

# Mammalian sex determination—insights from humans and mice

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**Abstract** Disorders of sex development (DSD) are congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical. Many of the genes required for gonad development have been identified by analysis of DSD patients. However, the use of knockout and transgenic mouse strains have contributed enormously to the study of gonad gene function and interactions within the development network. Although the genetic basis of mammalian sex determination and differentiation has advanced considerably in recent years, a majority of 46,XY gonadal dysgenesis patients still cannot be provided with an accurate diagnosis. Some of these unexplained DSD cases may be due to mutations in novel DSD genes or genomic rearrangements affecting regulatory regions that lead to atypical gene expression. Here, we review our current knowledge of mammalian sex determination drawing on insights from human DSD patients and mouse models.

**Keywords** Disorders of sex development · sex determination · gonad development · gonad dysfunction

## Abbreviations

ACD Acampomelic campomelic dysplasia  
AMH Anti-Muellerian hormone  
AR Androgen receptor

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BMP2 Bone morphogenic protein 2  
BPES Blepharophimosis/ptosis/epicanthus  
inversus syndrome  
CAH Congenital adrenal hypoplasia  
CBLN4 Cerebellin 4 precursor  
CBX2 Chromobox homolog 2  
CHD Congenital heart defect  
CK-1 Casein kinase 1  
CNV Copy number variation  
CTNNB1  $\beta$ -catenin  
CYP19A1 Cytochrome P450 family XIX, sub-  
family, A, polypeptide 1 or aromatase  
DAX1 Dosage sensitive sex reversal adre-  
nal, hypoplasia critical region, X,  
chromosome gene 1  
DHH Desert hedgehog  
DMRT1 2, 3 Doublesex and mab-3 related,  
transcription factor 1, 2, 3  
Dmy DM-domain gene on the Y  
chromosome  
Dpc Day post coitum  
DSD Disorders of sex development  
E Embryonic day  
EMX2 Empty spiracles homeobox 2  
ems Empty spiracles  
ERK1 Mitogen-activated protein kinase 3  
ERK2 Mitogen-activated protein kinase 1  
FGF9 Fibroblast growth factor 9  
FGFR1 2 Fibroblast growth factor receptor 1, 2  
FOG2 Zinc finger protein multitype 2  
FOXD4 Forkhead box protein D4  
FOXL2 Forkhead box L2  
FST Follistatin  
GATA4 GATA binding protein 4

GNRHR	Gonadotropin-releasing hormone receptor
GSK3- $\beta$	Glycogen synthase kinase 3 $\beta$
HMG	High mobility group
HSD3B2	3 beta-hydroxysteroid dehydrogenase/Delta 5-4-isomerase type 2
IGFs	Insulin-like growth factors
IHH	Idiopathic hypogonadotrophic hypogonadism
IHH	Indian hedgehog
INHA	Inhibin $\alpha$ -subunit
INSL4 6	Insulin-like 4, 6 protein
LHX9	LIM homeobox 9
LMX1B	LIM homeobox transcription factor 1- $\beta$
LRP5 6	Low density lipoprotein receptor related, protein 5, 6
M33	Mouse Polycomb group member M33
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
Map3k4	Mitogen-activated protein kinase kinase kinase 4
MAPK	Mitogen-activated protein kinase
MPS	Massively parallel sequencing
NEIL2	Endonuclease VIII-like 2
NGS	Next generation sequencing
NR0B1	Nuclear receptor subfamily 0 group B, member 1
NR5A1	Nuclear receptor subfamily 5 group A member 1
Ods	Odsex
P	Postnatal day
P38	Mitogen-activated protein kinases 11, 12, 13 and 14
PIS	Polled/intersex syndrome
PTCH1	Patched 1
PGDS	Prostaglandin-D synthase
RHOA	Ras homolog gene family member A
RSPO1	R-spondin 1
SF1	Steroidogenic factor-1
SHH	Sonic hedgehog
SOX3 8, 9, 10	SRY-related HMG box 3, 8, 9, 10
STAR	Steroidogenic acute regulatory protein
SRY	Sex-determining region Y
TCF/LEF/ BCL9	Transcription factor/lymphoid enhancer-binding factor/B cell, CLL/Lymphoma 9 protein
TES	Testis-specific enhancer of <i>Sox9</i>
TESCO	Testis enhancer sequence core element
VNN	Vanin-1

WAGR syndrome	Wilms' tumor aniridia, genitourinary, anomalies and mental retardation, syndrome
WNT4	Wingless-type MMTV integration site family, member 4
WT1	Wilms' tumor 1
ZFPM2	Zinc finger protein multitype 2

## Introduction

In mammals, biological differences between males and females are determined genetically during embryonic development. These differences have a significant impact on the physical, reproductive, psychological, and social life of an individual. Sex development can be divided into two processes, “sex determination”, which is the developmental decision that directs the undifferentiated gonad to develop as a testis or ovary and “sex differentiation”, which occurs once the gonad has developed and is induced by the products of the gonad to establish the phenotypic sex. In mammals, sex determination equates to gonad development. Factors influencing sex determination tend to be transcriptional regulators, whereas factors influencing sex differentiation are most often secreted hormones and their receptors.

Most of our current knowledge on genes and their proteins involved in sex development comes from mutation studies in human patients with disorders of sex development (DSD) and mouse models. Here, we will discuss the current knowledge of gonad development and how changes to this complex developmental network result in cases of DSD. We will highlight genes that have been implicated in embryonic mouse gonad development and finally, discuss how whole genome sequencing approaches are going to improve our current understanding of the etiology of DSD.

