (Gex3) and full-length Sf1 (pGex-Sf1).

AmhrII promoter fragment probes were amplified by PCR and gel-purified employing QIAEX II gel extraction kit (Qiagen) or ordered as oligonucleotides for both strands and annealed. Fragments as well as oligonucleotide probes were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase for 1h at 37°C and subsequently purified using Nick columns (Amersham). Binding reactions were performed in a final volume of 30 µl in binding buffer. Reaction mixtures, containing approximately 20 ng of purified protein, were preincubated for 10 min at room temperature followed by the addition of 3000 cpm of end-labeled probe. After a 30 min incubation at room temperature, DNA-protein complexes were resolved by electrophoresis on 8% polyacrylamide gels (containing 2.5% glycerol) at 150V for 2 h in 0.5x TBE buffer.

Binding buffer:	100 mM KCl; 1 mM $MgCl_2$; 10 µM $ZnSO_4$; 10 mM Tris pH 7.5; 4% glycerol; 0.1% Triton X-100; 1 mg/ml BSA; 1 µg of poly(dIdC)/poly(dAdT); 0.5 mM DTT
0.5x TBE buffer:	90 mM Tris; 90 mM boric acid; 2,5 mM EDTA

2.2.9 Chromatin immunoprecipitation (ChIP)

UB27 inducible cells were grown to 80-90% confluence in 15 cm cell culture dishes. For Wt1 expression, cells were induced 24h before fixation, by removal of tetracycline. Cells were fixed by addition of a 33.75% formaldehyde/10% methanol stock into the cell culture medium to a final volume of 1% formaldehyde and incubation for 10 min. Fixation was stopped by addition of Glycine to a final concentration of 0.1425 M and incubation for 5 min. After removal of medium and two

[90] Wilhelm, D., and Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16,** 1839-51.

[91] 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., and et al. (1991a). Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* **67,** 437-47.

washings with PBS, cells were harvested by scraping in 1.5 ml L1-buffer. To disrupt the cell membranes efficiently, this solution was sucked through a 27G needle for seven times on ice. After centrifugation with 3000 rpm at 4°C in a tabletop centrifuge for 5 min, the resulting nuclei were resuspended in 800 µl L2-buffer. Genomic DNA was sheared by sonication (50% intensity, ten 15 s pulses, 30 s pause) on ice. After removal of nuclear debris by centrifugation at 13000 rpm and 4°C for 10 min, the solution was distributed into 180 µl aliquots. Aliquots which were not needed immediately were stored at -80°C. For IPs 180 µl aliquots were diluted 1:10 in dilution buffer. One aliquot was stored as input control. The lysates were precleared by incubation with 80 µl proteinA-agarose for 2-3 h on a rotator at 4°C. After removal of proteinA-agarose by centrifugation with 10000 rpm at 4°C for 1 min and addition of 2 µl antibody (see 1.1.3), immunoprecipitation was performed on a rotator in the cold room over night.

Immunocomplexes were collected with 30 µl proteinA-agarose for 1 h at 4°C. After centrifugation (800 rpm, 4°C, 1 min), removal of the supernatant, washing in 1 ml washing buffer and TE three times each, immunocomplexes were eluted with 300 µl EB at 65°C in a shaker for 15 min. A second elution step with 250 µl EB was performed after centrifugation (5000 rpm, 2 min) and transfer of 250 µl EB into a fresh tube. Crosslinking was reversed by addition of 30 µl 5 M NaCl to the combined eluates and over night incubation at 65°C in a shaker. Stored input controls were diluted to 500 µl with EB and treated like IP samples from this step onwards.

The isolated DNA was purified by treatment with 40 µg proteinase K at 50°C for 2 h, phenol/chloroform extraction and ethanol precipitation. The DNA was dissolved in 100 µl water and used as template for PCR analysis. Efficiency of DNA shearing was checked by analyzing part of the input controls on a 1.5% agarose gel.

L1-buffer:	50 mM Tris pH 8.0; 2 mM EDTA pH 8.0; 0.1% NP-40; 10% glycerol; proteinase inhibitor cocktail
L2-buffer:	50 mM Tris pH 8.0; 1% SDS; 5 mM EDTA; proteinase inhibitor cocktail
Dilution buffer:	50 mM Tris pH 8.0; 0.5 mM EDTA, 0.5% NP-40; 200 mM NaCl; proteinase inhibitor cocktail
Washing buffer:	20 mM Tris pH 8.0; 2 mM EDTA; 0.1% SDS; 1% NP-40; 500 mM NaCl
TE:	10 mM Tris pH 8.0; 1 mM EDTA
Elution buffer (EB):	10 mM Tris pH 8.0; 1 mM EDTA; 1% SDS

3 Results

3.1 Wt1

3.1.1 Microarray analysis indicates significant changes of gene expression in urogenital ridges of Wt1$^{-/-}$ mouse embryos

To analyse differences in gene expression between *Wt1*$^{+/+}$ (hereafter termed wild-type or wt) and *Wt1*$^{-/-}$ (hereafter termed knock-out or ko) urogenital ridges, RNA was isolated from pooled urogenital ridges of five wt and five ko E11.5 embryos with 11-12 tail somites (ts), a stage immediately before the beginning of sex differentiation. Microarray analysis, with three measurements for each of the two RNA samples, was performed by Amersham Biosciences. The results contained information about 20289 mRNAs. After exclusion of results with bad quality, mostly due to low expression in wild-type and knock-out, data for 12043 mRNAs remained. Out of these mRNAs, 142 showed differences in expression level between knock-out and wild-type higher than factor three. 100 of those showed higher expression in wt urogenital ridges and 42 showed higher expression in ko urogenital ridges (see supplementary data).

To get an idea about the functions of Wt1 regulated genes, an EASE analysis of the microarray results was performed (http://david.niaid.nih.gov/david/ease.htm). With this bioinformatics tool database information on a group of mRNAs can be compared to a reference group, regarding the three categories: biological function, molecular function and cellular localization. Using this method, genes with higher expression in ko and/or wt urogenital ridges were compared to a group of 3070 mRNAs, which showed no differential expression in the microarray analysis (ko/wt = 1±0.1). In table 3 the four best hits in each category are shown. The results of this analysis show that genes with higher expression in ko urogenital ridges have mainly developmental functions and code for proteins with hormone or transcription factor activity, whereas most of the genes with higher expression in wt urogenital ridges code for proteins with enzymatic functions and proteins located in membranes or extracellular space. Interestingly, genes involved in muscle development and neurogenesis, two tissues neighbouring the gonads, seem to be affected especially in ko urogenital ridges.

	higher expression in wt	higher expression in wt or ko	higher expression in ko
biological function	hormone metabolism	hormone metabolism	muscle development
	catabolism	development	development
	proteolysis and peptidolysis	neurogenesis	organogenesis
	one-carbon compound metabolism	cell adhesion	cellular process
molecular function	metallopeptidase	hormone activity	transcription factor activity
	intramolecular isomerase	intramolecular isomerase	hormone activity
	exopeptidase activity	metallopeptidase	binding
	protease inhibitor	transcription factor activity	DNA binding
cellular component	extracellular space	extracellular space	transcription factor complex
	plasma membrane	extracellular matrix	nucleoplasm
	extracellular matrix	transcription factor complex	extracellular
	apical plasma membrane	apical plasma membrane	muscle fiber

Table 3. Ease analysis results.
EASE analysis results for three different data sets; genes with more than three times higher expression in ko and/or wt were compared to a group of 3070 genes with a wt/ko ratio =1±0.1. For each comparison the four best hits for biological function, molecular function and cellular localization are shown.

3.1.2 Microarray analysis and real-time RT-PCR lead to similar results

To get an idea about the biological significance of the microarray data, a second method, quantitative real-time RT-PCR (qRT-PCR), was used.

The qRT-PCR method was established using the known gonadal Wt1 target genes *Sf1*[92] and *Sry*[93,94] together with the housekeeping genes *Actb* (beta actin), *Tbp* (TATA box binding protein), *Hprt* (hypoxanthine guanine phosphoribosyl transferase) and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). To minimize the number of embryos needed, pairs of urogenital ridges of single embryos were used. As shown in Fig 5 the Wt1 target genes showed clear differences in expression in ko and wt

[92] Wilhelm, D., and Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16,** 1839-51.
[93] Hossain, A., and Saunders, G. F. (2001). The human sex-determining gene SRY is a direct target of WT1. *J Biol Chem* **276,** 16817-23.
[94] Hammes, A., Guo, J. K., Lutsch, G., Leheste, J. R., Landrock, D., Ziegler, U., Gubler, M. C., and Schedl, A. (2001). Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. *Cell* **106,** 319-29.

urogenital ridges from single embryos, while no differences were observed for the housekeeping genes (only Tbp is shown).

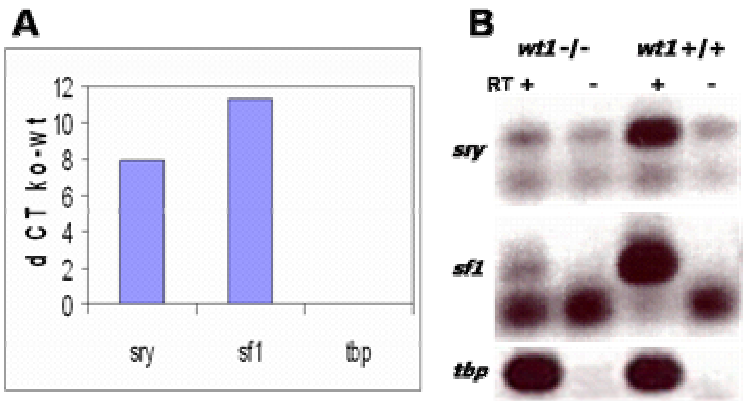

Fig. 5. Differential expression of *Sry* and *Sf1* in *Wt1*$^{-/-}$ and *Wt1*$^{+/+}$ urogenital ridges detected by qRT-PCR.
Differential expression of the known Wt1 dependent genes *Sry* and *Sf1* in wt and ko urogenital ridges of single male embryos with 19 ts measured by qRT-PCR. The results are shown as differences of the threshold cycles (ΔC_T values, see 1.2.4.3) in a bar diagram (A) and as DNA bands on an 1.5% agarose gel (B). ΔC_T values were normalized to *Tbp*. While Tbp is unchanged, *Sry* and *Sf1* show more than 8 cycles difference between Wt1$^{-/-}$ and Wt1$^{+/+}$ urogenital ridges. RNA samples that were not treated with reverse transcriptase (RT) were used as negative controls.

After having shown, that qRT-PCR is a reliable method to compare gene expression in *Wt1* wt and ko urogenital ridges, expression levels of several genes, found in the microarray results, were determined. For some of these genes only qRT-PCR results of bad quality, due to weak or unspecific signals, most likely caused by unsuitable primer pairs, were obtained. In table 4 only genes showing good quality qRT-PCR results, are listed. The list contains part of the top 13 upregulated genes in ko embryos (*Zic1* to *Dmrt2*), the top 12 upregulated genes in wt embryos (*AmhrII* to *Aldh1a1*), genes known to be involved in muscle development, an aspect that showed up in the EASE analysis, (*Myf5*, *Gdf8*, *En1*), genes known or thought to be involved in gonad development (*Gata4*, *Sf1*, *Wnt4*, *Vnn1*, *Gata6*, *Dax1*, *Lhx9*, *Cbln1*, *Aldh1a1*, *AmhrII*) and housekeeping genes as negative controls (*Actb*, *Tbp*, *Hprt*). Overall, a concordance between the microarray analysis and qRT-PCR data sets was found in about 70% of the analysed genes. However, several genes showed differences in expression levels detected with one of the two methods and no difference detected with the other method. There is also a group of three genes, that shows higher expression in ko according to the microarray analysis, but higher expression in wt according to the qRT-PCR results. For *Sf1,* the microarray results were false negative, as reduced expression of *Sf1* in ko gonads was shown before. For all other genes without concordance differences are as yet unexplained.

Name	Description	Microarray	qRT-PCR	
Zic1	zinc finger protein of the cerebellum 1 (Zic1), mRNA	**0,05**	**0,11**	+
Tnnc2	troponin C2, fast (Tnnc2), mRNA	**0,05**	**3,48**	-
AI426026	AI426026.1 mg47f12.x1 Soares embryo NbME13.5 14.5 cDNA clone	**0,07**	**0,04**	+
Gdf8	growth differentiation factor 8 (Gdf8), mRNA	**0,08**	**0,11**	+
Tlx1	T-cell leukemia, homeobox 1, mRNA	**0,15**	**8,00**	-
Dmrt2	doublesex and mab-3 related transcription factor 2 (Dmrt2), mRNA	**0,16**	**0,11**	+
Myf5	myogenic factor 5 (Myf5), mRNA	**0,21**	**12,55**	-
En1	engrailed 1 (En1), mRNA	**0,33**	**0,00**	+
Actb	actin, beta, cytoplasmic (Actb), mRNA	0,48	1,00	+
Tbp	TATA box binding protein (Tbp), mRNA	1,00	0,57	+
Gata4	GATA binding protein 4 (Gata4), mRNA	1,04	**11,31**	o
Hprt	hypoxanthine guanine phosphoribosyl transferase (Hprt), mRNA	1,29	1,23	+
Sf1	nuclear receptor subfamily 5, group A, member 1 (Nr5a1, Sf1), mRNA	1,32	**630,35**	o
Wnt4	wingless-related MMTV integration site 4 (Wnt4), mRNA	2,14	**3,48**	o
Vnn1	vanin 1 (Vnn1), mRNA	2,21	0,50	+
Gata6	transcription factor GATA-6 mRNA, partial cds	2,34	2,64	+
Dax1	nuclear receptor subfamily 0, group B, member 1 (Nr0b1, Dax1), mRNA	**3,48**	**39,40**	+
Lhx9	LIM homeobox protein 9 (Lhx9), mRNA	**3,89**	1,41	o
H19	H19 fetal liver mRNA (H19), mRNA	**5,54**	2,46	o
Cbln1	cerebellin 1 precursor protein (Cbln1), mRNA	**6,63**	**42,22**	+
Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1), mRNA	**8,01**	**19,70**	+
Cst9	cystatin 9 (Cst9), mRNA	**8,24**	**6,50**	+
Tac2	tachykinin 2 (Tac2), mRNA	**8,44**	**14,93**	+
AK003577	AK003577.1 (18-day embryo whole body cDNA, RIKEN clone:1110008I14)	**13,02**	**776,05**	+
Tulp2	tubby-like protein 2 (Tulp2), mRNA	**13,39**	**11,31**	+
Ptprv	protein tyrosine phosphatase, receptor type, V (Ptprv), mRNA	**15,42**	**24,25**	+
Cd59a	CD59a antigen (Cd59a), mRNA	**16,27**	1,74	o
Slc9a3r1	solute carrier family 9, isoform 3 regulator 1 (Slc9a3r1), mRNA	**19,75**	0,66	o
NM_027582	NM_027582.1 (RIKEN cDNA 4921521F21 gene (4921521F21Rik), mRNA)	**19,76**	**36,76**	+
Cst8	adult male testis cDNA, RIKEN clone:1700108L10 product:cystatin 8	**23,23**	**362,04**	+
Neo	L0208E07-3 NIA Newborn Ovary cDNA Library cDNA clone	**35,65**	**274,37**	+
Amhrll	anti-Mullerian hormone type 2 receptor (Amhr2), mRNA	**63,89**	**194,01**	+

Table 4. Comparison of microarray and qRT-PCR results.
The expression levels of several genes in wild-type and *Wt1*-mutant gonads were compared on the basis of the microarray analysis and qRT-PCR data. The former are expressed as the ratio of the signal intensities in wild-type (wt) and mutant (ko) situation, respectively. The foldness of difference in the qRT-PCR experiments were calculated from the difference of the Ct values (see 1.2.4.3). qRT-PCR was performed using cDNAs from wt and ko embryos with the same number of tail somites, ranging from 9 to 13 ts (E10.5-E11.0). Most genes were measured several times, leading to virtually identical results. Only one experiment is listed for each gene. Genes are sorted according to the microarray results and divided into three groups. The top group contains genes with higher expression in ko compared to wt urogenital ridges. In the middle genes with no difference and in the bottom genes with higher expression in wt urogenital ridges are listed. The threshold defining differential expression was set to factor 3. Numbers above this threshold are printed in bold style. “+” in the last column indicates that microarray analysis and qRT-PCR lead to similar results; “o” marks genes that show differential expression measured with one method but no difference with the second method and “-” indicates higher expression in ko according to microarray analysis, but higher expression in wt according to qRT-PCR.