## Disorders of sex development

DSD are congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (Hughes et al. 2006). DSD covers a wide spectrum of different phenotypes with hypospadias being the most common defect with an average of 1 in 250–350 male births. In addition, 1 in 4,500 babies

worldwide is born with significant ambiguous genitalia (Hughes et al. 2006) and significantly, DSDs account for 7.5% of all birth defects. Furthermore, DSD phenotypes are often associated with other syndromes, such as Mayer–Rokitansky–Kuster–Hauser syndrome, Smith–Lemli–Opitz syndrome or genitopalato-cardiac syndrome (Porter 2008; Sultan et al. 2009). Since the discovery of the sex-determining region Y (*SRY*) in 1990 (Sinclair et al. 1990), there have been considerable advances in understanding the genetic factors involved in gonad differentiation. Nevertheless, it has been estimated that a molecular diagnosis is made in only approximately 20% of DSD cases (Hughes et al. 2006), and that up to 50% of 46,XY DSD patients cannot be provided with an accurate diagnosis. Furthermore, for approximately 80% of 46,XY DSD complete gonadal dysgenesis patients and about 20% of 46,XX testicular DSD patients, the causative mutation remains unknown (Hughes et al. 2006). DSD represents a major pediatric concern, due to the difficulty of clinical management of these complex conditions and their common sequelae of gonad cancer and infertility. The cause of these DSD conditions is most often the breakdown of the complex network of gene regulation and gene expression, essential for proper development of testes or ovaries in the embryo.

### Gonad development in humans and mice and the molecular basis of DSD

In humans, both males and females have 22 pairs of autosomal chromosomes (mice have 19 pairs of autosomal chromosomes) but differ in their sex chromosome complement. Females in both humans and mice have two X chromosomes (46,XX in humans, 40,XX in mice), whereas males have one X and one Y chromosome (46,XY in humans and 40,XY in mice).

At the beginning of gestation (first and second week in humans), embryos of the two sexes differ only by their sex chromosomes. The first visible sign of sexual dimorphism in mammalian embryos is when the bi-potential gonad starts to develop into either a testis or an ovary in XY and XX individuals, respectively. This decision is made around embryonic day 10.5 (E10.5) in mice and 6 weeks of development in humans. Differentiation of the gonads leads to testicular and ovarian hormone

production and subsequent induction of anatomical and psychological differences.

Both testis and ovarian development involve sex-specific pathways that appear to act antagonistically to one another (see Fig. 1). The normal role of *SRY* in XY gonads is to tip the balance in favor of the testis-specific pathway (Sinclair et al. 1990; Lovell-Badge 1992). In mouse, *Sry* is transiently expressed in the bi-potential XY gonad (Koopman et al. 1990) and in both species, *SRY* expression initiates an up-regulation of *SOX9* expression. In mice, *SOX9* has been shown to stimulate *Fgf9* expression and subsequently, both *FGF9* and *SOX9* act together in a positive feedback loop, which are thought to suppresses *Wnt4* (by unknown mechanisms) and leads to the establishment of the testis-specific pathway.

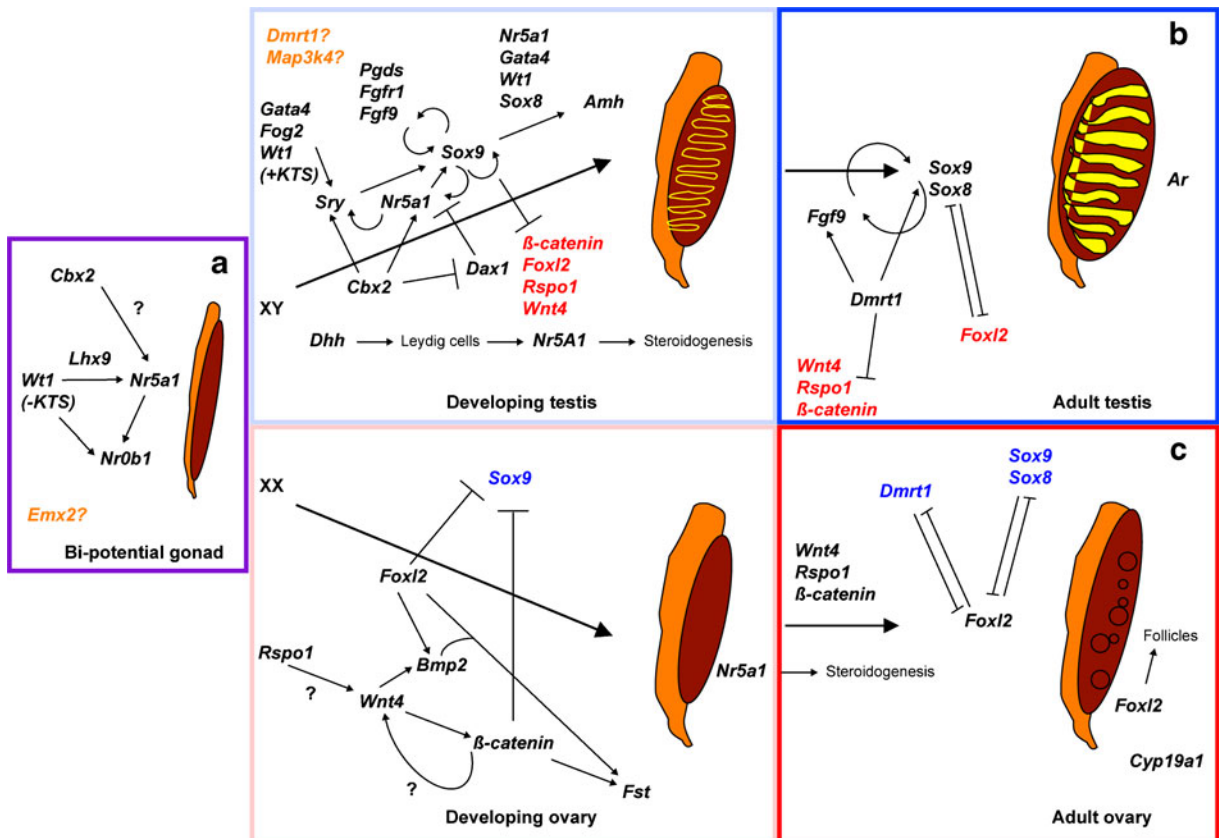
In the absence of *SRY* in XX individuals, *RSPO1* and *WNT4* are expressed at high levels and stabilize cytoplasmic  $\beta$ -catenin, which is then translocated into the nucleus, where it binds to the TCF/LEF (transcription factor/lymphoid enhancer-binding factor) and activates the transcription of target genes. Both *WNT4* and  $\beta$ -catenin suppress (by unknown mechanisms) the *SOX9/FGF9* positive feedback loop, allowing the ovarian-specific pathway to progress.

### Genes required for the development of the bi-potential gonad

Mammalian gonads arise in both sexes from a bilateral, bi-potential gonad (also called genital ridges), an organ that has the potential to develop either as an ovary or as a testis, depending on differentially expressed genes (reviewed by (Capel 1998; Swain and Lovell-Badge 1999; Capel 2000)). In mouse, the bi-potential gonads are first visible at E9.5, 1 day before the onset of *Sry* expression, which is critical for initiating testis development in XY individuals. A number of genes have been shown to be required for the development of the bi-potential gonad (Fig. 1a; see also Table 1 for a summary of mouse and human phenotypes of genes described in this section).

#### Empty spiracles homeobox 2

Empty spiracles homeobox 2 (*Emx2*) is a homolog of the *Drosophila* head gap gene empty spiracles (*ems*)



**Fig. 1** Overview of the key genes and regulatory networks leading from the bi-potential gonad to either testis or ovary development in mouse. **a** Genes that are essential for the development of the bi-potential gonad have been identified due to the total lack of gonads in the corresponding knockout mouse strain and they have been shown to be expressed in the bi-potential gonad. Functional studies revealed that specifically the WT1 –KTS isoform binds to and activates the *Nr5a1* promoter in conjunction with LHX9. In the spleen and adrenal gland, CBX2 has been shown to regulate *Nr5a1*, leading to the hypothesis of a similar function in the bi-potential gonad. **b** Various genes have been implicated in the pathways leading to testis development in mouse. In XY mouse embryos, *Sry* is transiently expressed in the bi-potential gonad, whereas in human embryos, *SRY* expression is periodic. However, in both species, *Sry* expression initiates an increase of *Sox9* expression, which then stimulates *Fgf9* expression. Both FGF9 and SOX9 act in a positive feedback loop, which act to suppress the female specific genes, especially *Wnt4* and subsequently lead to the manifestation of the testis-specific program. Numerous other genes and their gene products, such as *Gata4*, *Fog2*, *Wt1* (+KTS isoform), *Nr5a1*, *Pgsd*, *Fgf1*, *Cbx2*, *Sox8*, *Amh*, *Dax1*, and *Dhh* are necessary for the regulation (positive as well as negative) and maintenance of this

crucial male determining pathway. *Dmrt1* has recently been shown to be required for the maintenance of gonadal sex, especially to prevent female reprogramming in postnatal mouse testis. **c** In XX individuals, *Sry* is absent and ovary-specific genes, such as *Rspo1*, *Wnt4*, and *Foxl2* are expressed. *Rspo1* –/– ovaries show reduced levels of WNT4, suggesting that *Rspo1* acts upstream of *Wnt4*. However, a synergistic action of WNT4 and RSP01 to activate  $\beta$ -catenin has also been suggested. WNT4/ $\beta$ -catenin have been proposed to suppress the SOX9/FGF9 positive feedback loop, allowing the ovarian-specific pathway to progress. WNT4 and FOXL2 are also involved in the positive regulation of *Bmp2*. Together, FOXL2, RSP01, and WNT4 activate *Fst* expression. Genes of the female pathway that have been shown or suggested to interact with the male pathway are shown in red, genes of the male pathway interacting with the female pathway are highlighted in blue. In this figure, solid lines do not necessarily imply direct interactions. Question marks indicate that the position of that gene and the interaction with other genes has been proposed. Many pathways shown in this figure are similar or even identical between mouse and human; however, for some of them, there might be differences between the two species

and is essential for the development of dorsal telencephalon in mice (Yoshida et al. 1997). In addition, *Emx2* is expressed in the epithelial components of the developing urogenital system. The kidneys, ureters,

gonads, and genital tracts are completely absent in *Emx2* –/– mice, while the adrenal glands and bladder develop normally. However, a study by Taylor et al. (1999) failed to identify mutations within *EMX2* in

**Table 1** Genes involved in the development of the bi-potential gonad, testis development, and ovarian development in mice and humans