3.1.3 RNA *in situ* hybridization demonstrates reduced expression of *Amhrll*, *Ptprv* and *Gata4* in *Wt1*$^{-/-}$ gonads

Microarray and qRT-PCR are based on the usage of isolated urogenital ridges. Thus, for both methods contamination with cells from other tissues can not be completely excluded and both methods provide no information on the part of the urogenital ridge, in which the respective gene is expressed. A method that gives an idea about intensity and location of gene expression at the same time is RNA *in situ*

hybridization. To verify microarray analysis and qRT-PCR results, RNA *in situ* hybridization, using probes against the genes listed in table 5, was performed.

Gene	Specific signal	Difference
Zic1	+	-
AI426026 (Dbx1)	-	n.d.
Gdf8	+	-
Dmrt2	+	-
Cbln1	(+)	n.d.
Aldh1a1	?	n.d.
AK003577 (Muc16)	-	n.d.
Ptprv	+	+
Cst8	?	n.d.
Neo	-	n.d.
AmhrII	+	+
Gata4	+	+
Lhx9	+	(+)

Table 5. Genes analysed by RNA *in situ* hybridization.
Genes analysed by RNA *in situ* hybridization are listed with respect to signal specificity. If a specific signal could be detected expression in ko and wt embryos was compared. The genes are divided into three groups, according to the microarray analysis and qRT-PCR results: genes with higher expression in ko (top), genes with higher expression in wt (middle) and genes with ambiguous results (bottom). n.d.: No knock-out embryo was analysed for this gene.

Before comparing gene expression in wt and ko embryos all probes were tested by RNA *in situ* hybridization on paraffin sections of *Wt1* wild-type or heterozygous embryos in antisense and sense orientation to make sure that a specific signal is detected (Table 5). Specific signals were observed for probes against *Zic1*, *Gdf8*, *Dmrt2*, *Cbln1*, *Ptprv*, *AmhrII*, *Gata4* and *Lhx9* mRNA and resembled the published expression patterns of these genes, if available[95,96,97,98,99]. No expression of *Dbx1*, *Muc16* and *Neo* could be detected and signals for *Aldh1a1* and *Cst8* were very weak and not reproducible. Gonadal expression of *Cbln1* was detected only after sex determination, but not at stages used for microarray analysis and qRT-PCR analysis. All genes that showed a specific signal were analysed on paraffin sections of wt or heterozygous and ko embryos (Table 5). For all those genes that showed higher expression in ko according to microarray and qRT-PCR analysis (Table 5, top panel) no signal could be detected in the region of *Wt1* expression (Fig. 6A), neither in ko

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nor in wt/heterozygous embryos (data not shown). Expression was only detected in areas neighbouring the urogenital ridge, in the region of the somites and the neural tube, as expected for the respective gene. All other genes analysed showed clear expression in the gonadal part of the urogenital ridge of wt/heterozygous embryos, but reduced expression in ko embryos (Fig. 6B-I). Gonadal expression of *Lhx9* was only slightly reduced and still clearly detectable, as described before[92] and resembling the microarray analysis and qRT-PCR results (Fig. 6D, E). The persistence of Lhx9 expression demonstrates, that the gonadal part of the urogenital ridge is still existing in ko embryos, although the size may be reduced, even as early as at the 9 ts stage (approximately E10.5). Expression of *Gata4* was highly reduced with only very faint expression in the gonads and the dorsal mesentery of ko embryos (Fig. 6B, C) and *Ptprv* and *AmhrII* expression was not detectable at all in ko gonads (Fig. 6F-I). Differential expression of these four genes was also confirmed by whole mount *in situ* hybridization, as shown for *AmhrII* as an example (Fig. 6J, K).

In summary, only differential expression of genes with higher expression in wt urogenital ridges could be confirmed by RNA *in situ* hybridization. Expression of *Ptprv* and *AmhrII* is completely lost. *Gata4* shows highly reduced expression in gonads from *Wt1* knock-out mice, while *Lhx9* expression is only slightly reduced. After having confirmed the reduced expression of these genes in the *Wt1*$^{-/-}$ gonads with RNA *in situ* hybridization, *Gata4* and *AmhrII* seemed to be the most interesting candidates as they show dependence on Wt1 and have been shown to be important for sexual development[100,101].

Fig. 6. Analysis of *Wt1*, *Gata4*, *Lhx9, Ptprv* and *AmhrII* expression in Wt1$^{+/+,+/-}$ and Wt1$^{-/-}$ embryos by RNA *in situ* hybridization.
Analysis of the expression of *Wt1* (A), *Gata4* (B,C), *Lhx9* (D, E), *Ptprv* (F, G) and *AmhrII* (H-K) in urogenital ridges of *Wt1* wt (A, B, D, F) or heterozygous (H, J) and ko (C, E, G, I, K) embryos (E10.5-E11.5) with 6 tail somites (ts) (A-C), 9 ts (D-G), 14ts (H), 13 ts (I), 15 ts (J) or 16 ts (K) by RNA *in situ* hybridization on paraffin sections (A-I) and whole urogenital ridges (J, K). Each gene shows clear expression in the gonadal part of the urogenital ridge of wt and heterozygous embryos indicated by a blue staining (arrows in J). In ko gonads the expression is reduced to different extents. For each gene at least seven sections for at least two wt or heterozygous and two ko embryos with different numbers of tail somites have been analysed. Blue staining outside the urogenital ridge region is background staining, as similar staining was also found in sense controls (data not shown). The blue spots outside the gonadal ridge in F and G are most likely due to overstaining.

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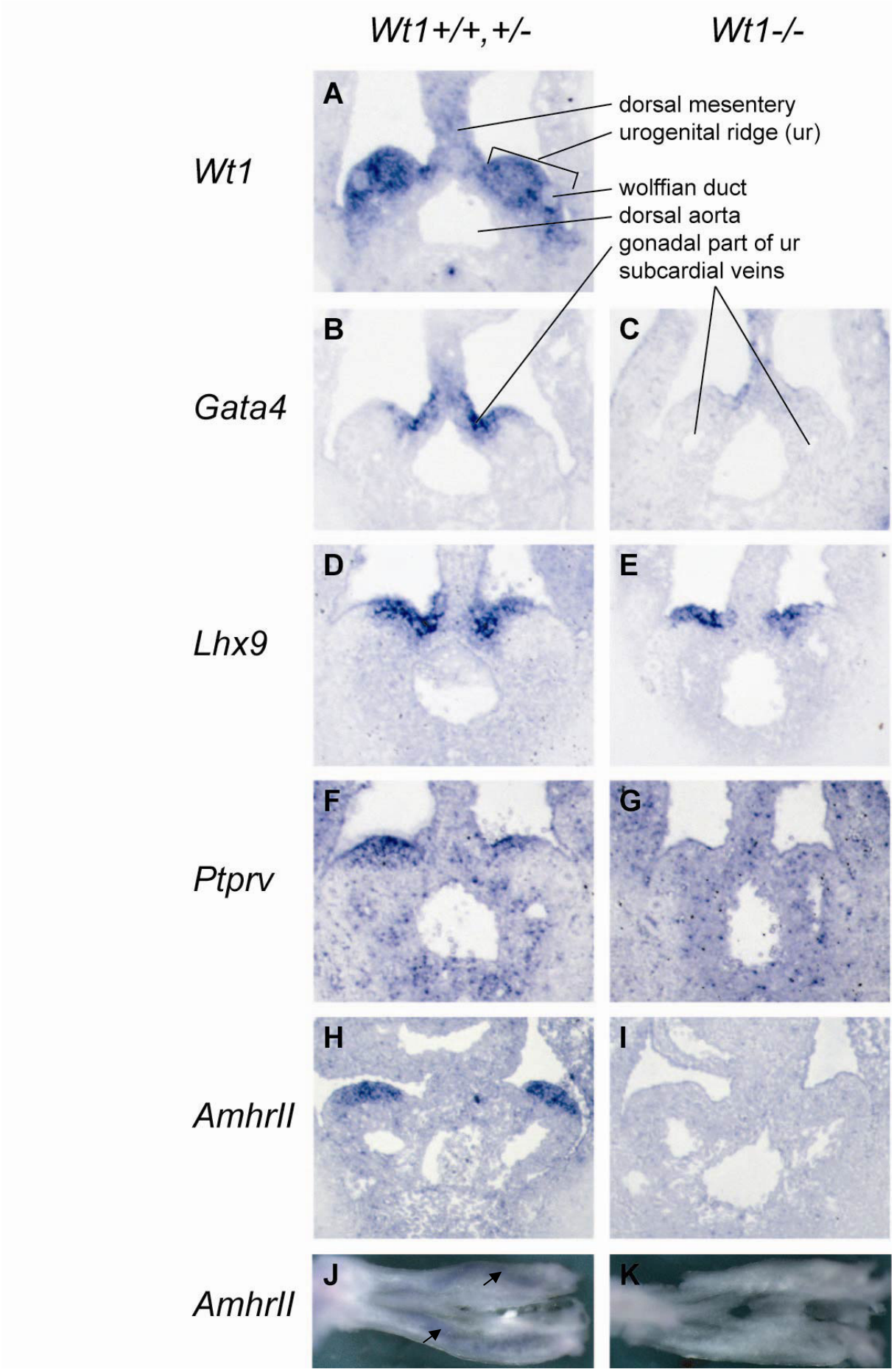
Wt1+/+,+/-
Wt1-/-
Wt1
Gata4
Lhx9
Ptprv
AmhrII
AmhrII
A
B
C
D
E
F
G
H
I
J
K
dorsal mesentery
urogenital ridge (ur)
wolffian duct
dorsal aorta
gonadal part of ur
subcardial veins

3.1.4 *Gata4* promoter constructs are activated by Sf1 and Gata4, but not Wt1

In order to investigate the influence of Wt1 on the *Gata4* promoter, a *Gata4* promoter construct was needed. Therefore a genomic mouse library was screened and a 10.6 kb fragment of *Gata4* (-10,534 to +72 from transcriptional start site), from one of the clones identified, was cloned into pGl3-basic vector (Promega).

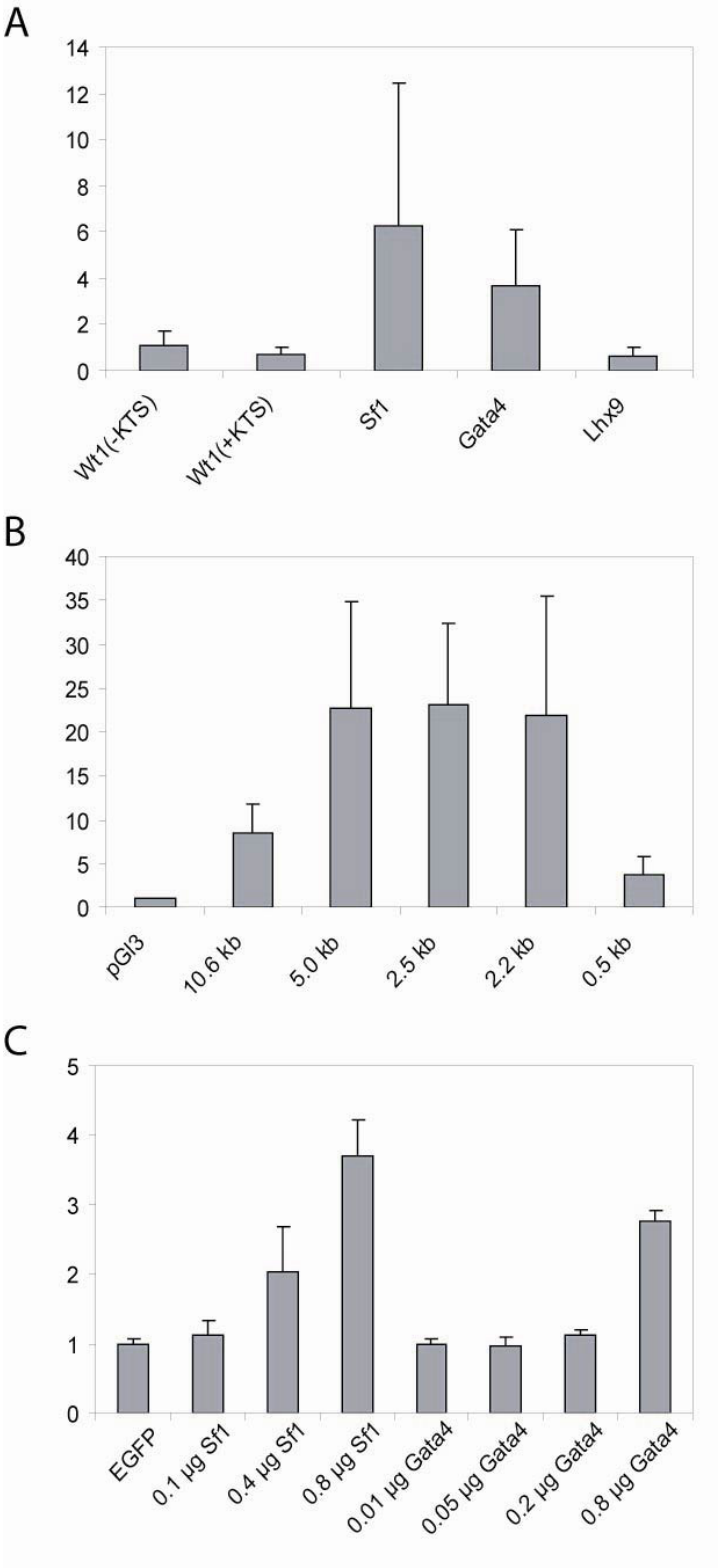

Fig. 7. Activation of the *Gata4* promoter by Sf1 and Gata4 in TM4 cells.
(A) Activation of the 10.6 kb *Gata4* promoter construct by Sf1 and Gata4, but not Wt1(-KTS), Wt1(+KTS) or Lhx9. Results are given as relative activation compared to the reporter construct cotransfected with the empty expression vector pRcCMV, set as one. All samples were measured in triplicates. (B) Activation of different *Gata4* promoter constructs by Sf1 compared to the respective promoter construct cotransfected with an EGFP expression construct. Activation of pGl3-basic was set 1. All samples were measured in duplicates and normalized to the activity of a SEAP control vector. (C) Activation of the 0.5 kb *Gata4* promoter construct by different concentrations of Sf1 and Gata4. TM4 cells were cotransfected with the reporter constructs and different amounts of Sf1 or Gata4 expression construct in addition to EGFP expression construct up to 0.8 µg in total. Activity of the reporter vector cotransfected with EGFP alone was set as 1. All samples were measured in triplicates and normalized to the activity of a Renilla-CMV control vector.
All experiments were performed with TM4 cells. Error bars represent standard deviations.

Smaller constructs containing 5 kb, 2.5 kb, 2.2 kb and 0.5 kb of the *Gata4* 5' region were obtained by religation of this construct after digestion with appropriate enzymes. The 10.6 kb promoter fragment showed transactivation by Sf1 or Gata4, but not by Wt1(+/-KTS) or Lhx9 in a luciferase reporter assay after cotransfection in TM4 cells (Fig. 7A). Transactivation by Sf1 was also seen for the smaller constructs to various extents (Fig. 7B) and even the 0.5 kb construct could be activated in a concentration dependent manner by Sf1 and Gata4 (Fig. 7C). Although an activation of *Gata4* by Wt1 in other cell lines or under different conditions than those used in this experiments can not be excluded, the results obtained here suggest, that *Gata4* is not a direct target of Wt1, but of the Wt1 target Sf1 and therefore indirectly dependent on Wt1. As the aim of this work was to identify direct Wt1 targets, no further analysis of *Gata4* regulation was undertaken.

3.1.5 *AmhrII* expression is altered after *Wt1* knock-down or *Wt1* induction in mammalian cell lines

To analyse Wt1 dependence of the second potential target gene, *AmhrII,* its expression was analysed after Wt1 knock-down and Wt1 induction in mammalian cell lines.