Gene	Protein function	Gonad phenotype in mouse models	Human syndrome/phenotype (DSD related)
<b>Genes involved in the development of the bi-potential gonad</b>			
<i>Emx2</i>	TXNF	<i>Emx2</i> <i>-/-</i> lacking kidneys, ureters, gonads and genital tracts	<sup>a</sup>
<i>Lhx9</i>	TXNF	<i>Lhx9</i> <i>-/-</i> fail to develop bi-potential gonad	<sup>a</sup>
<i>M33/CBX2</i>	TXNF	<i>Cbx2</i> <i>-/-</i> XY male-to-female sex reversal XX impaired ovary development	XY ovarian DSD
<i>Nr5a1</i>	Nuclear receptor/TXNF	<i>Nr5a1</i> <i>-/-</i> fail to develop bi-potential gonad	Embryonic testicular regression syndrome; XY gonadal dysgenesis XX premature ovarian failure (POF)
<i>Wt1</i>	TXNF	<i>Wt1</i> <i>-/-</i> mice fail to develop bi-potential gonad	Denys-Drash, WAGR, and Fraiser syndromes
<b>Genes involved in testis development</b>			
<i>Dhh</i>	Signaling molecule	<i>Dhh</i> <i>-/-</i> disruption of testis cord formation due to abnormal peritubular tissue	LOF: XY partial or complete gonadal dysgenesis
<i>Dmrt1</i>	TXNF	<i>Dmrt1</i> <i>-/-</i> impaired testis development (P2) and loss of Sertoli and germ cells	Hemizygosity: XY gonadal dysgenesis Deletion of 9p24 (including <i>DMRT1</i> ): XY gonadal dysgenesis (varying degrees)
<i>Fgf9</i>	Signaling molecule	<i>Fgf9</i> <i>-/-</i> XY male-to-female sex reversal and impaired development of Sertoli cells	<sup>a</sup>
<i>Fog2</i>	Cofactor of Gata4	<i>Fog2</i> <i>-/-</i> Reduced Sry levels, XY male-to-female sex reversal	Translocation including <i>FOG2</i> : XY hypergonadotropic hypogonadism combined with congenital heart defects (CHD)
<i>Gata4</i>	TXNF	<i>Gata4</i> <i>-/-</i> embryo lethality (E7–E9.5)  <i>Gata4</i> <sup>ki</sup> sever anomalies of testes	LOF: XY ambiguous genitalia or reduced phallus length Deletion downstream of <i>GATA4</i> (also including <i>NEIL2</i> ): XY complete gonadal dysgenesis combined with adrenal hypoplasia congenital (CAH)
<i>Map3k1</i>	Kinase	<sup>b</sup> (dependent on genetic background)	XY partial or complete gonadal dysgenesis
<i>Map3k4</i>	Kinase	XY male-to-female sex reversal	<sup>a</sup>
<i>Nr0b1</i>	Nuclear receptor	XY impaired testis cord formation and spermatogenesis (dependant on genetic background)	LOF/Deletions: Congenital adrenal hypoplasia (CAH) Duplications: XY gonadal dysgenesis with disorganized testis cords and hypogonadotropic hypogonadism
<i>Sox3</i>	TXNF	<sup>b</sup>	Duplications (including <i>SOX3</i> ) and deletion upstream of <i>SOX3</i> : XX testicular DSD
<i>Sox8</i>	TXNF	<i>Sox8</i> <i>-/-</i> reduced fertility <i>Sox8</i> <i>-/-</i> , <i>Sox9</i> <i>-/-</i> XY variable degree of male-to-female sex reversal	<sup>a</sup>
<i>Sox9</i>	TXNF	<i>Sox9</i> <i>-/-</i> XY male-to-female sex reversal  Ods (deletion upstream of <i>Sox9</i> ) XX female-to-male sex reversal Conditional knockout: XY ovarian development XX testicular development	LOF: XY gonadal dysgenesis combined with campomelic dysplasia (CD) GOF: XX female-to-male sex reversal (GOF) Duplications including <i>SOX9</i> : XX testicular DSD Translocation upstream of <i>SOX9</i> : XY ovotesticular DSD Duplication/triplication upstream of <i>SOX9</i> : XX testicular DSD Deletion upstream of <i>SOX9</i> : XY ovarian DSD with acampomelic campomelic dysplasia

**Table 1** (continued)

Gene	Protein function	Gonad phenotype in mouse models	Human syndrome/phenotype (DSD related)
<i>Sox10</i>	TXNF	<sup>b</sup>	(ACD), gonadal dysgenesis, female or ambiguous external genitalia Duplication encompassing <i>SOX10</i> (includes also other genes): XX masculinized or incompletely feminized
<i>Sry</i>	TXNF	<i>Sry</i> - XY male-to-female sex reversal <i>Sry</i> translocation: XX female-to-male sex reversal	LOF: XY ovarian DSD GOF/Translocation: XX testicular DSD
<b>Genes involved in ovary development</b>			
<i>Ctnnb1/β-catenin</i>	TXNF	Conditional knockout in Sertoli cells XX similar to <i>Wnt4</i> <sup>-/-</sup> and <i>Rspo1</i> <sup>-/-</sup>	<sup>a</sup>
<i>Foxl2</i>	TXNF	<i>Foxl2</i> <sup>-/-</sup> Premature ovarian failure <i>Foxl2</i> <sup>-/-</sup> , <i>Wnt4</i> <sup>-/-</sup> XX female-to-male sex reversal	BPES and premature ovarian failure (POF)
<i>Fst</i>	Inhibitor of activin	XX partial female-to-male sex reversal, coelomic vessel formation	<sup>a</sup>
<i>Rspo1</i>	Signaling molecule	XX partial female-to-male sex reversal, similar to <i>Wnt4</i> <sup>-/-</sup> and conditional <i>Ctnnb1</i> knockout	XX testicular and ovotesticular DSD Duplication of 1p (including <i>WNT4</i> and <i>RSPO1</i> ): XY gonadal dysgenesis (GOF)
<i>Wnt4</i>	Signaling molecule	XX Müllerian duct agenesis, testosterone synthesis, and coelomic vessel formation	Duplication of 1p (including <i>WNT4</i> and <i>RSPO1</i> ): XY gonadal dysgenesis (GOF) (male-to-female sex reversal) LOF: XX Müllerian duct agenesis, testosterone synthesis, and coelomic vessel formation

TXNF transcription factor

<sup>a</sup>No mutations described so far

<sup>b</sup>No gonadal phenotype

120 patients with either Kallmann syndrome or idiopathic hypogonadotrophic hypogonadism (IHH).

### LIM homeobox 9

The LIM homeobox 9 (*Lhx9*) is a member of the LIM homeobox gene family. In *Lhx* <sup>-/-</sup> mice, germ cells migrate normally, but somatic cells of the genital ridge fail to proliferate and a discrete gonad fails to form. As a result of the absence of testosterone and anti-Müllerian hormone, genetically male mice develop as phenotypic females (Luo et al. 1994; Birk et al. 2000). The expression of steroidogenic factor 1 (*Sf1* also known as nuclear receptor subfamily 5 group A member 1 (*Nr5a1*)), a nuclear receptor essential for gonadogenesis, is reduced to minimal levels in the *Lhx9*-deficient genital ridge, indicating that *Lhx9* may lie upstream of *Nr5a1* in a developmental cascade in mouse (Birk et al. 2000). Furthermore, an in vitro biochemical analysis showed that LHX9 has the potential to bind and trans-activate the

*Nr5a1* promoter in conjunction with WT1 (Wilhelm and Englert 2002). However, mutation analysis of *LHX9* in a range of human DSD patients (including bilateral gonadal agenesis) did not reveal any mutations (Ottolenghi et al. 2001).

### Mouse polycomb group member M33/chromobox homolog 2

Mouse polycomb group member M33 (*M33*) or chromobox homolog 2 (*Cbx2*) is the mouse ortholog of the *Drosophila Polycomb* gene. *Cbx2* <sup>-/-</sup> mice show male-to-female sex reversal in most XY animals and XX animals have either smaller or absent ovaries (Katoh-Fukui et al. 1998). Katoh-Fukui et al. (1998) suggested a role for *Cbx2* early during gonad development, prior to the critical time of sex determination and before expression of *Sry*. As CBX2 has been implicated in the regulation of *Nr5a1* expression in the adrenal gland (Katoh-Fukui et al. 2005), it has been

speculated that CBX2 may play a similar role in gonad development. A recent study by Biason-Lauber et al. (2009) identified a compound heterozygous loss-of-function mutation in the coding sequence of *CBX2* in a 46,XY girl with female external and internal genitalia (including ovaries). This study also supports a role for CBX2 in the trans-activation of *NR5A1*, and thus a role early during gonad development.

#### Nuclear receptor subfamily 5 group A member 1

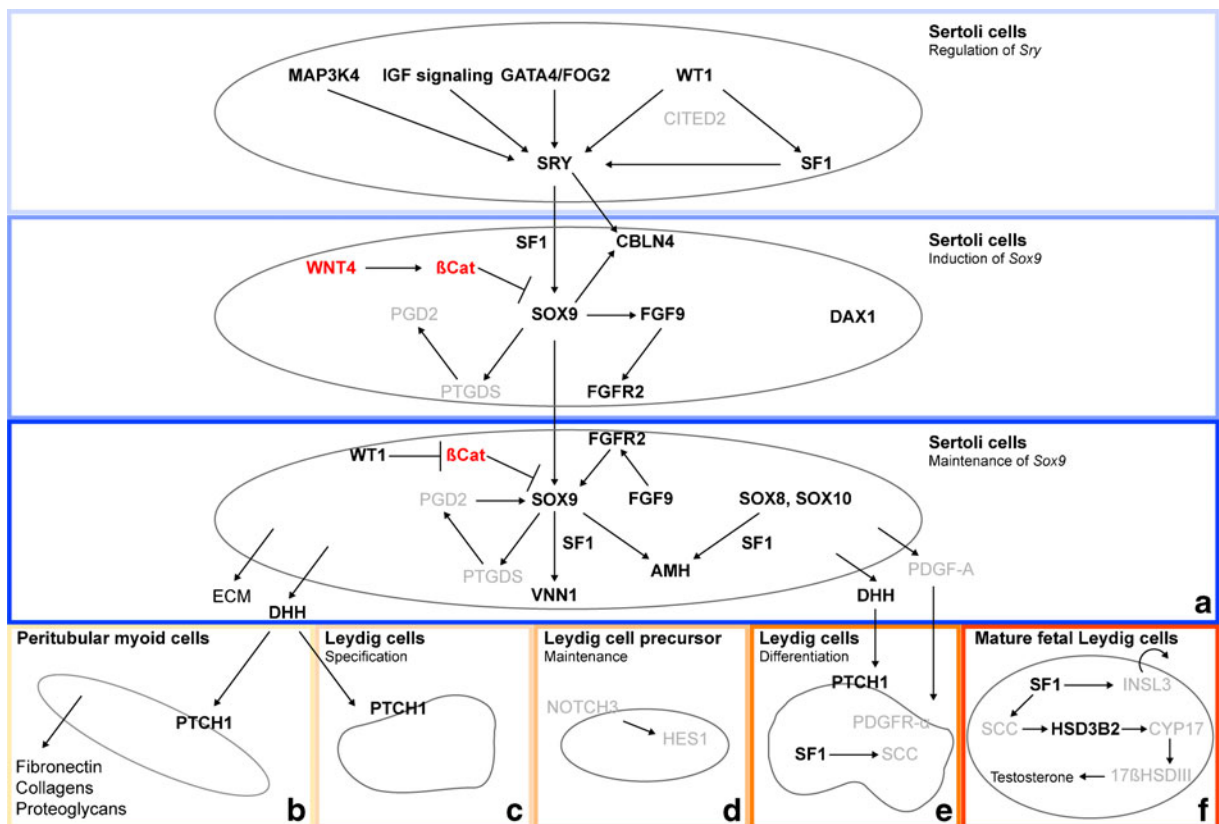
*NR5A1* encodes the nuclear receptor family member nuclear receptor subfamily 5 group A member 1, also known as steroidogenic factor-1 (SF1), which plays a role in gonadal and adrenal development (Val and Swain 2010). *NR5A1* is expressed in the urogenital ridge, the developing hypothalamus, and the anterior pituitary gland. Expression in the bi-potential gonad can be detected prior to *SRY* onset and functional reporter studies suggest that *NR5A1* acts to mediate the up-regulation of *SRY* (de Santa Barbara et al. 2001). Like the *Lhx9*  $-/-$  mice, *Nr5a1*  $-/-$  mice lack adrenal and gonadal development. Gonad development does not proceed beyond the early indifferent stage, which results in complete male-to-female sex reversal of XY animals, with the Müllerian ducts differentiating into uteri, oviducts, and upper vagina (Luo et al. 1994; Shinoda et al. 1995; Sadovsky and Dorn 2000). *NR5A1* also has additional roles in the gonad post-differentiation (see Fig. 2). NR5A1 is expressed in Sertoli, and Leydig cells of the developing testis. Together with SOX9, NR5A1 regulates the levels of *AMH* (Arango et al. 1999) and other genes associated with gonad development (Burriss et al. 1995). Furthermore, Sekido and Lovell-Badge (2008) have shown that NR5A1 binds synergistically with SRY to a testis specific enhancer region (TESCO) to up-regulate *SOX9* expression. SOX9 then displaces SRY and, together with NR5A1 causes further up-regulation of itself. Mutations in and around *NR5A1* cause 46,XY DSD gonadal dysgenesis (Achermann et al. 1999), whereas mutations in *NR5A1* in 46,XX individuals are associated with primary ovarian insufficiency (Lourenco et al. 2009).