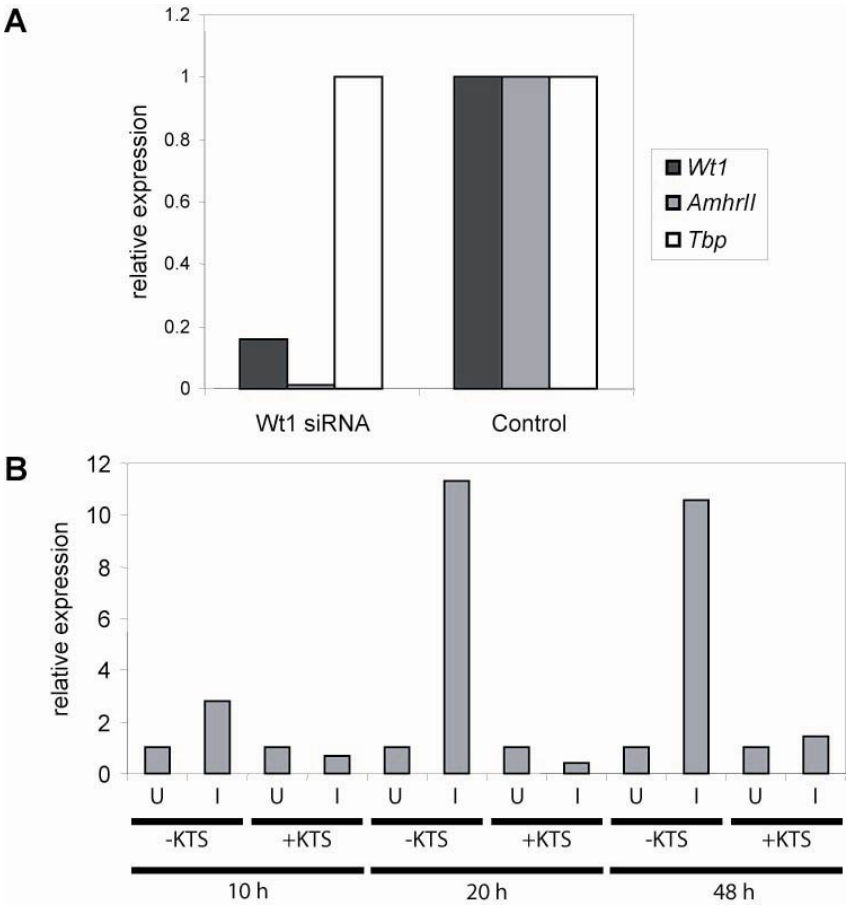

Fig. 8. *AmhrII* expression after Wt1 knock-down and Wt1 induction.
(A) *AmhrII*, *Wt1* and *Tbp* expression in M15 cells measured by qRT-PCR 55 h after transfection with *Wt1* siRNA (left) and control cells treated with transfection reagent without siRNA only (right). Relative expression compared to the controls and normalized to *Tbp* was calculated using the double delta method (see 2.2.4.3).
(B) *AMHRII* expression in uninduced (U) and induced (I) cells harbouring an inducible allele of Wt1(-KTS) or (+KTS) measured by qRT-PCR 10 h, 20 h and 48 h after induction. Relative expression compared to uninduced cells and normalized to *TBP* was calculated using the double delta method (see 2.2.4.3).

As shown by Ralph Sierig within his diploma work, Wt1 can be efficiently knocked down on RNA and protein level by RNA interference[102]. M15 cells treated with siRNA directed against *Wt1* mRNA show highly reduced *AmhrII* expression compared to control cells treated only with transfection reagent (Fig. 8A).

After having shown that *AmhrII* expression is decreased by inactivation of *Wt1* in gonads and M15 cells, the human osteosarcoma cell lines UB27 and UD28 that express Wt1(-KTS) or Wt1(+KTS) respectively under the control of a tetracycline regulated promoter[103] were used to test whether *AMHRII* expression can be increased by induction of Wt1. *AMHRII* was upregulated in a time dependent manner after induction of *Wt1(-KTS),* but not *Wt1(+KTS).* Ten hours after Wt1 induction *AMHRII* expression was about three times higher than in uninduced cells and reached a plateau at about eleven times higher expression after 20 h, that was also observed 48 h after induction (Fig. 8B).

Thus, expression of endogenous murine and human *AmhrII* shows an immediate response upon changes in Wt1 concentration in the respective cell line. This suggests a direct regulation of the murine and the human gene by Wt1.

3.1.6 The *AmhrII* promoter contains potential Wt1 binding sites

As *AmhrII* expression was changed by Wt1(-KTS) activation as well as inactivation, the question arose whether Wt1(-KTS) regulates *AmhrII* directly by binding to its promoter. An optimized binding site for Wt1 similar to the binding site described for early growth response 1 EGR-1 has been characterized and called WTE[104]. Since then several naturally occurring Wt1 binding sites similar to WTE have been characterized[92,93,105,106,107,108,109,110,111,112,113,114,115,116]. To get a reliable consensus

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binding site, a thorough literature search for published WTE like Wt1 binding sites was performed (Fig. 9A). All binding sites found contain 5 highly conserved Gs in positions 3, 5, 6, 7 and 9, while all other positions were less conserved (Fig. 9B, C). This consensus binding site was used to search for potential Wt1 binding sites in a *AmhrII* promoter sequence published by Teixeira et al.[117]. This sequence was published as the *AmhrII* promoter from rat. However, an intensive database search, done by Ralph Sierig, showed that it matches only published murine sequences, but no other rat sequence and has therefore to be considered as the mouse *AmhrII* promoter[102]. Teixeira et al. identified Sf1, Gata and Sp1 consensus binding sites in this promoter sequence (Fig. 9, 10) and showed that the Sf1 and Sp1 binding sites are protected in a DNase footprinting assay. In addition to these sites two more sites were protected in the DNase footprinting assay, for which the binding factor was not identified[117]. These two sites overlap with three Wt1 consensus binding sites (Fig. 9D). No other perfect Wt1 consensus binding site can be found in the promoter region analysed by Teixeira et al. All three potential Wt1 binding sites are highly conserved in human and rat[102]. 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[102,117].

[109] Lee, S. B., Huang, K., Palmer, R., Truong, V. B., Herzlinger, D., Kolquist, K. A., Wong, J., Paulding, C., Yoon, S. K., Gerald, W., Oliner, J. D., and Haber, D. A. (1999). The Wilms tumor suppressor WT1 encodes a transcriptional activator of amphiregulin. *Cell* **98,** 663-73.

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A

site1	GCGTGGGCGG	Hamilton et al., 1995
site2	GCGTGGGCGT	
site3	GCGTGGGAGT	Nakagama et al., 1995
site4	GCGGGGGCGG	Ward et al., 1996
site5	CGGCGGGCGC	
site6	GTGGGGGCGG	Moshier et al., 1996
site7	CCGGGGGAGG	
site8	GCGTGGGAGT	Hamilton et al., 1998
site9	CCGTGGGTGG	Lee et al., 1999
site10	GCGGGGGAG	Li et al., 1999
site11	GCGTGGGCGG	Dejong et al., 1999
site12	GCGCGGGCGG	Oh et al., 1999
site13	CCGGGGGCGG	Hosono et al., 2000
site14	GAGGGGGTGT	Hossain et al., 2001
site15	GTGGGGGCGG	Loeb et al., 2002
site16	GGGTGGGAGT	Wilhelm & Englert, 2002
site17	GGGTGGGGGC	
site18	ACGTGGGGGC	
site19	GGGTGGGGGG	
site20	GTGTGGGCGT	Han et al., 2003
site21	CCGGGGGCGG	Gross et al., 2003

B

Position	A	T	C	G
1	1	0	5	15
2	1	3	13	4
3	0	0	0	21
4	0	11	2	8
5	0	0	0	21
6	0	0	0	21
7	0	0	0	21
8	5	2	11	3
9	0	0	0	21
10	0	6	3	11

C

D

```
-375 CTGTTCAGGA TGCTACAGAT CTGAGTTCTC CATCCATGGG AACCATCTTG

-325 GACAGAGCTG AGCGCCCTAA ATGTTTGACA TCTTTCTGAA AAAGGGCAGA

                          Sf1
-275 AGGTCCAGCA CCTTCTTCCA AGGTCAGTAG GGGTAGAGAT TTCGTAGTCA

                                   Sf1
-225 ATTATCACAG GGTTCTCAGC TGGACAGCCA AGGTCCCTTC CTCCCCTCTC

-175 CGAGGAGAAA AAGAGGAGGT AGCTGAAGAA AAGATTGATT CTCTGCTCCT

                                Wt1        Wt1
-125 CCCTTTCTCT GCCTGTTTCC CTCACACCCA CCTCTCCCCC ACAGCAGCCT

      Wt1                       Gata          Sp1
 -75 TCTCCCCCAC AGAGACCGGG ATAGGACAGA GCGGGGCGGA GTTGGGGATT

                                   +1
 -25 GAAGGCTTGG AAGCCCCACA GTGCCCTGTA TTTGTAGGAA GCCTTGGCTG

 +26 AGGTAGCTGT GCTGGTTTTT GCTGGTTTAT GCTGTCCAGC TGGCATCCTT
```

Fig. 9. The *Amhrll* promoter contains three Wt1(-KTS) consensus binding sites.
(A) Published EGR-1 like binding sites of Wt1(-KTS). In all cases the more G-rich strand is shown in 5' to 3' direction. (B) Matrix calculated from the alignment shown in (A) starting with the most 5' position as position 1. (C) Graphical representation of the Wt1(-KTS) consensus binding site designed with weblogo.berkeley.edu. All binding sites contain guanidine nucleotides at positions 3,5,6,7 and 9. (D) Part of the murine *Amhrll* promoter sequence, containing transcription factor binding sites for Sf1, Gata, Sp1 and two unknown factors (dark grey) published by Teixeira et al.[104]. This sequence contains consensus Wt1(-KTS) binding sites (boxed) in three positions overlapping with the two binding sites for unknown factors described by Teixeira et al. Sequences matching four of the five conserved G/Cs in the Wt1 consensus binding site are shaded in light grey. The transcriptional start site is labelled +1.

3.1.7 Wt1(-KTS) binds to the *AmhrII* promoter *in vitro*

As analysed by Ralph Sierig within his Diploma work, the *AmhrII* promoter published by Teixeira et al.[117] showed significant transactivation by Wt1(-KTS), but not Wt1(+KTS), Sf1, Gata4 or Lhx9, in M15 cells[102]. If all three potential Wt1 binding sites in the AmhrII promoter were mutated by replacement of three of the five conserved cytosines with adenines, the activity of the promoter was highly reduced, when measured with a luciferase reporter assay in M15 or TM4 cells. The activity of the mutated promoter could not be enhanced by cotransfection with a Wt1(-KTS) expression construct in TM4 cells. In M15 cells, however, a transactivation of the mutated promoter by Wt1(-KTS) could still be observed. In both cell lines deletion of the Sf1 binding sites has no negative effect on the activity of the promoter construct. It is not clear from these results if the observed reduction of activity is caused by a loss of Wt1(-KTS) binding to these sites or by mutation of binding sites for other unknown factors[102].

Therefore, electrophoretic mobility shift assays (EMSA) were used to investigate *AmhrII* promoter binding by Wt1. First of all, a 0.4 kb fragment of the *AmhrII* promoter, containing all identified potential binding sites, was used (Fig. 10A). As shown in Fig. 10B, this fragment was bound by Wt1(-KTS) and Sf1. A mutated form of Wt1(-KTS), which contains the most common Denys Drash Syndrome causing mutation in zinc finger III, did not bind to this fragment as well as did not Wt1(+KTS) and GST (Fig. 10B, left). If all potential Wt1 binding sites were mutated by alteration of three conserved cytosines into adenines, binding by Wt1(-KTS) was still observed (Fig. 10B, right) indicating that Wt1(-KTS) binds not or not only to the identified consensus binding sites, but also to one or more additional sites. To determine the region bound by Wt1(-KTS) in more detail, the 0.4 kb fragment was divided into six overlapping fragments (F1-F6, Fig. 10A). As expected, the most efficient binding by Wt1(-KTS) was observed for F4, which contained the Wt1 consensus binding sites. Sf1 bound most efficiently to F2 and F3, the fragments containing the published Sf1 binding sites[104]. However, weaker binding of Wt1(-KTS) to F2 and F3 as well as of Sf1 to F5 and F6 was also observed. In contrast to the results for the 0.4 kb fragment, Wt1(-KTS) binding to F4 could be highly reduced by mutating the three potential binding sites (Fig. 10D). In Fig. 10D also weak band shifts for GST and Wt1(+KTS) can be observed. The band in the GST lane is most likely due to contamination from the neighbouring Wt1(-KTS) lane. Wt1(+KTS) binding, however,

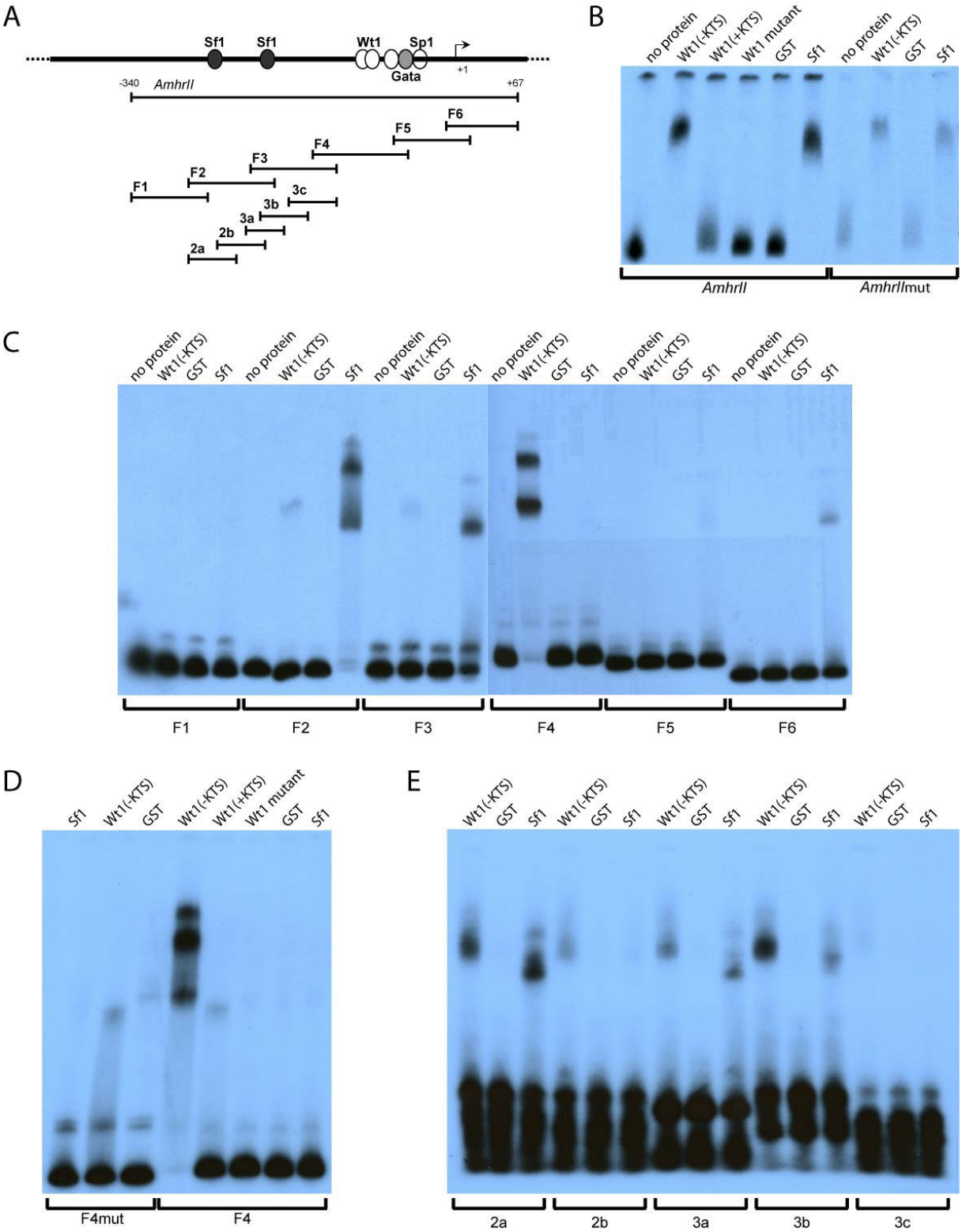

Fig. 10. Wt1(-KTS) and Sf1 bind to the murine *AmhrII* promoter *in vitro*.
(A) Schematic representation of the *Amhrll* promoter, containing potential transcription factor binding sites, and the fragments used for EMSA. (B) EMSA analysis using the 0.4 kb fragment in wild-type and a mutant form, in which all three potential Wt1 binding sites have been mutated by replacing three of the five conserved cytosines with adenines, as probes. The labelled fragments were incubated with recombinant GST, GST-Wt1(-KTS), GST-Wt1(+KTS), a mutant form of GST-Wt1(-KTS) harbouring a point mutation at position 394 and GST-Sf1. (C-E) EMSA analysis using fragments F1 to F6 (C), wild-type and mutated F4 (D) and fragments 2a to 3c (E) as probes. More than one shifted band with different sizes in one lane (C,D,E) are most likely due to different numbers of bound binding sites or dimerization of proteins.

is in line with previously published weak binding of Wt1(+KTS) to Wt1(-KTS) EGR-1-like binding sites[118]. F2 and F3 were subsequently divided into smaller fragments, to find out where Wt1(-KTS) binds to these fragments (Fig. 10A). Of these fragments Sf1 bound only to fragments containing published Sf1 binding sites, while Wt1(-KTS) bound all fragments to various extents (Fig. 10E). As shown in Fig. 9D, the *AmhrII* promoter contains several G or C rich sites with four of the five Gs or Cs of the Wt1(-KTS) consensus binding site in the right positions. It is most likely, that also these imperfect Wt1(-KTS) binding sites are weakly bound by Wt1(-KTS), in addition to the perfect consensus binding sites, as this would explain the shifts of the other fragments than F4 (Fig. 10C, E) as well as the incomplete loss of Wt1(-KTS) binding to the mutated fragments (Fig. 10B, D). This may also cause the incomplete loss of transactivation by Wt1(-KTS), observed in M15 cells with luciferase reporter assays, when the mutated form of the *AmhrII* promoter construct was used.
In summary, the EMSA shows, that Wt1(-KTS) binds to the *AmhrII* promoter *in vitro.* The binding occurs at the predicted binding sites and, to a weaker extent, also at several additional sites.

3.1.8 Wt1(-KTS) binds to the *AmhrII* promoter in living cells

To show that the *AmhrII* promoter is also bound by Wt1(-KTS) in living cells, chromatin immunoprecipitation (ChIP) was employed. For this purpose DNA and DNA-binding proteins of induced and uninduced human osteosarcoma UB27 cells, containing an inducible allele of Wt1(-KTS), were crosslinked by formaldehyde treatment. The so treated cells (input) were used for ChIP with different antibodys directed against Wt1, Sf1 and cMyc. After the IP, crosslinking of DNA and DNA-binding proteins was reversed and DNA was isolated from the precipitates. This DNA was tested for *AMHRII* promoter fragments by PCR with *AMHRII* specific primer pairs. When DNA was isolated directly from the formaldehyd treated cells, without previous ChIP, a specific PCR product could be amplified (Input, Fig. 11). This positive control shows that the selected primers and PCR conditions are suitable to amplify the desired PCR fragment from UB27 genomic DNA fragments. If an antibody against cMyc, a protein which does not bind to the *AMHRII* promoter, or no antibody was used for ChIP, no *AMHRII* promoter fragment was amplified. However,

[118] Rauscher, F. J., 3rd, Morris, J. F., Tournay, O. E., Cook, D. M., and Curran, T. (1990). Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* **250,** 1259-62.

AMHRII promoter fragments could be detected, when a Wt1 antibody was used with material from induced UB27 cells, but not when uninduced cells were used. Thus, when Wt1 is present in UB27 cells, it binds to the *AMHRII* promoter. ChIP assays using an Sf1 antibody were negative, although Sf1 binding to the *AMHRII* promoter was shown before[119]. The most likely explanation is that Sf1 is not expressed in UB27 cells. Indeed, Sf1 expression in UB27 cells could not be detected by RT-PCR.

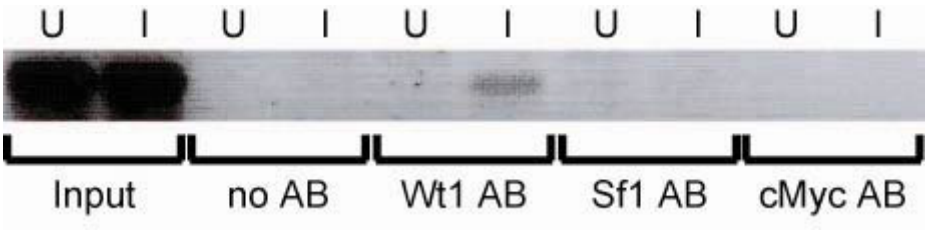

Fig. 11. Wt1(-KTS) binds to the human *AMHRII* promoter in living cells.
PCR analysis of DNA purified after ChIP with uninduced (U) and induced (I) UB27 cells, using antibodies against Wt1, Sf1, cMyc and no antibody, and an input control. The PCR was done with two different primer pairs with identical results. Only results for one pimer pair are shown.

In conclusion the ChIP assay shows that Wt1(-KTS) binds to the *AMHRII* promoter in UB27 cells. This demonstrates that Wt1(-KTS) binds the *AmhrII* promoter not only *in vitro* but also in living cells. Furthermore, as UB27 cells are of human origin, Wt1(-KTS) binds not only the murine *AmhrII* promoter, used for EMSA, but also the human promoter.

The presented analyses showed, that in addition to the reduced *AmhrII* expression in *Wt1* knock-out mice, *AmhrII* expression is also Wt1-dependent in mammalian cell lines like M15 and UB27 cells. The murine *AmhrII* promoter contains three Wt1(-KTS) consensus binding sites, which are bound by Wt1(-KTS) *in vitro*. This consensus binding sites are also conserved in the human *AMHRII* promoter. Accordingly, Wt1(-KTS) binds also to the *AMHRIII* promoter in living human UB27 cells. The presented results demonstrate that the approach used in this work is suitable to identify new Wt1 target genes.

[119] Barbara, P. S., Moniot, B., Poulat, F., Boizet, B., and Berta, P. (1998). Steroidogenic factor-1 regulates transcription of the human anti-mullerian hormone receptor. *J Biol Chem* **273,** 29654-60.

3.2 Dmrt8

3.2.1 Murine *Dmrt8.1* and *Dmrt8.2* show a sexually dimorphic expression

Our collaboration partners Anne-Marie Veith and Jean-Nicolas Volff (Department of Physiological Chemistry I, University of Würzburg) found out that *Dmrt8* is conserved on the X chromosome of placental mammals and possesses a DM-like domain in some species, although this domain is absent in mouse and man. The conservation of this gene on the X chromosome despite the lack of a proper DM domain suggests that there is a DM domain independent function of this gene. While *Dmrt8* exists as a single copy in most mammals, three copies of this gene, called *Dmrt8.1*, *Dmrt8.2* and *Dmrt8.3*, have been identified in mouse, rat and rabbit.

To investigate the expression of the three *Dmrt8* genes in mouse, Anne-Marie Veith performed RT-PCR analyses on cDNA, which I had previously isolated from several tissues of adult mice (Fig. 12). In contrast to human *Dmrt8,* which is expressed in various tissues including brain, lung, kidney, pancreas, and gonads of both sexes[120], *Dmrt8.1* and *Dmrt8.2* are exclusively expressed in testes of adult mice. Three different isoforms could be identified for *Dmrt8.1* and *Dmrt8.2* (Fig. 12). No expression of *Dmrt8.3* could be detected (not shown).

Furthermore I analysed embryonic expression of *Dmrt8.1* and *Dmrt8.2* by qRT-PCR on cDNA from different tissues of single embryos at E13.5 with primers recognizing all isoforms of *Dmrt8.1* and *Dmrt8.2*, respectively. While in adult mice, *Dmrt8.1* is exclusively expressed in testis (Fig. 12), expression of *Dmrt8.1* could be detected at low levels in all embryonic tissues tested with no sex-specific expression pattern (Fig. 13A). In contrast, expression of *Dmrt8.2* was detectable exclusively in testis and ovary with a significantly higher expression in testis at E13.5 (Fig. 13B). In order to investigate the temporal expression of *Dmrt8.2* during gonadal development, qRT-PCR analysis was performed on cDNA from male and female urogenitial ridges/ gonads starting from E8.5 to five weeks after birth (Fig. 13C). *Dmrt8.2* shows a dynamic expression only for a short period of time during gonad development and is first visible from E11.5 to E12.5. At E13.5 *Dmrt8.2* expression shows a peak in testes, while expression strongly decreases in ovary. Expression of *Dmrt8.2* in testis also decreases from E14.5 to E15.5 and reappears exclusively in testis after birth.

[120] Ottolenghi, C., Fellous, M., Barbieri, M., and McElreavey, K. (2002). Novel paralogy relations among human chromosomes support a link between the phylogeny of doublesex-related genes and the evolution of sex determination. *Genomics* **79,** 333-43.

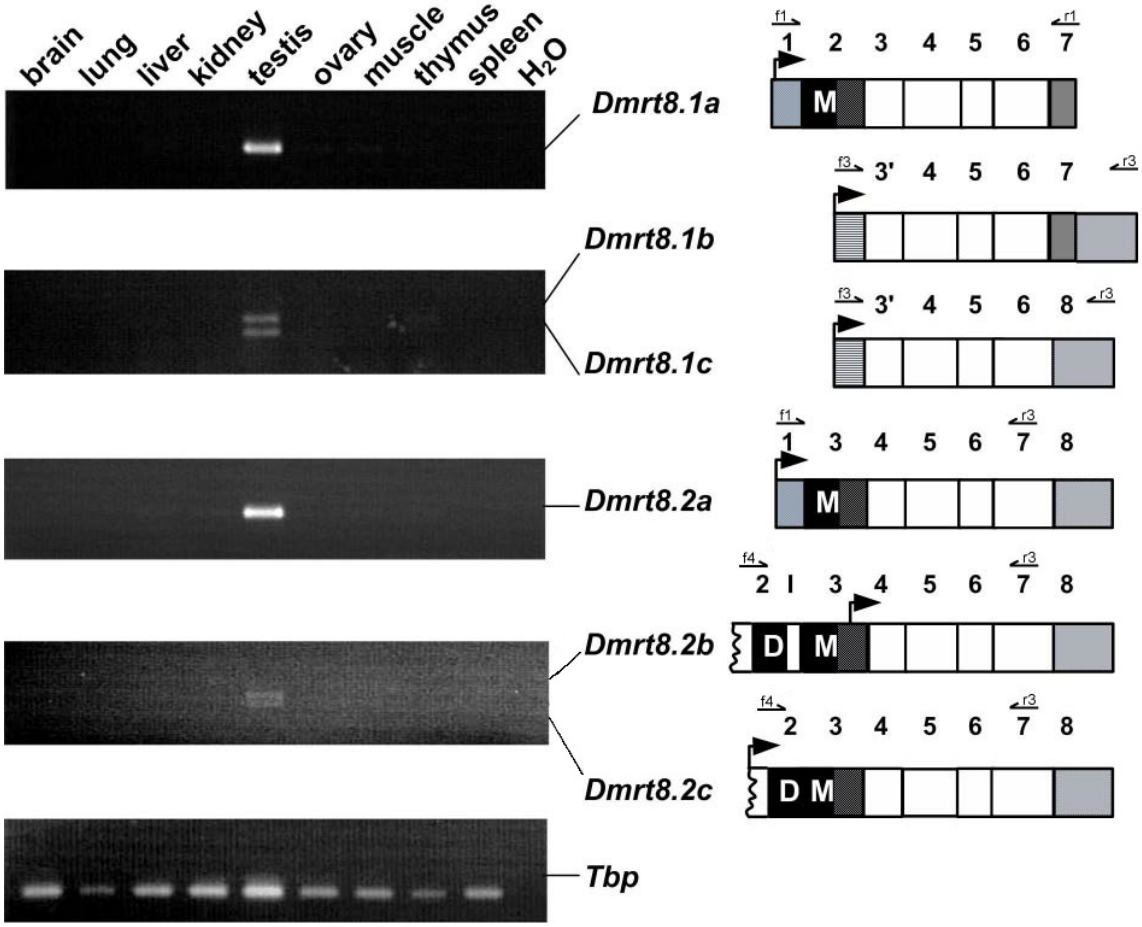

Fig. 12. Analysis of the expression of *Dmrt8.1* and *Dmrt8.2* isoforms in adult mice.
RT-PCR-analysis of the expression of *Dmrt8.1* and *Dmrt8.2* isoforms in different tissues of adult mice. The splice variants represented by the respective bands are depicted on the right. *Tbp* (TATA box binding protein) was used as a control. As negative control, water instead of cDNA was added to the PCR. Following primers were used; *Dmrt8.1a*, f1, r1; *Dmrt8.1b*, c f3, r3; *Dmrt8.2a* f1,r3, *Dmrt8.2b*, c, f4,r3.

The spatial expression pattern of *Dmrt8* genes in adult mice was analysed by RNA *in situ* hybridization on testis sections. Expression of *Dmrt8.1* was detected only in Sertoli cells, a somatic key cell type in testis (Fig. 14A). This Sertoli cell specific expression was confirmed by comparison to the expression pattern of *Wt1* (Fig. 14B), a marker for Sertoli cells in adult mice[121]. We were not able to detect *Dmrt8.2* expression in adult testis, or Dmrt8.1 and Dmrt8.2 expression in embryonic gonads by *in situ* hybridization, although expression was clearly detectable by RT-PCR. However, the qRT-PCR data indicates that in comparison to *Dmrt8.1*, *Dmrt8.2* is expressed at about ten fold lower level in adult testis (data not shown). Expression levels of *Dmrt8.1* and *Dmrt8.2* in embryos are lower than those in adult testis (Fig.

[121] 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., and et al. (1991a). Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* **67,** 437-47.

13). Therefore, apart from *Dmrt8.1* in adult testes, the expression levels might be below the detection limit of RNA *in situ* hybridization, a less sensitive detection method compared to qRT-PCR.

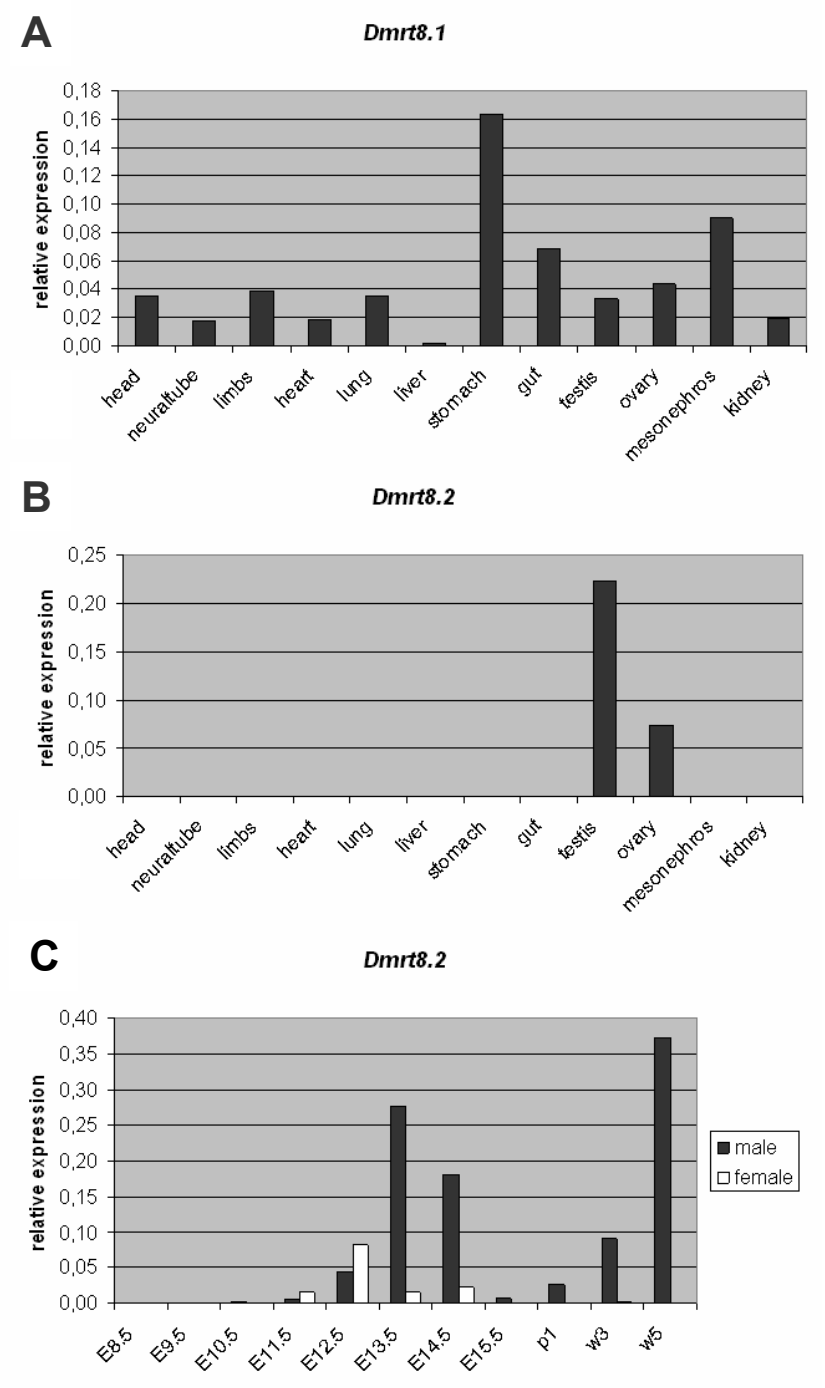

Fig. 13. Analysis of Dmrt8.1 and Dmrt8.2 expression in embryonic tissues by qRT-PCR.
(A) Analysis of *Dmrt8.1* and (B) *Dmrt8.2* expression in different organs at E13.5. (C) Analysis of *Dmrt8.2* expression at different stages of male and female gonad development. p1: one day after birth; w3: three weeks after birth; w5: five weeks after birth. Experiments were at least performed twice, with similar results. In each case, the result of one experiment is shown. Numbers on the y axes refer to relative expression levels. To define a point of reference the expression level in adult testis was set as 1.