A large deletion (3 Mb in size) of chromosome 9q34 that includes *NR5A1* and *LMX1B* (LIM homeobox transcription factor 1-beta) was identified in a 46,XY girl with clinical features of genitopatellar syndrome and ovotesticular DSD (Schlaubitz et al. 2007). van

Silfhout et al. (2009) found a smaller, 970 kb deletion which includes *NR5A1* in a 46,XY DSD female without skeletal features.

#### Wilms' tumor 1

Wilms' tumor 1 (*WT1*) encodes a zinc finger transcription factor, which is primarily expressed in embryonic mesodermal tissues such as the urogenital ridge, gonads, and mesonephros (Armstrong et al. 1993). *WT1* mutations in humans were first identified in patients with Wilms' tumor, a form of kidney cancer occurring primarily in children (Haber et al. 1990). This tumor is also seen as part of WAGR syndrome (Wilms' tumor, Aniridia, genitourinary anomalies, and mental retardation), a more complex syndrome including other clinical features such as aniridia, genitourinary anomalies, and mental retardation. The gonadal phenotype is recapitulated in other disorders, where mutations in *WT1* have been described such as Denys-Drash syndrome (including gonadal abnormalities and renal failure) (Pelletier et al. 1991; Lee et al. 2011) and Frasier syndrome (46,XY gonadal dysgenesis together with glomerulopathy) (Barboux et al. 1997). The Frasier patients have been found to carry mutations that results in the loss of the WT1+KTS isoform. This isoform has an additional three amino acids (K—lysine; T—threonine, and S—serine), which are located between the third and fourth zinc finger of WT1. The two different isoforms –KTS and +KTS have been shown to play distinct roles during embryogenesis. The –KTS isoform has been shown to bind to the *SRY* promoter region, leading to the transactivation of *SRY* (Hossain and Saunders 2001). Furthermore, the –KTS isoform of WT1 was shown to bind sequences within the *Nr5a1* promoter (Wilhelm and Englert 2002). Conversely, the +KTS isoform seems to play a role in the regulation of the *SRY* transcript. Knocking out this +KTS isoform of WT1 specifically, results in reduced *Sry* levels (Hammes et al. 2001) presumably mediated by its RNA binding affinity. There is also evidence that this isoform may function synergistically with NR5A1 to increase *Amh* expression (Arango et al. 1999), which is essential for inhibiting the development of the female Mullerian structures. Mice carrying a mutated *Wt1* gene fail to develop kidneys and gonads and often die at embryonic stages due to heart defects (Kreidberg et al. 1993).



**Fig. 2** Known genes and pathways of the different cell types of the developing testis in mouse (modified from Wainwright and Wilhelm 2010). **a** Postulated molecular pathways underlying Sertoli cell specification, including the regulation of *Sry*, induction of *Sox9* expression, and the maintenance of *Sox9* expression. **b** Differentiation of peritubular myoid (PM) cells is regulated by signaling from Sertoli cells via desert hedgehog (DHH) and its receptor patched 1 (PTCH1). In addition, *Dax1* expression in Sertoli cells is required for PM cell differentiation, but the molecular mechanism still remains to be elucidated. The interaction between Sertoli cells and PM cells results in the secretion of extracellular matrix (ECM) molecules by both cell types, which finally leads to the formation of basement membrane (BM). Both cell types contribute distinct components to the ECM, with PM cells secreting fibronectin, collagens, and proteoglycans. **c–f** The regulation of Leydig cell development via Sertoli cell and Leydig cell interactions. The morphogen DHH is secreted by Sertoli cells and induces Leydig cell specification through its receptor PTCH1. Signaling via the receptor NOTCH3 and its effector HES1 is crucial for the maintenance of the progenitor population and the

restriction of their differentiation to fetal Leydig cells. Platelet-derived growth factor A (PDGFA) signaling via the receptor PDGFR $\alpha$  plays a role in Leydig cell differentiation. In addition, Sertoli cell expressed DAX1/NR0B1 has been implicated in Leydig cell survival. As Leydig cells start to differentiate, they start expressing genes required for steroid synthesis, such as side chain cleavage (*Sc*). Differentiated Leydig cells synthesize testosterone using the four enzymes P450 side chain cleavage (SCC), 3- $\beta$ -hydroxysteroid dehydrogenase/Delta-5-4 isomerase type 2 (HSD3B2), cytochrome P450 17-hydroxylase (CYP17), and 17 $\beta$ -hydroxysteroid dehydrogenase 3 (17 $\beta$ HSDIII). The biosynthesis of testosterone leads to the masculinization of the developing embryo. Furthermore, fetal Leydig cells express insulin-like factor 3 (INSL3), which regulates testis descent. Both INSL3 and SCC are regulated by SF1/NR5A1 at the transcriptional level. Gene products from the female pathways known to interact with the male-specific pathway are shown in red. Solid lines in this figure do not indicate if the interactions occur in a direct or indirect manner. Genes/gene products shown in gray are not mentioned in

### Genes required for testis development

The following section will summarize key genes involved in testis development and their corresponding DSD phenotypes (see Table 1 for a summary of the human and mouse phenotypes of the genes described

below). Figure 1b illustrates known molecular pathways (from mouse studies) that are important for testis development and putative inhibitory interactions between testis and ovary pathways. Figure 2 shows genes involved in testis determination and maintenance of different testis cell populations.



## GATA binding protein 4 and zinc finger protein multitype 2

The evolutionary conserved GATA family of tissue- and organ-specific vertebrate transcription factors (GATA-binding proteins 1 to 6; GATA1 to GATA6) consists of two zinc finger domains. The two zinc fingers have been shown to be necessary for DNA recognition and binding (C-terminal zinc finger), the stabilization of the protein–DNA interaction (N-terminal zinc finger) and the protein–protein interactions of the GATA family members with other transcription co-factors (Evans and Felsenfeld 1989; Molkenkin 2000). All family members recognize the consensus target sequence (T/A)GATA(A/G). Of these six family members, two genes, *Gata2* and *Gata4*, are known to be expressed in the fetal mouse gonads. *Gata2* expression is detected between E10.5–15.5 in XX gonads and the mesonephros of XX and XY gonads, but its expression is missing in XX gonads lacking germ cells. At E13.5 the expression is restricted to germ cells of XX gonads, suggesting a role for GATA2 in ovarian germ cell development (Siggers et al. 2002). *Gata4* is the only GATA family member that is expressed in somatic cells (and not germ cells) in the bi-potential gonad (Heikinheimo et al. 1997; Viger et al. 1998). At E11.5, *Gata4* is expressed in the somatic cells of the bi-potential gonad of both sexes but becomes sexually dimorphic at E13.5, with its expression being up-regulated in XY Sertoli cells and down-regulated in interstitial cells. By contrast, *Gata4* expression is down-regulated in all cell types in XX gonads. *Gata4* expression is maintained in Sertoli cells postnatally and regained in adult ovaries (predominantly in granulosa cells) (Heikinheimo et al. 1997; Viger et al. 1998). GATA4 cooperatively interacts with several proteins, including NR5A1 and zinc finger protein multitype 2 (FOG2) (friend of GATA protein 2 or zinc finger protein, multitype 2; encoded by the *Zfpm2* gene) to regulate the expression of several sex-determining genes. Amongst those are *SRY*, *SOX9*, and *AMH*. Those complexes regulate key steroidogenic genes, such as *STAR* (encoding steroidogenic acute regulatory protein), *CYP19A1* (encoding aromatase), *INHA* (encoding inhibin  $\alpha$ -subunit), and *HSD3B2* (encoding 3 beta-hydroxysteroid dehydrogenase/delta 5-4-isomerase type 2) (Viger et al. 2008; Nishida et al. 2008; Miyamoto et al. 2008).

*Gata4*  $-/-$  mice die at around E7.0–E9.5 due to abnormalities in ventral morphogenesis and heart tube formation (Kuo et al. 1997; Molkenkin et al. 1997) so

analysis of gonadal differentiation is not possible. The role of GATA4 in gonad development has been highlighted by the use of *Gata4*<sup>ki</sup> mice that have a p.V217G mutation in the N-terminal zinc finger domain, which disrupts the protein–protein interaction of GATA4 with its cofactor FOG2 (Bouma et al. 2007). These mice have severe anomalies of testis development (Crispino et al. 2001; Tevosian et al. 2002).

In humans, mutations in *GATA4* have been associated with congenital heart defects (CHD), whereas other organs were described as being normal in all cases (Garg et al. 2003; Hirayama-Yamada et al. 2005; Nemer et al. 2006; Tomita-Mitchell et al. 2007). A recent study describes a heterozygous loss-of-function mutation in *GATA4* (p.G221R) in a family with three 46,XY DSD cases (with either ambiguous genitalia or reduced phallus length) and two 46,XX females with CHD. Functional studies showed failure of the mutated protein to bind and activate the *AHM* promoter and to bind to FOG2 (Lourenco et al. 2011). A 35-kb deletion downstream of *GATA4* was identified by White et al. (2011) in a patient with 46,XY complete gonadal dysgenesis and adrenal hypoplasia congenita. The authors argue that the deletion might affect a regulatory region of *GATA4*, which could in turn explain the lack of cardiac malformations in this patient.