In summary, two of three murine *Dmrt8* genes show testis specific expression in adult mice. One of the two genes - Dmrt8.1 was detectable by *in situ* hybridization in Sertoli cells. Additionally, *Dmrt8.2* also shows gonad specific expression during embryonic development. Thus, a role of these genes in gonadal development and testis function is suggested.

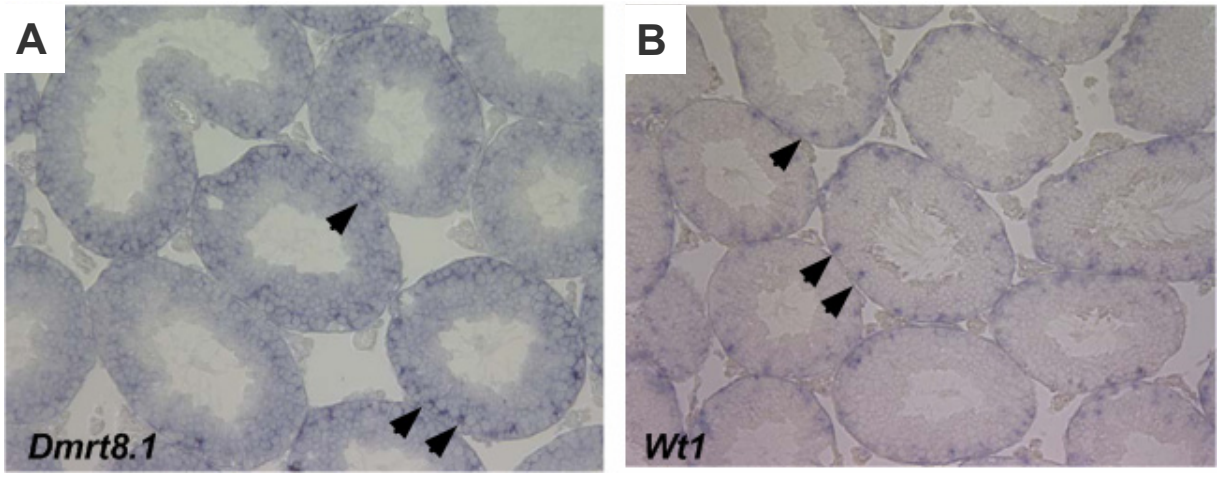

Fig. 14. RNA *in situ* hybridization on paraffin sections of adult testis.
Dmrt8.1 (A) and *Wt1* (B) are expressed in Sertoli cells (some Sertoli cells are marked by arrows). *Wt1* is a marker for expression in Sertoli cells, a somatic cell type located in the outer layer of testis cords that surrounds the spermatogonia.

3.3 Sox9

3.3.1 Gene expression analyses show complete sex reversal of *Ck19:Cre;Sox9flox/flox* XY embryos

Our collaboration partners Francisco Barrionuevo and Gerd Scherer (Institute of Human Genetics and Anthropology, University of Freiburg) used the CRE/*loxP* system to generate a homozygous knock-out of *Sox9* during early gonadal development. For this, they crossed a *Sox9^{flox}* mouse line, where exons 2 and 3 of *Sox9* are flanked by *loxP* sites[122], with the *Ck19:Cre* mouse line[123]. This line expresses the CRE recombinase throughout the early postimplantation mouse embryo, including the embryonic ectoderm, mesoderm and definitive endoderm[124]. Usage of the CRE reporter line R26R, in which CRE activity activates a *LacZ* gene[125], revealed intense lacZ staining of *Ck19:Cre* urogenital ridges at E10.5 and E11.5. Thus, the *Ck19:Cre* transgene must be efficiently expressed in this tissue already before the upregulation of *Sox9* expression in XY embryos.

Cre/+;Sox9$^{flox/+}$ animals survive, are fertile, and can be backcrossed to *Sox9$^{flox/flox}$* mice to obtain *Cre/+;Sox9$^{flox/flox}$* offspring. These mice show developmental defects caused by reduced *Sox9* expression in several tissues in addition to gonads. As a consequence of these various defects, *Cre/+;Sox9$^{flox/flox}$* embryos show significant early embryonic lethality. But in contrast to *Sox9$^{-/-}$* embryos, ~1/8 of the expected number of embryos survived after the timepoint of sex determination at E11.5. Whereas *Cre/+*;*Sox9$^{flox/flox}$* XX and *Cre/+;Sox9$^{flox/+}$* XY embryos showed normal ovary and testis development, respectively (not shown), *Cre/+;Sox9$^{flox/flox}$* XY embryos (hereafter termed mutants) showed morphological complete male-to-female sex reversal (data not shown).

To examine the efficiency of *Sox9* inactivation and the sex reversal phenotype, quantitative real-time RT-PCR (qRT-PCR), using RNA isolated from mutant and from control gonads, was performed. In wt male gonads *Sox9* and *Amh* expression

[122] Kist, R., Schrewe, H., Balling, R., and Scherer, G. (2002). Conditional inactivation of Sox9: a mouse model for campomelic dysplasia. *Genesis* **32,** 121-3.
[123] Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *Embo J* **18,** 5931-42.
[124] Means, A. L., Chytil, A., Moses, H. L., Coffey, R. J., Jr., Wright, C. V., Taketo, M. M., and Grady, W. M. (2005). Keratin 19 gene drives Cre recombinase expression throughout the early postimplantation mouse embryo. *Genesis* **42,** 23-7.
[125] Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21,** 70-1.

increased at about E11.5, as expected (Fig. 15A), correlating with an increasing number of tail somites. *Sox9* upregulation started at about 16ts (Fig. 15A, left), while *Amh* expression started a little bit later, after 20 ts (Fig. 15A right). In contrast, male *Sox9* mutant gonads and female controls showed no enhanced expression for none of the two genes. The expression pattern of *Sox9* and *Amh* also indicated that a threshold of *Sox9* has to be reached to initiate *Amh* expression (Fig. 15A), in accordance with the known role of *Sox9* in this process[126,127]. Also at E12.5, *Sox9* expression levels in male mutant embryos were drastically below the expression levels in male controls and even below the levels in female controls (Fig. 15B, left).

The ovary-specific marker *Foxl2* that is required for granulosa cell differentiation starts to be expressed in female gonads around E12.5[128]. Accordingly, significant E12.5 *Foxl2* expression was detected in control female gonads as well as male *Sox9* mutant gonads, but not in male controls (Fig. 15B, middle). The mirror-image expression pattern was seen for *Amh* in the same gonads. Male control gonads expressed high levels of *Amh*, whereas in female controls and male *Sox9* mutants almost no expression was detectable (Fig. 15B, right).

Wild-type *Sry* expression starts at E10.5, peaks at E11.5, and disappears around E12.5[129]. In our analysis, control and mutant XY gonads showed similar *Sry* levels at E11.5 (Fig. 15C, left). Thus, *Sry* expression is independent of *Sox9* dosage, illustrating that *Sry* acts upstream of *Sox9*. At E12.5, *Sry* is down-regulated in control male gonads but remains at least 5-fold higher in the mutant XY gonads (Fig. 15C, right), pointing to a negative regulation of *Sry* by Sox9. Similarly, at E13.5, 4.5-fold higher *Sry* levels were detected in *Sox9* mutant embryos compared to male controls (data not shown).

In summary the qRT-PCR data demonstrate, that male *Sox9* mutant embryos show female-specific gene expression in the presence of *Sry* transcripts and are therefore completely sex reversed.

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[126] De Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F., and Berta, P. (1998). Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. *Mol Cell Biol* **18,** 6653-65.

[127] Arango, N. A., Lovell-Badge, R., and Behringer, R. R. (1999). Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. *Cell* **99,** 409-19.

[128] Schmidt, D., Ovitt, C. E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A. C., and Treier, M. (2004). The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* **131,** 933-42.

[129] Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. Ibid.**121,** 1603-14.

The loss of *Sox9* expression and the sex reversal phenotype in *Sox9* mutant gonads was confirmed by additional analyses using histological sections, immunohistochemistry and RNA *in situ* hybridization done by the collaboration partners in Freiburg (data not shown).

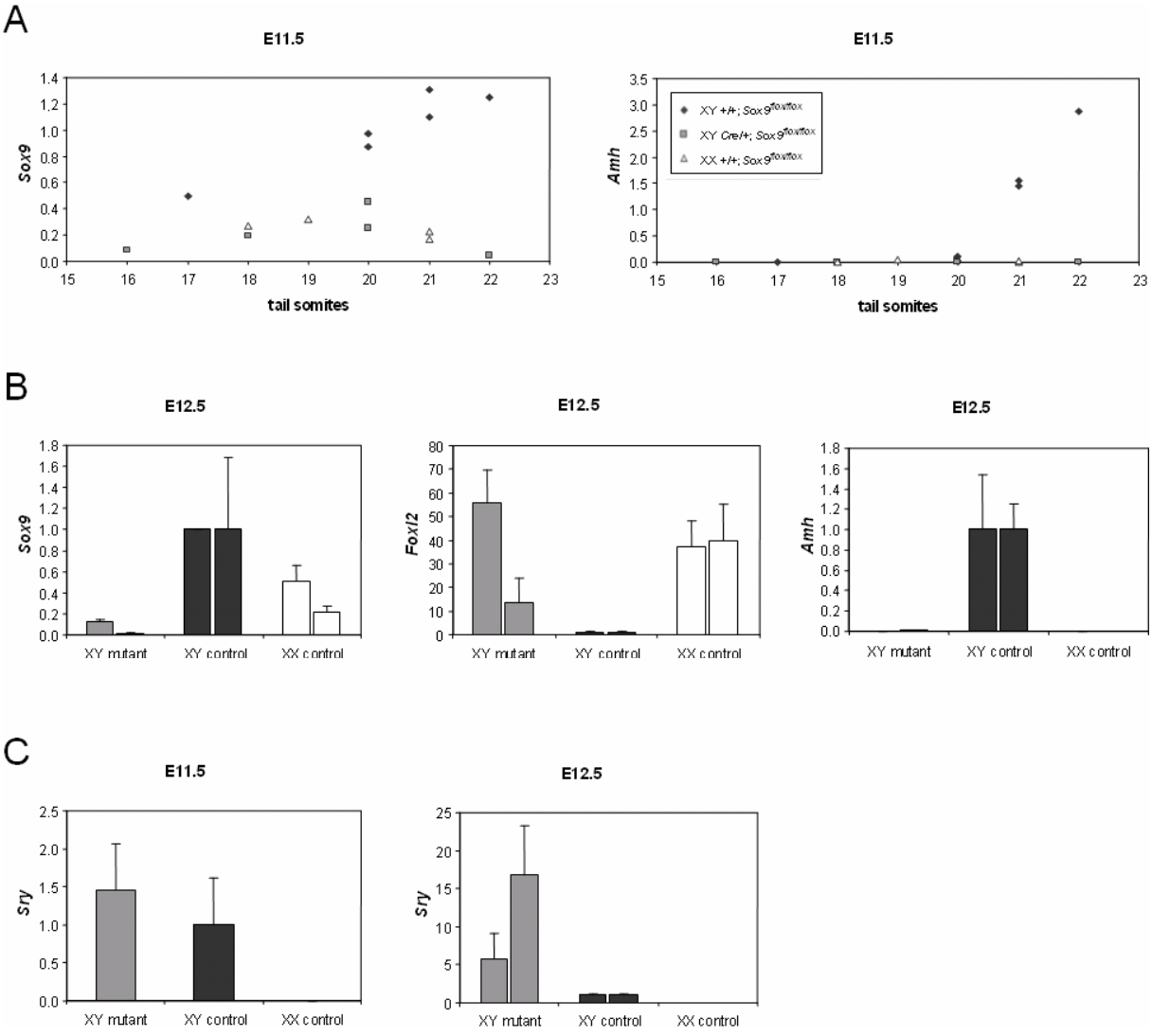

Fig. 15. Gene expression analysis of XY *Sox9* mutant and control gonads by qRT-PCR.
(A) Analysis of *Sox9* (left) and *Amh* (right) expression in control and mutant gonads at E11.5. (B) Analysis of *Sox9* (left), of the female-specific gene *Foxl2* (middle) and of the male-specific gene *Amh* (right) in control and mutant gonads at E12.5. (C) Determination of *Sry* expression levels at E11.5 and E12.5. Analysis was done on a total of five mutant samples of E11.5 together with six male and four female controls. In case of E12.5 (in B and C) to experiments were done. The left bars correspond to experiment 1, the right bars to experiment 2, performed with two and four samples of each genotype, respectively. All samples were measured at least as duplicates. Experiments were at least performed twice, yielding virtually identical results. In each case, the result of one measurement is shown. Numbers on the y axes refer to relative expression levels. Note that to define a point of reference, the mean values for male controls are set as 1. Error bars denote standard deviation.

3.3.2 *Prostaglandin D synthase* (*Ptgds*) expression is Sox9 dependent

After having demonstrated that *Sox9* mutant mice show a complete male-to-female sex reversal, we started a collaboration with Francis Poulat (Institut de Gene´tique Humaine, CNRS UPR1142, Montpellier Cedex, France) and used the *Ck19:Cre;Sox9$^{flox/flox}$* mouse model to investigate the expression of Prostaglandin D synthase (Ptgds), another gene known to be important for male sex determination. Ptgds synthesizes the signalling molecule prostaglandin D_2 (PGD_2), secreted by pre-Sertoli cells, which express *Sry* and *Sox9*. PGD_2 is able to recruit cells without Sry expression as additional Sertoli cells by inducing up-regulation of *Sox9*[130]. Therefore the question arises whether *Ptgds* is activated by Sox9 or by Sry in pre-Sertoli cells. Induced by Sry, PGD_2 could also act as activator of Sox9 in a cell-autonomous way, as has been proposed[131].

To find out whether *Ptgds* is activated by Sry or Sox9, *Ptgds* expression in *Cre/+;Sox9$^{flox/flox}$* XY gonads was analysed using qRT-PCR (Fig. 16). At E11.5 *Ptgds* showed an expression pattern similar to *Amh* with an increase in male controls after 20 ts, while expression remained low in *Sox9* mutants and female controls. Similarly, high expression in XY controls and almost only low expression in XY *Sox9* mutants and XX controls was detected at E12.5 and E13.5. As XY *Sox9* mutants do express *Sry* at even higher levels than male controls (Fig. 15C), the reduced *Ptgds* expression in XY mutants shows, that *Ptgds* is not directly activated by Sry but depends on Sox9.

In summary, gene expression analysis demonstrated, that XY *Ck19:Cre;Sox9$^{flox/flox}$* embryos are completely sex reversed in the presence of *Sry* expression. This confirms that *Sox9* is necessary for male sex determination. *Sox9* seems to be the master sex determining gene, which activates male sex differentiation, inhibits female sex differentiation and turns off *Sry*, after *Sox9* itself has been activated by Sry. A new potential target gene of Sox9 is the *Prostaglandin D synthase gene* (*Ptgds*).

[130] Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Beverdam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev Biol* **287,** 111-24.

[131] Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F., and Boizet-Bonhoure, B. (2005). Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *Embo J* **24,** 1798-809.

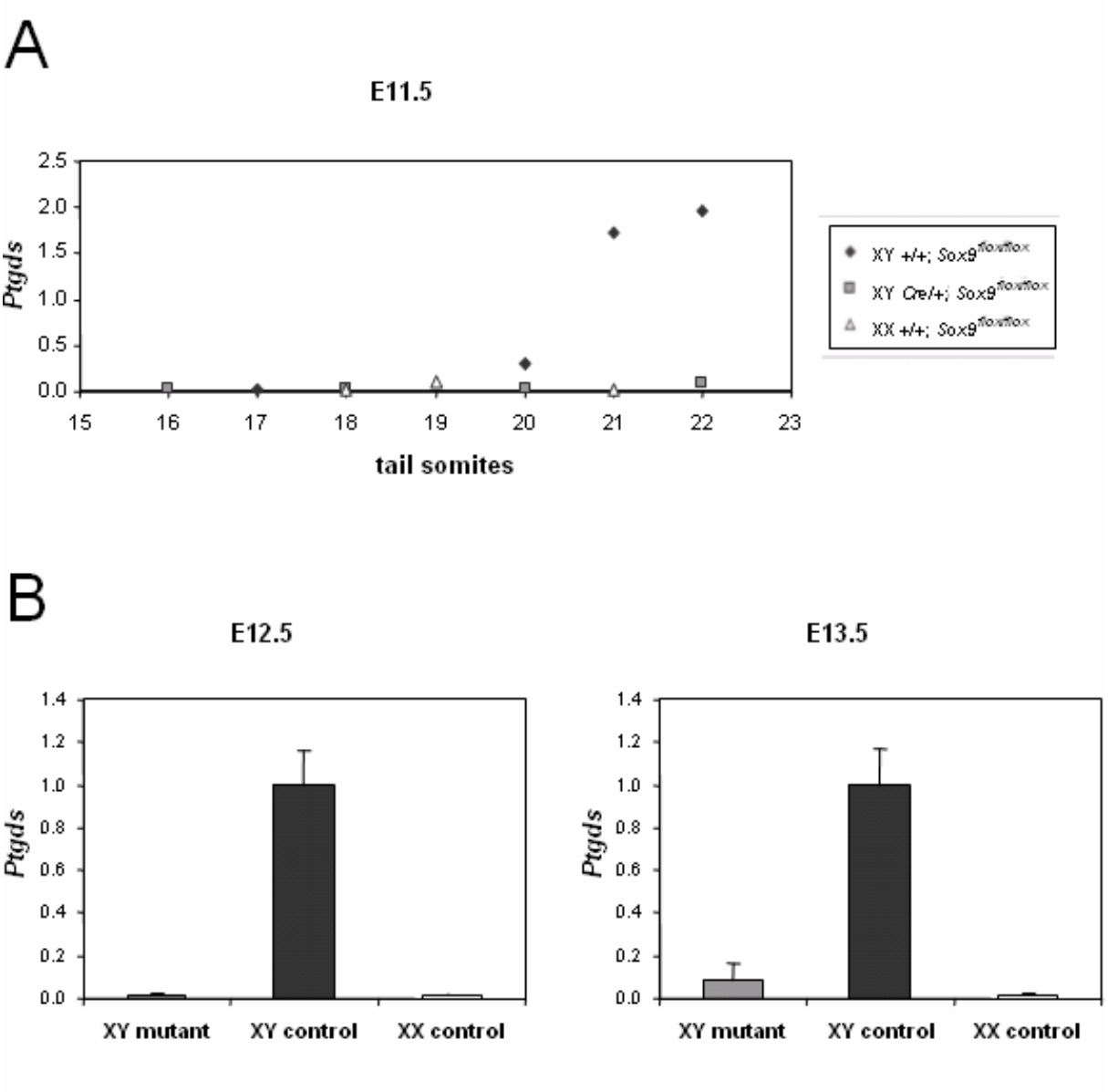

Fig. 16. Analysis of Ptgds expression in control and mutant gonads by qRT-PCR.
Analysis of *Ptgds* expression in control and mutant gonads at E11.5 (A), E12.5 (B, left) and E13.5 (B, right). Analysis was done on four, six and three samples of E11.5, E12.5 and E13.5 gonads of each genotype, respectively. All samples were measured as duplicates. Numbers on the y axes refer to relative expression levels. Note that to define a point of reference, the mean values for male controls are set as 1. Error bars denote standard deviation.

4 Discussion

4.1 Wt1

4.1.1 Differential gene expression analysis

In order to identify unknown Wt1 target genes in gonad development, gene expression in urogenital ridges of *Wt1*$^{-/-}$ and *Wt1*$^{+/+}$ embryos was compared by microarray and qRT-PCR analysis. As secondary effects, such as the upregulation of indirect targets, accumulate at later time points, identification of direct targets by these methods is limited to time points early after the mutated gene starts to be expressed in a certain organ.

In the developing urogenital ridge *Wt1* mRNA can be detected from E9.5 on[132,133], even before the gonads start to form at about E10.0. It is very difficult to isolate urogenital ridges immediately after they become visible. To reduce the possibility of contamination with parts of other tissues embryos with 11-12 ts were chosen for microarray analysis. At this time point urogenital ridges are clearly visible. Furthermore this time point lies perfectly in between the start of *Sry* expression at 8 ts[134] and the start of sex differentiation at 16ts[135] and matches the time point when Sry protein is first detectable[136]. It is known that *Wt1*(*+KTS*) knock-out leads to sex reversal due to reduced *Sry*, and resulting *Sox9* and *Amh*, expression[137]. Thus, secondary effects of missing *Wt1* expression have to be expected after the time point of sex determination at the latest.

It has to be mentioned, that all embryos used for microarray analysis were dissected from *Wt1*$^{+/-}$ mothers at E11.5. Although the expected number of tail somites is about 18 ts at this time point[134], the mean number of tail somites isolated from *Wt1*$^{+/-}$

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mothers at E11.5 was found to be 12 ts. E11.5 embryos of *Wt1*$^{+/-}$ mothers are in average 5 ts smaller than embryos isolated from wild-type mothers (data not shown). These differences in the number of tail somites correlate only with the genotype of the mother but not with the genotype of the embryo. The observed *Sry* expression pattern in gonads of these embryos correlates with the number of tail somites as published (data not shown)[134]. Thus, the embryos show an overall retardation in embryonic development and not only in somite development. The reason for this retardation is unknown. *Wt1* is expressed in the uterus near placentas (own observations and published data[138]). Therefore, one possible reason for the growth retardation could be insufficient nutrition of the embryos, caused by a *Wt1* haploinsufficiency effect in the maternal uterus.

The transcription factor Wt1 was shown to be able to activate and inhibit gene expression in reporter gene assays. However, most of the physiological relevant targets were found to be activated by Wt1[139]. In line with this, the microarray analysis showed genes with decreased as well as increased expression in *Wt1*$^{-/-}$ urogenital ridges. 100 genes showed higher expression in wt and only 42 genes showed higher expression in ko. Thus, more than two times more genes seem to be activated by Wt1 than inhibited.

As the presented microarray data reveal, genes involved in muscle development seem to belong to the group of genes upregulated in Wt1$^{-/-}$ urogenital ridges. Wilms tumours, especially those with *Wt1* mutations, sometimes differentiate into structures not normally seen during nephrogenesis. Most often differentiation into primitive skeletal muscle is seen[140]. In addition to this Myagawa et al. found that transfection of a murine myoblastic cell line with *Wt1* expression construct leads to inhibition of myogenic differentiation. Therefore, they suggested that Wt1 inhibits myogenesis[141]. However, a second group could not confirm inhibition of myogenic differentiation by

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Wt1 in the same cell line nor in human rhabdomyosarcoma[142]. The upregulation of genes, which play a role in muscle development, in Wt1$^{-/-}$ urogenital ridges, found in the EASE analysis of the microarray data, would be in line with an inhibitory effect of Wt1 on myogenesis. However, differentially expression of only a part of these genes could be confirmed by qRT-PCR and only *Gdf8* (*growth differentiation factor 8*, also known as *myostatin*) was further analysed by RNA *in situ* hybridization. No difference in the expression of *Gdf8* could be seen with this method. As discussed below reasons for these inconsistent results could be contaminations with other tissues and differences in the sensitivity of the detection methods.

Of the previously described in vivo targets of Wt1, namely *p21*, *Bcl2*, *E-Cadherin* and *Sprouty homolog 1*[139], none showed differences in expression higher than factor 2. However, some of these genes could be false negative as observed for known gonadal targets of Wt1, like *Sf1*.

Of the genes known to be involved in early gonad development, namely *Lhx9*, *M33*, *Emx2*, *Gata4*, *Fog2*, *Sf1*, *Wnt4*, *Dax1*, *Fgf9*, *Fgfr2*, *Ir*, *Irr* and *Igf1r*[143], only *Lhx9* and *Dax1* showed a difference in expression slightly higher than factor three in the microarray analysis. However, another gene known to be involved in male differentiation, *AmhrII*[144], is the gene which shows the highest difference in expression between ko and wt urogenital ridges. Interestingly, *Sf1* showed no significant difference although a clear reduction of the expression of this gene in ko gonads has been shown before[145].

Since it is known that microarray analyses lead to a certain amount of false positive and false negative data, qRT-PCR was used, to analyse expression of genes of interest by a second method.

All analysed genes which were described as gonadal Wt1 targets before, namely *Sf1*, *Wnt4* and *Dax1*, showed no or relatively mild differences according to the microarray data. In each case qRT-PCR showed a higher difference in the expression levels in knock-out and wild-type. Especially the results for *Sf1* and *Dax1*

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matched previously published data in a much better way[145]. Why the microarray results for these genes were false negative, although the same kind of material was used for both methods, is not clear. It has to be taken into account, that the microarray results may be also falsely negative for many yet unknown Wt1 dependent genes. One example described here is *Gata4*, which showed reproducible differences when qRT-PCR or RNA *in situ* hybridization was used.

It is striking that none of the genes with higher signals for ko urogenital ridges, according to the microarray and qRT-PCR analysis, could be confirmed by RNA *in situ* hybridization, while higher expression in wt urogenital ridges could be seen for four genes. In addition only genes of the first group showed contradictive results in qRT-PCR. As can be seen in the EASE results this group contains many genes involved in muscle development and to a lesser extent neurogenesis, two processes that take place in neighbouring tissues of the urogenital ridge, namely the somites and the neural tube. One explanation for the unreproducible results may be that there is a bias in preparation which leads to higher contamination of ko gonads with neighbouring tissues. It has been observed for later stages that ko urogenital ridges are more difficult to preparate, as the ko phenotype becomes obvious and the gonads start to degenerate. Maybe this is, to a lesser extent, also the case at the earlier stage used for the microarray and qRT-PCR analysis. A reduced size of the gonadal ridge as soon as at the 9 ts stage is indicated by the reduced area of Lhx9 expression observed with RNA *in situ* hybridization (Fig. R1D, E). In general ko urogenital ridges seem to be smaller even at 6-9 ts stage (Fig. R1C, E, G) in comparison to wt urogenital ridges (Fig. R1B, D, F). Another explanation for the negative RNA *in situ* hybridization results could be that this method is less sensitive than microarray analysis or qRT-PCR. Maybe the respective genes are upregulated in $Wt1^{-/-}$ gonads, but only to a level below the detection limit of this less sensitive method. All in all this is in line with the observation that it is more difficult to identify genes inhibited by Wt1, although Wt1 has been described as an activator as well as an inhibitor of gene expression[139].

The slight differences in size of the wt and ko urogenital ridges described above indicate that the ko phenotype starts to become obvious before the 11 ts stage. Therefore the time point used for the microarray analysis may not be optimal to look for primary effects of Wt1. This is also indicated by the results obtained for *Gata4.* *Gata4* seems to be activated by Sf1 and is therefore most likely a secondary target of

Wt1. To reduce these secondary effects, embryos with less than 6 ts should be used for a new microarray analysis. However, as it is very complicated to isolate urogenital ridges without contamination at this early time point, larger portions of the embryo would have to be used.

The observed size differences of wt and ko gonads could be explained by increased apoptosis or reduced proliferation of ko gonads. Especially enhanced apoptosis has been described for several tissues of *Wt1* knock-out embryos, including gonads[146,147,148,149]. Apoptosis may occur generally throughout the gonad or only in a certain cell type. Apoptosis of a certain cell type would result in reduction of the expression of all genes specifically expressed in this cell type. Sex reversal effects due to a reduced number of pre-Sertoli cells have been discussed for *Wt1* mutants[150], *Gata4/Fog2* mutants and *Ir*, *Igf1r* and *Irr* triple knock-outs[151]. Together with *Gata4, Wt1* is one of the first genes upregulated in Sertoli cells after sex determination[152]. Reduced size, enhanced apoptosis and a reduced number of *Sox9* and *Amh* expressing cells has been described especially for *Wt1(-KTS)* knock-out embryos[147]. As shown here, *Gata4* expression seems to depend indirectly on this variant of Wt1. Wt1 could be also involved in the definition or generation of the Pre-Sertoli cell type, as the Wt1 target gene *Sf1* is known to be an early marker of this cell type[150,153]. A reduced number of such important cells as the supporting cells of both sexes (Sertoli cells, Granulosa cells) could also be the reason for gonadal regression in *Wt1*$^{-/-}$ embryos.

Differential expression of *Lhx9*, *Ptprv*, *Gata4* and *AmhrII* could be confirmed by RNA *in situ* hybridization. In contrast to the three other genes, *Lhx9*, a gene known to be

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involved in gonadal development[154], shows only a slight difference. As no difference in the intensity of the staining but only in the size of the stained area is observed, this difference is most likely due to the reduced size of the ko gonadal ridge and not due to regulation of *Lhx9* by Wt1. This difference in the size of the *Lhx9* expression area has been described before[155]. Expression of *Lhx9* shows that *Wt1*$^{-/-}$ embryos of this time point have gonadal anlagen. Therefore the severely reduced expression observed for other genes is not due to a loss of the gonad, but to a specific loss of the expression of these genes, or the complete loss of a certain cell type (see above).

PtprV is a transmembrane receptor-like protein-tyrosine-phosphatase and also known as osteotesticular phosphatase (Ost-PTP) or embryonic stem cell phosphatase (Esp). *Ptprv* has been reported to be expressed in bones and gonads[156]. Its expression is detectable throughout gonad development of both sexes from E10.5. In adult gonads it is localized to Sertoli cells of the testes and coelomic epithelial cells of the ovaries and overlaps therefore with *Wt1* expression. No gonadal phenotype of *Ptprv* knock-out mice has been published and a gonadal function of this gene is not known. Ptprv plays a role in osteoblast differentiation[156,157] and in p53-dependent cell cycle arrest[158]. Ptprv is thought to be a transcriptional target of p53 and mediates cell cycle arrest in a similar way as p21[158], a known Wt1 target[159]. However, function of Ptprv has been lost in humans[160]. As this gene does not seem to play a conserved or essential role during mammalian gonad development, its dependence on Wt1 has not been further investigated.

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4.1.2 Gata4

Gata4 is known to be expressed very early in the developing gonad together with *Wt1*, *Lhx9* and *Sf1*[161]. Like *Wt1* and *Sf1*, *Gata4* is expressed in the gonads of both sexes[162], upregulated in differentiating Sertoli cells[163,164] and seems to play a role in *Amh* activation[165]. It has been shown that interaction of Gata4 and a second factor, Fog2 (Friend of Gata 2), is important for proper gonad development of both sexes and for normal expression of *Sry*[166].

Gata gene expression is known to be regulated by genomic regions spanning several 10 or 100 kb[167]. For example, a 14.8 kb fragment upstream of the transcriptional start site is necessary to drive expression of zebrafish *Gata4* in the heart[168]. Nothing is known about the regulation of the murine *Gata4* promoter yet. For this work a construct spanning half of the noncoding exon 1 and 10.6 kb 5' flanking region has been cloned. This construct and its shortened derivates contain some promoter activity, as they drive luciferase expression above background level and can be activated by Sf1 and Gata4 itself, as well as inhibited by Hey transcription factors (this work and data not shown[169]). However, it was not clarified, whether the promoter constructs can drive gonad specific expression. A transactivation of these constructs by Wt1 was not observed. The focus of this project was to identify direct targets of Wt1. As the obtained results suggested that *Gata4* is not directly regulated by Wt1, but by the Wt1 target gene *Sf1*, *Gata4* regulation was not further analysed.

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4.1.3 AmhrII

The most promising Wt1 target, identified by microarray analysis, qRT-PCR and RNA *in situ* hybridization, is *AmhrII*. A further hint at a direct link between *AmhrII* and Wt1 is the dependence of *AmhrII* expression on Wt1 in mammalian cell lines. Knock-down of *Wt1* by RNAi in M15 cells or induction of *Wt1* in UB27 cells leads to decrease or increase in *AmhrII* expression, respectively.

An important step in sex differentiation is regression of Müllerian ducts in male embryos. As the formation of both kinds of sexual ducts, the Wolffian ducts, which differentiate into epididymes, vasa deferentia and seminal vesicles, and the Müllerian ducts, which form Fallopian tubes, the uteri and the upper third of the vagina, is initiated in both sexes, one pair of ducts has to regress in each sex during sex differentiation. Regression of Müllerian ducts in male embryos is initiated by the action of Amh, a member of the Transforming growth factor β superfamily. Amh is secreted by differentiating Sertoli cells and binds to a heterodimeric receptor complex consisting of a type I and a type II serine/threonine kinase receptor, expressed in mesenchymal cells surrounding the Müllerian duct epithelium[170]. The type II receptor binds the ligand and phosphorylates the type I receptor, which then phosphorylates a receptor-regulated Smad (R-Smad). R-Smads interact with the common Smad4 and this complex is then translocated to the nucleus, where it binds to promoters of target genes[171]. Müllerian duct regression is most likely mediated by a parakrine factor which induces apoptosis of the Müllerian duct epithelium[172,173,174]. By now, only the ligand and the type II receptor of this signalling pathway have been clearly identified, while several type I receptors and R-Smads are discussed as possible candidates[170]. In addition to the mesenchymal cells surrounding the epithelium of the Müllerian ducts[175,176], *AmhrII* expression is also detected in granulosa cells of the ovary from

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fetal life to adulthood and Sertoli and Leydig cells of the testis from fetal life to puberty[170]. In these cells it plays a role in the regulation of various steroidogenic proteins[177]. Gonadal expression in both sexes starts before sex determination. While the exact role of *Amhrll* expression in gonadal cells is not absolutely clear, its role in Müllerian duct regression has been shown in a mouse model. Like *Amh* deficient mice, male *Amhrll* knock-out mice show internal pseudohermaphroditism with fully developed male genitalia, but additional oviducts and uteri[178,179]. Mutations of *Amh* or *Amhrll* are also found in human patients with a rare form of male pseudohermaphroditism, the persistent Müllerian duct syndrome (PMDS), which is characterized by the persistence of Müllerian derivatives in otherwise normally virilized males[180].

Regulation of *Amhrll* expression has been investigated by several groups, using luciferase reporter assays and biochemical promoter analysis. De Santa Barbara et al. identified two SP1 sites, one SOX9 site, two GATA motifs and a perfect consensus SF1 response element in a 1.1 kb promoter fragment of the human *AMHRII* gene[181]. Using EMSA and luciferase reporter assays they could show, that SF1 binds to its consensus binding site and transactivates the promoter fragment in NT2/D1 and HeLa cells. Teixeira et al. analysed a 1.6 kb murine *Amhrll* promoter fragment (erroneously referred to as rat sequence) in a similar way and identified one conserved Sp1 and one conserved GATA site at the position of the respective human sites identified by De Santa Barbara et al.[182]. They also found the respective Sf1 binding site, a second not conserved Sf1 site and two C-rich binding sites, for which the binding factor was not identified. They showed that the Sp1 site, the C-rich sites and the Sf1 sites are protected in a DNase I footprinting assay with R2C cell nuclear

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extract and that the Sf1 binding sites are bound by a protein in this extract, which is recognized by an Sf1 antibody. However, the promoter fragment is not transactivated by Sf1 in JEG-3, UGR1 or COS cells, although mutagenesis of both Sf1 binding sites abrogates activity of the promoter fragment in R2C cells. Activity of this promoter fragment is reduced when one of the C-rich sites is mutated. Hossain and Saunders found that β-catenin activates a 0.8 kb fragment of the human *AMHRII* promoter together with TCF4 via four TCF4-binding sites in HeLa cells and showed binding of TCF4 to these sites by EMSA and ChIP[183]. They also found that β-catenin and TCF4 act synergistically with SF1 and that SF1 interacts with β-catenin. This suggests that β-catenin is the missing cofactor in those cell lines where Sf1 is not able to transactivate the *AmhrII* promoter alone. However, in contrast to the Sp1, GATA, Sf1 and C-rich sites described by Teixeira et al., the TCF4 sites are poorly conserved in mouse and rat (data not shown).

The data presented here show that Wt1(-KTS) seems to be another important factor for *AmhrII* activation. A murine *AmhrII* promoter fragment is transactivated by Wt1(-KTS) in M15 and TM4 cells[184]. Transactivation by Wt1, to the same or even higher extent, can be also observed when the Sf1 binding sites are deleted, suggesting a Sf1 independent transactivation of *AmhrII* by Wt1. The C-rich sites described by Teixeira et al. overlap with Wt1(-KTS) consensus binding sites, which are bound by Wt1(-KTS) *in vitro*. Mutagenesis of these sites abrogates Wt1(-KTS) binding and transactivation of the *AmhrII* promoter by Wt1(-KTS), at least partially. Binding and transactivation of the mutated promoter is most likely due to several additional G or C rich sites, which are similar to Wt1(-KTS) consensus binding sites. As shown by ChIP, Wt1 binds the *AMHRII* promoter also in human UB27 cells. This demonstrates that Wt1 binds the *AmhrII* promoter not only *in vitro*, but also in living cells and that Wt1 binds the promoter of the murine as well as the human *AmhrII* gene. The EMSA analysis presented here also shows that the Sf1 sites described by Teixeira et al. are actually bound by Sf1, as was shown for the human promoter by De Santa Barbara et al. Similar to the observations by Teixeira et al. the proximal Sf1 site, which is not conserved in the human promoter, showed weaker Sf1 binding than the distal site, which is conserved (Fig. 9C, E).

[183] Hossain, A., and Saunders, G. F. (2003). Synergistic cooperation between the beta-catenin signaling pathway and steroidogenic factor 1 in the activation of the Mullerian inhibiting substance type II receptor. *J Biol Chem* **278,** 26511-6.

[184] Sierig, R. (2005). Die Rolle von Wt1 in der Regulation der AmhrII-Expression, Vol. Diploma Thesis. University of Würzburg.

None of the three fragments used for *Amhrll* promoter analysis in previous publications was shown to be sufficient to drive specific *Amhrll* expression *in vivo*. One possibility to make sure that these fragments contain all regulatory elements of the *Amhrll* promoter is to investigate expression of a *LacZ* gene driven by one of the promoter fragments in transgenic mice. Respective experiments are currently performed in our lab.

It has been described, that internal Müllerian duct structures are found in some male DDS patients with differentiated testes[185,186]. As these patients have differentiated testes, they express most likely *AMH*. The data presented here show that lack of *AMHRII* expression, due to inefficient activation by WT1, is a possible explanation for this phenotype in DDS patients.

The presented results show that the strategy used is suitable to identify yet unknown Wt1 target genes and provide a good basis for the identification of other new targets in addition to *Amhrll*. However, identification of potential target genes by microarray analysis is hampered by the fact that this technique leads to many false negative and false positive results. Therefore, microarray results have to be checked by independent methods, like qRT-PCR and RNA *in situ* hybridization. Despite this problem, microarray analysis is a potent tool to get an idea about new potential target genes. As mentioned before, this is restricted to early targets, if a constitutive knock-out mouse is used. To identify Wt1 targets during late gonad development, a conditional knock-out mouse would be needed.

[185] Goldman, S. M., Garfinkel, D. J., Oh, K. S., and Dorst, J. P. (1981). The Drash syndrome: male pseudohermaphroditism, nephritis, and Wilms tumor. *Radiology* **141,** 87-91.

[186] Manivel, J. C., Sibley, R. K., and Dehner, L. P. (1987). Complete and incomplete Drash syndrome: a clinicopathologic study of five cases of a dysontogenetic-neoplastic complex. *Hum Pathol* **18,** 80-9.

4.2 Dmrt8

Analyses done by Anne-Marie Veith show that, based on currently available data from public databases, *Dmrt8* is present in placental mammals only. Structural and sequence similarity of *Dmrt7* and *Dmrt8* are consistent with the hypothesis that *Dmrt8* originated by duplication of *Dmrt7*[187]. The analyses also indicate that the common ancestor of all placental mammalian *Dmrt8* genes was located on the X chromosome. Most species have one copy of *Dmrt8*, while at least three copies are present in rabbit, mouse and rat. Analysis of the DM domain encoding sequence of *Dmrt8* genes from different mammalian species shows that during evolution of *Dmrt8* genes several mutations have occurred within the DM domain encoding sequence. As a result, none of the analysed *Dmrt8* genes encodes a typical DM domain. While *Dmrt8* genes of cat, dog, cow, pig and rabbit encode a DM-like domain, a complete or partial loss of the DM domain has occurred in primates, mouse and rat (data not shown).

At present it can not be excluded that *Dmrt8* genes are only pseudogenes, as the existence of Dmrt8 protein has not been shown yet and no function of *Dmrt8* is known. However, the expression analysis presented here shows that *Dmrt8.1* and *Dmrt8.2* are expressed in a time and tissue specific manner. This is a first hint at a possible role of these genes in gonadal development and function. Despite the lack of the DM domain, *Dmrt8* genes are maintained on the X chromosome in the placental mammalian lineage. Therefore, potential Dmrt8 proteins might exhibit a DM domain independent function. Other functional domains located downstream of the DM domain might be present and important for Dmrt8 proteins. However, in vertebrates not much is known about the presence of other functional domains in Dmrt proteins, as there is only little conservation outside the DM domain[188]. In *Drosophila* DSX, carboxy-terminal domains are important for protein dimerization and recruitment of co-regulatory factors[189,190]. Thus, one possible role for DM domain-less

[187] Ottolenghi, C., Fellous, M., Barbieri, M., and McElreavey, K. (2002). Novel paralogy relations among human chromosomes support a link between the phylogeny of doublesex-related genes and the evolution of sex determination. *Genomics* **79,** 333-43.

[188] Volff, J. N., Zarkower, D., Bardwell, V. J., and Schartl, M. (2003). Evolutionary dynamics of the DM domain gene family in metazoans. *J Mol Evol* **57 Suppl 1,** S241-9.

[189] Erdman, S. E., Chen, H. J., and Burtis, K. C. (1996). Functional and genetic characterization of the oligomerization and DNA binding properties of the Drosophila doublesex proteins. *Genetics* **144,** 1639-52.

[190] Bayrer, J. R., Zhang, W., and Weiss, M. A. (2005). Dimerization of doublesex is mediated by a cryptic ubiquitin-associated domain fold: implications for sex-specific gene regulation. *J Biol Chem* **280,** 32989-96.

Dmrt8 proteins could be to act as negative regulator by competition for dimerization-partners or co-regulatory factors.

As the presented results demonstrate, *Dmrt8.1* and *Dmrt8.2* show a male-biased expression exclusively in testes of adult mice. *Dmrt8.1* is expressed in the testicular somatic Sertoli cells. Thus, *Dmrt8.1* might be involved in some Sertoli cell-mediated aspects of testicular function. In embryos, *Dmrt8.2* shows a dynamic expression exclusively in male and female gonads with a peak at E13.5 in males, two days after the peak of *Sry* expression. Thus, the time course of *Dmrt8.2* expression suggests a potential role of *Dmrt8.2* during male sex differentiation. After birth, expression of *Dmrt8.2* becomes restricted to testis, suggesting that *Dmrt8.2* might, similar to *Dmrt8.1*, also be expressed in Sertoli cells.

This is in line with the hypothesis that in addition to *Dmrt1*, other *Dmrt* genes are involved in sexual development in mouse[191]. Interestingly *Dmrt1* is expressed at similar levels in gonads of male and female mouse embryos from E11.5 to E13.5 and becomes male-specific at E14.5[192,193], an expression pattern similar to that observed for *Dmrt8.2*. In adults *Dmrt1* is expressed in Sertoli cells and germ cells[192,194]. Hence, one potential function of *Dmrt8* genes in mouse could be the direct or indirect regulation of the activity of *Dmrt1* or other *Dmrt* genes and thus promote male sexual development.

[191] Kim, S., Kettlewell, J. R., Anderson, R. C., Bardwell, V. J., and Zarkower, D. (2003). Sexually dimorphic expression of multiple doublesex-related genes in the embryonic mouse gonad. *Gene Expr Patterns* **3,** 77-82.

[192] Raymond, C. S., Kettlewell, J. R., Hirsch, B., Bardwell, V. J., and Zarkower, D. (1999a). Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. *Dev Biol* **215,** 208-20.

[193] Bouma, G. J., Hart, G. T., Washburn, L. L., Recknagel, A. K., and Eicher, E. M. (2004). Using real time RT-PCR analysis to determine multiple gene expression patterns during XX and XY mouse fetal gonad development. *Gene Expr Patterns* **5,** 141-9.

[194] Smith, C. A., McClive, P. J., Western, P. S., Reed, K. J., and Sinclair, A. H. (1999). Conservation of a sex-determining gene. *Nature* **402,** 601-2.

4.3 Sox9

The analysis of the role of Sox9 in sex determination and the search for Sox9 target genes in this process have been hampered by the early death of $Sox9^{-/-}$ embryos and the incomplete and/or late deletion in a first conditional knock-out. Our collaboration partners in Freiburg achieved efficient, homozygous inactivation of a conditional *Sox9* allele at the earliest stages of gonadal development by use of the *Ck19*:*Cre* line. In contrast to constitutively inactivated $Sox9^{-/-}$ embryos which die at E11.5 at the onset of testicular morphogenesis, some of the *Ck19*:*Cre*;$Sox9^{flox/flox}$ embryos survive up to at least E15.5. This allowed us to follow the fate of $Sox9^{-/-}$ XY gonads *in vivo* throughout this entire phase of testicular development and circumvented the necessity for organ culture as in the case of constitutively inactivated $Sox9^{-/-}$ XY gonads[195]. The results to which we contributed and the ex vivo organ culture study[195] both show that, in contrast to the situation in humans, complete XY sex reversal in mice requires inactivation of both *Sox9* alleles, and that *Sox9* is essential for testis induction to occur.

Efficient CRE-mediated excision of the *Sox9* alleles in mutant embryos is demonstrated by the qRT-PCR analysis. At E11.5, *Sox9* expression in the mutant gonads was not completely extinguished and was similar to the low levels detected in female controls, whereas at E12.5, *Sox9* expression in the mutants was even lower than in female embryos, while the results for female controls resemble the published expression pattern[196,197]. This indicates that CRE-mediated inactivation of *Sox9* was not complete but efficient enough to reduce *Sox9* expression to a level that causes complete, immediate XY sex reversal. Expression analyses by qRT-PCR have been performed using 5 mutant samples of E11.5 and 2 and 4 mutant samples of E12.5, respectively. As exemplified by *Sry*, gene expression during gonad development and differentiation is a very dynamic and stage-dependent process. In the presented results, this can be observed in case of *Sox9*, *Amh* and *Ptgds* expression in male E11.5 controls, which show a linear correlation with developmental stage, indicated

[195] Chaboissier, M. C., Kobayashi, A., Vidal, V. I., Lutzkendorf, S., van de Kant, H. J., Wegner, M., de Rooij, D. G., Behringer, R. R., and Schedl, A. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* **131,** 1891-901.

[196] Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., and Lovell-Badge, R. (1996). Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat Genet* **14,** 62-8.

[197] Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996). A male-specific role for SOX9 in vertebrate sex determination. *Development* **122,** 2813-22.

by the number of tail somites (Fig. 15A, 16A). This dynamic gene expression might be an explanation for the variability between the two experiments for E12.5 in which case the embryos were staged according to plug formation (Fig. 15B). Lower *Sox9* and *Foxl2* and higher *Sry* levels in the mutant gonads of the second experiment might be due to a slightly earlier developmental stage of the respective embryos.

The qRT-PCR results also show that, in contrast to wild-type male gonads, *Sry* expression is not turned off at E12.5 (and E13.5) but remains high in mutant XY gonads. These findings are in line with the previous observation of persistent *Sry* expression in E13.5 XY gonads of *Sf1*:*Cre*;*Sox9*$^{flox/flox}$ mice with low levels of *Sox9*[182]. It appears that one function of Sox9 is to down-regulate *Sry* expression, directly or indirectly, after it has itself been up-regulated by Sry.

The fact that the mutant XY gonads express *Foxl2* in a female-specific manner even though *Sry* expression persists beyond E11.5 furthermore indicates that downregulation of these early ovary-specific marker in wild-type XY gonads is not dependent on Sry but rather directly or indirectly dependent on Sox9, or both factors.

Recently it has been shown that Pre-Sertoli cells, expressing *Sry* and *Sox9*, can recruit other cells which do not express *Sry* as Sertoli cells by activation of *Sox9* expression via secreted prostaglandin D_2[198]. *Ptgds,* the gene coding for prostaglandin D synthase, the enzyme responsible for PGD_2 synthesis, is upregulated in Pre-Sertoli cells around the time point of sex determination. It has been speculated that PGD_2 acts cell autonomously upstream of Sox9 and leads to Sox9 nuclear import after induction by Sry and is therefore the missing link or one of the missing links between Sry and Sox9[199]. However, this has been doubted by others, as no change in the subcellular localization of Sox9 could be observed and Sox9 could not be detected in the cytoplasm of gonadal cells[198,200]. Inhibition of PGD_2 receptor inhibits only recruitment of additional Sertoli cells[185]. It does not inhibit testis differentiation, as does not knock-out of this receptor or knock-out of *Ptgds*[198,201,202]. This makes it

[198] Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Beverdam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev Biol* **287,** 111-24.

[199] Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F., and Boizet-Bonhoure, B. (2005). Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *Embo J* **24,** 1798-809.

[200] Sekido, R., Bar, I., Narvaez, V., Penny, G., and Lovell-Badge, R. (2004). SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. *Dev Biol* **274,** 271-9.

[201] Eguchi, N., Minami, T., Shirafuji, N., Kanaoka, Y., Tanaka, T., Nagata, A., Yoshida, N., Urade, Y., Ito, S., and Hayaishi, O. (1999). Lack of tactile pain (allodynia) in lipocalin-type prostaglandin D synthase-deficient mice. *Proc Natl Acad Sci U S A* **96,** 726-30.

unlikely that PGD_2 plays a role in cell autonomous activation of *Sox9*. However, the question remained whether *Ptgds* is upregulated directly by Sry or indirectly via Sox9 action. Using the *Ck19*:*Cre*;*Sox9*$^{flox/flox}$ mouse line we could show, that the expression of *Ptgds* is clearly dependent on Sox9, as its expression is very low in *Sox9* mutant and female gonads. This makes it unlikely that *Sox9* activity depends on PGD_2. It is more likely that PGD_2 acts downstream of Sox9 and is secreted by Pre-Sertoli cells after *Ptgds* was activated by Sox9 that itself was induced by Sry. *Ptgds* expression in wild-type XY gonads is therefore, similar to *Foxl2* inhibition, directly or indirectly dependent on Sox9.

In conclusion the data presented here demonstrate that *Sox9* is not only sufficient but also necessary for sex determination in mice. This suggests that Sox9, and not Sry, activates all downstream elements of the male sex determination cascade as well as inhibits female development and is therefore the master sex determining factor. This is in line with the more conserved role of *Sox9* in other vertebrate species[196,197], while Sry is only found in mammals[203]. The acquisition of Sry as an activator of the autosomal *Sox9* seems to be the consequence of the XY-chromosomal sex determination mechanism. Our results present an additional hint that the only function of Sry is to switch on *Sox9* expression and that *Sry* itself is turned off by Sox9 directly or indirectly as soon as its job is done.

[202] Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y., Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., and Narumiya, S. (2000). Prostaglandin D2 as a mediator of allergic asthma. *Science* **287,** 2013-7.

[203] Spotila, J. R., Spotila, L. D., and Kaufer, N. F. (1994). Molecular mechanisms of TSD in reptiles: a search for the magic bullet. *J Exp Zool* **270,** 117-27.

5 Appendices

5.1 Abbreviations

A	Adenine
Arg	Arginine
ATP	Adenosine triphosphate
bp	Base pairs
C	Cytosine
°C	Degrees Celsius
cDNA	copy DNA
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalo virus
C_T	Threshold cycle
C-Terminus	Carboxy-Terminus
Cys	Cystidine
DDS	Denys-Drash syndrome
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
E	day of embryonal development
EDTA	Ethylendiamin-tetra-acetic acid
E. coli	*Escherichia coli*
e.g.	exempli gratia
et al.	et alii
Fig.	Figure
g	Gram
G	Guanine
GST	Glutathione-S-transferase
HA	Hemaglutinin
His	Histidine
h	Hour

IP	Immunoprecipitation
IPTG	Isopropyl-thio-galactoside
K	Lysine
k	Kilo
kb	Kilo base pairs
ko	knock-out
M	Molar
mg	Milligram
µg	Microgram
min	Minute
ml	Millilitre
µl	Microlitre
mm	Millimetre
mM	Millimolar
µM	Mikromolar
mRNA	messenger-RNA
N-Terminus	Amino-Terminus
ng	Nanogram
nM	Nanomolar
PFA	Paraformaldehyde
PCR	Polymerase chain reaction
pH	potentium Hydrogenium
qRT-PCR	quantitative real-time RT-PCR
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcriptase
S	Serine
SDS	Sodium dodecylsulfate
sec	Sekunde
siRNA	Short interfering RNA
SV40	Simian virus 40
MCS	Multiple cloning site
T	Thymine
T	Threonine

Taq	Thermus aquaticus
Tris	Tris-hydroxymethyl-aminomethane
Trp	Tryptophan
ts	tail somites
V	Volt
v/v	Volume to volume
WAGR	Wilms Tumour, Aniridia, genitourinary malformations, mental retardation
w/v	Weight to volume
wt	Wild-type
z.B.	Zum Beispiel

5.2 Oligonucleotides

All sequences are shown in 5'=>3' orientation.

Type	Name	Sequence	Reference
Genotyping primers	Wt1-pgk	CTACCGGTGGATGTGGAATGTGT	Wilhelm & Englert[204]
	Wt1-shared	TCCCGAACAATTTCACCTTGAATC	Wilhelm & Englert[191]
	Wt1-wt	AGCCTAACTTTGGGGCTTATCTCC	Wilhelm & Englert[191]
	Zfy 5'	GACTAGACATGTCTTAACATCTGTCC	Hogan et al.[205]
	Zfy 3'	CCTATTGCATGGACTGCAGCTTATG	Hogan et al.[192]
qRT-PCR primers	actb_1f	TGTTACCAACTGGGACGACA	this work
	actb_2r	GGGGTGTTGAAGGTCTCAAA	this work
	AI426026-1f	ATACACAGTAGAGCACATCTCAGGT	this work
	AI426026-2r	GCTCTGCTTGATGTCCTTCTGC	this work
	AK003577-f1	TCGGCGAGTAGACAGAGTTGC	this work
	AK003577-r2	GAATGATAGCCCAGAAAGGAAGACC	this work
	aldh1a1-1f	GCTATATCATGTTGTCAGCCCAGT	this work
	aldh1a1-2r	TGCTGGTTACTATAGGAGATGTGT	this work
	amh-3f	ACCCTTCAACCAAGCAGAGA	this work
	amh-4r	CCTCAGGCTCCAGGGACA	this work
	amhr-1f	CCCAACATCCCATCCACTT	this work
	amhr-2r	GCTGAAAGGCAGTTCTCTGG	this work
	hAmhrII-1f	GGACCCCTACTCAACCACAA	this work
	hAmhrII-2r	ACAGGAGCAGAGCCAAAGAG	this work
	cbln1-1f	CAGAACGCAGCACTTTCATCGC	this work
	cbln1-2r	GCTCGGTCGCCTTTCTCCATC	this work
	cd59a-1f	TGATGGTACTTGCTTCCTTTGCTTAG	this work
	cd59a-2r	GTCCTAGAACTGGCTGGCTTCC	this work
	cst8-1f	TGTTTGGTTTGCCATGAAAG	this work
	cst8-2r	AGGGAAGTGCTCCTACCAGA	this work
	cst9-1f	CATTCAACCAGGAAAGTCAGG	this work
	cst9-2r	CAGTAAACAGGCAGGTGAAGG	this work
	dax1_3f	GGAGTCTGAACATTGACACCA	this work
	dax1-5r	ATGATGGGCCTGAAAAAGAG	this work
	dmrt2-1f	AGGCCACAGAGGACAAGAAG	this work
	dmrt2-2r	ACTGACAGGCGGAGGTAGAG	this work
	en1-3f	ACACAACCCTGCGATCCTAC	this work
	en1-4r	GCCGCTTGTCTTCCTTCTC	this work
	foxL2-1f	GCAGAAGCCCCCGTACTC	this work

[204] Wilhelm, D., and Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16,** 1839-51.

[205] Hogan, B. (1994). "Manipulating the mouse embryo: A laboratory manual." Cold Spring Harbor Laboratory Press, New York.

foxL2-2r	ATGCTATTCTGCCAGCCCTTC	this work
GAPDH-1f	AACTTTGGCATTGTGGAAGG	this work
GAPDH-2r	ACACATTGGGGGTAGGAACA	this work
gata4-1f	GCAGCAGCAGTGAAGAGATG	this work
gata4-2r	GCGATGTCTGAGTGACAGGA	this work
gata6-1f	CAGGCAGTGTGAGTGGAGGT	this work
gata6-2r	GAGTAGGTCGGGTGATGGTG	this work
gdf8-1f	CTCAAACAGCCTGAATCCAAC	this work
gdf8-2r	AGTCAAGCCCAAAGTCTCTCC	this work
h19-1f	AGACTAGGCCAGGTCTCCAG	this work
h19-2r	AGCCCATGGTGTTCAAGAAG	this work
hpgrt-1f	CCTAAGATGAGCGCAAGTTGAA	http://medgen31.rug.ac.be/primerdatabase/
hpgrt-2r	CCACAGGACTAGAACACCTGCTAA	http://medgen31.rug.ac.be/primerdatabase/
lhx9_1f	CAGCAGCCTTATCCACCTTC	this work
lhx9_2r	TATCAACACCCCCATTCTCC	this work
myf5-1f	CTGAGGGAACAGGTGGAGAA	this work
myf5-2r	GCTGGACAAGCAATCCAAG	this work
neo-3f	GAGTTCTTTATTTAATCTGGTGACTGA	this work
neo-4r	CATGGTTTCTGTCTTTATAAGTTCTG	this work
nm_027582-f1	GCAGCGTGGGATTGTCATTGTC	this work
nm_027582-r2	AGCATATCGGTAGTTTCTGTTCAGC	this work
Ptprv-f1	CAGTGGATGTCTTCAGTGTGGC	this work
Ptprv-r2	TGTTCCTCAATGCGCTGTTCAG	this work
qL-Ptgds-f	GGGCAGCATCCACTCCGTGTC	Malki et al.[206]
qL-Ptgds-r	GGGTGGCCATGCGGAAGT	Malki et al.[193]
sf1-7f	CCAGGAGTTCGTCTGTCTCAA	this work
sf1-8r	TCCACCAGGCACAATAGCA	this work
hSf1-3f	GGAGTTTGTCTGCCTCAAGTTCA	http://medgen31.rug.ac.be/primerdatabase/
hSf1-4r	CGTCTTTCACCAGGATGTGGTT	http://medgen31.rug.ac.be/primerdatabase/
Slc9a3r1-f1	CGAACAAGCCGAGCCTCCAG	this work
Slc9a3r1-r2	GCAGGTTGAAGCCATAGCCATTG	this work
sox9-3f	CGGAGGAAGTCGGTGAAGA	this work
sox9-4r	GTCGGTTTTGGGAGTGGTG	this work
sry_7f	GCAAACAGCTTTGTGGTCAA	this work
sry_8r	GGAAAAGGGGATGAAATGGT	this work

[206] Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F., and Boizet-Bonhoure, B. (2005). Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *Embo J* **24,** 1798-809.

	tac2-3f	TCTACGACAGCCGCCCTG	this work
	tac2-4r	TCGGTGGGAGTGTCTGGTT	this work
	tbp-1f	GGCCTCTCAGAAGCATCACTA	http://medgen31.rug.ac.be/primerdatabase/
	tbp-2r	GCCAAGCCCTGAGCATAA	http://medgen31.rug.ac.be/primerdatabase/
	tlx1-1f	GAGAGTAACCGCAGATACACAAAGG	this work
	tlx1-2r	GCTCCGCCGAAGCCAAGT	this work
	tnnc2-1f	CGGAGGAGACAACCCACA	this work
	tnnc2-2r	TCTGCCCTAGCATCCTCATC	this work
	tulp2-f1	GGGCCTCGGAAAATGACTGTG	this work
	tulp2-r2	TCTGTACGCGACTCAATATGGAATC	this work
	Vnn1-1f	GAGAAGCGAGCAGATGAGGT	this work
	Vnn1-2r	CATACCGGGTTCCAAAAGTG	this work
	wnt4-3f	ACTCCTCGTCTTCGCCGTGTT	this work
	wnt4-4r	ACATCTGCACCTGCCTCTGGAT	this work
	wt1-1f	AGTTCCCCAACCATTCCTTC	this work
	wt1-5r	TTCAAGCTGGGAGGTCATTT	this work
	zic1-1f	GGACACACACAGGGGAGAAG	this work
	zic1-2r	CTGTGAGCCCTGAGAAGAGG	this work
Riboprobes	AmhrII-1	CGTTTCTCCCAGGTAATCCA	Sierig[207]
	AmhrII-2	ATGGCGCATGACCTATCTTC	Sierig[194]
	cbln1-3f	ATGCTGGGCGTCGTGGAG	this work
	cbln1-4r	TCAGAGGGGAAACACGAGGAAT	this work
	dbx1-1f	TTATTTGGCATTTGGCCATT	this work
	dbx1-2r	TCCAGTGCTCTGCTTGATGT	this work
	dmrt2-3f	AGACATGATGCCACCCAAA	this work
	dmrt2-4r	GTGAGTGACGGCAGATGAAG	this work
	gdf8-3f	GGCCCAGTGGATCTAAATGA	this work
	gdf8-4r	CAGGCTGTTTGAGCCAATTT	this work
	muc-2r	AGGTAGAATCACTGTAACTTCCTCA	this work
	muc-EcoRV-1f	GATATCTGGCTCCACAGTGGTC	this work
	neo-3f	GAGTTCTTTATTTAATCTGGTGACTGA	this work
	neo-4r	CATGGTTTCTGTCTTTATAAGTTCTG	this work
	Ptprv-3f	GGAGCGCTCATTTTGTCTTC	this work
	Ptprv-4r	GATGCCCATTGCATATCTCC	this work
Gata4 expression construct	gata4-EcoRI	ATAGAATTCAAAGCCTGGCCATGGCCG	this work
	gata4-ApaI	ATTAGGGCCCTGATTACGCGGTGATTATGTCCG	this work
siRNA-template	Wt1si5	AAGGATACAGCACGGTCACTTCCTGTCTC	Sierig[194]
	Wt1si6	AAAAGTGACCGTGCTGTATCCCCTGTCTC	Sierig[194]

[207] Sierig, R. (2005). Die Rolle von Wt1 in der Regulation der AmhrII-Expression, Vol. Diploma Thesis. University of Würzburg.

ELISA	amhrII-p1	ATGGGAACCATCTTGGACAG	this work
	amhrII-p2	CAGCTGGACAGCATAAACCA	this work
	amhrII-p3	CAGTGCCCTGTATTTGTAGGA	this work
	amhrII-p4	CACAGAGACCGGGATAGGAC	this work
	amhrII-p5	GCCAAGGCTTCCTACAAATACA	this work
	amhrII-p6	TTGGAAGAAGGTGCTGGA	this work
	amhrII-p7	AGGTCCAGCACCTTCTTCC	this work
	amhrII-p8	AAGGGACCTTGGCTGTCC	this work
	amhrII-p9	CAGCTGGACAGCCAAGGTC	this work
	amhrII-p10	AAGGGAGGAGCAGAGAATCA	this work
	amhrII-p11	GAAAAGATTGATTCTCTGCTCCTC	this work
	amhrII-p12	GTCCTATCCCGGTCTCTGTG	this work
	amhrII-2a-f	AGGTCCAGCACCTTCTTCCAAGGTCAGTAG GGGTAGAGATTTCGTAGTCA	this work
	amhrII-2a-r	TGACTACGAAATCTCTACCCCTACTGACCT TGGAAGAAGGTGCTGGACCT	this work
	amhrII-2b-f	GGGTAGAGATTTCGTAGTCAATTATCACAG GGTTCTCAGCTGGACAGCCA	this work
	amhrII-2b-r	TGGCTGTCCAGCTGAGAACCCTGTGATAAT TGACTACGAAATCTCTACCC	this work
	amhrII-3a-f	GGTTCTCAGCTGGACAGCCAAGGTCCCTTC CTCCCCTCTC	this work
	amhrII-3a-r	GAGAGGGGAGGAAGGGACCTTGGCTGTCCA GCTGAGAACC	this work
	amhrII-3b-f	AGCCAAGGTCCCTTCCTCCCCTCTCCGAGG AGAAAAAGAGGAGGTAGCTG	this work
	amhrII-3b-r	CAGCTACCTCCTCTTTTTCTCCTCGGAGAG GGGAGGAAGGGACCTTGGCT	this work
	amhrII-3c-f	AGAAAAAGAGGAGGTAGCTGAAGAAAAGAT TGATTCTCTGCTCCTCCCTT	this work
	amhrII-3c-r	AAGGGAGGAGCAGAGAATCAATCTTTTCTT CAGCTACCTCCTCTTTTTCT	this work
ChIP	Amhrp-1f	GGTTCTCTGCTCCTCCCTTT	this work
	Amhrp-2r	GGCTTCTGATCCCTCAGTCC	this work
	Amhrp-3f	CCTCACATTGGTTTCCTCCT	this work
	Amhrp-4r	TGCAACCTCCTCTTTGCTCT	this work

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Lebenslauf

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Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.
Ich versichere außerdem, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Doctor rerum naturalium beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

Jena, den 02.01.2006

Jürgen Klattig