In vitro, FOG2 represses GATA4-dependent transactivation of *AMH* in primary Sertoli cell cultures (Tremblay et al. 2001). Whether FOG2 acts as a transcriptional repressor or activator in the context of gonad development in vivo, in mouse as well as in human, still has to be elucidated, but a role in early gonadogenesis in mouse has been determined using *Fog2* null mice. These mice die mid gestation (around E14.5) from a cardiac defect and exhibit a block of gonad development (Crispino et al. 2001; Tevosian et al. 2002). Finelli et al. (2007) identified a translocation including *FOG2* in a male patient with hypergonadotropic hypogonadism, supporting a role for *FOG2* in mammalian sex determination.

## Sex-determining region Y

Testis development is characterized by the expression of *SRY*, the testis-determining gene on the Y chromosome, in the supporting cell lineage of the genital ridge (Bullejos and Koopman 2001; Wilhelm et al. 2005). *SRY* was identified using 46,XX testicular DSD male patients, who each had small translocations of material

from the Y to the X chromosome (Sinclair et al. 1990). Further studies with 46,XY DSD gonadal dysgenesis female patients revealed numerous point mutations in the *SRY* gene, demonstrating it was required for testis development. Finally, studies of transgenic mice, showed *Sry* was sufficient, on its own, to cause XX sex reversal (Koopman et al. 1991). Since then, numerous studies have shown that translocations of the *SRY* gene account for approximately 90% of 46, XX testicular DSD, whereas point mutations in *SRY* account for approximately 15% of 46,XY DSD gonadal dysgenesis cases (Cameron and Sinclair 1997). 46,XX testicular DSD is a condition in which genotypically female individuals develop as males. That is the gonads develop varying degrees of testicular tissue and may produce testosterone, leading to virilization of external genitalia. In 46, XY DSD gonadal dysgenesis patients, a dysgenic streak gonad develops instead of a testis.

The expression pattern of *SRY* and its resulting protein differs between the two species. In mice, *Sry* expression is limited to a transient period in the genital ridge of male embryos from E10.5 to E12.5, whereas *SRY* expression in human embryos starts at E41, peaks shortly after, and then remains present at low levels beyond embryogenesis (Hanley et al. 2000). In all mammals studied, *SRY* is a single-exon gene, but the protein can vary in size. *SRY* is part of the *SOX* gene family (*SRY*-related HMG box), a family of 20 genes in human and mice (Bowles et al. 2000; Schepers et al. 2002), which contain a highly conserved high mobility group (HMG) DNA-binding domain. Outside the HMG domain, *SRY* shows limited sequence conservation between species, suggesting that this DNA-binding domain is of critical importance. In DSD patients, the vast majority of *SRY* mutations are located within the HMG domain (Cameron and Sinclair 1997), suggesting that this is the most important domain. The *SRY* HMG domain contains signal sequences that can direct *SRY* to the nucleus, where it is assumed to play a role in transcriptional regulation (Poulat et al., 1995). This domain also mediates the sequence-specific DNA-binding ability of *SRY*, which results in the alteration of chromatin conformation (Giese et al. 1992). However, an additional C-terminal CAG repeat domain, which is not present in the human homolog, has been shown to be essential for male sex determination in the mouse (Bowles et al. 1999).

*SRY*-related HMG-box genes 9, 8, and 10—the *SoxE* genes

The *SOX* gene family consists of 20 members, which all encode for transcription factors. These genes have been divided into 10 subgroups, A to J, according to their sequence homologies (Bowles et al. 2000). Besides *SRY*, three other members of this gene family are expressed in the XY gonad (Cory et al. 2007; Polanco et al. 2010; Schepers et al. 2003). These members are *SOX8*, *SOX9*, and *SOX10*, which together form the subgroup E within this gene family.

*SOX9* was first shown to be associated with testis development in 1994, when mutations in and around *SOX9* were identified in human DSD patients (Foster et al. 1994; Wagner et al. 1994).

In mice, *Sox9* is expressed in the genital ridge at low levels at E10.5 (Morais da Silva et al. 1996) shortly after the expression of *Sry* in XY embryos and then shows a dramatic increase in expression levels. This temporal and spatial expression profile in mouse suggested that *Sox9* is either a direct or indirect downstream target of *SRY*. This is supported by the findings that only *Sry* expressing precursor cells of the bi-potential gonad show an increase in *Sox9* expression and subsequently develop into Sertoli cells (Sekido et al. 2004). *Odsex* (*Ods*) mice carry a 150-kb deletion, 1.3 Mb upstream of *Sox9* (Bishop et al. 2000). These mice show XX female-to-male sex reversal (dependent on the genetic background of mice) (Poirier et al. 2004) mediated by the up-regulation of *Sox9* and failed down-regulation in XX gonads, suggesting the presence of a distal repressor element that is deleted in *Odsex* mice. The existence of distal, long-range regulatory elements of *Sox9/SOX9* was confirmed by Sekido and Lovell-Badge 2008, who identified a testis-specific regulatory element in mouse. Subsequent findings in human DSD patients with genomic re-arrangements upstream of *SOX9* also point to novel gonad-specific *SOX9* regulatory elements (see below).

Downstream targets of *SOX9* have been identified, including the genes encoding anti-Müllerian hormone (*Amh*; (Arango et al. 1999)), Vanin-1 (*Vnn*; (Wilson et al. 2005)), *Pgds* (Wilhelm et al. 2007) and cerebellin 4 precursor (*Cbln4*; (Bradford et al. 2009)) (see Fig. 2).

Due to its role in chondrocyte differentiation, *SOX9* loss of function mutations lead to a combined phenotype of 46,XY DSD gonadal dysgenesis and campomelic dysplasia, a syndrome characterized by various

severe skeletal malformations (Foster et al. 1994; Wagner et al. 1994; Hanley et al. 2000). Duplications including *SOX9* have also been described in a 46,XX testicular DSD patient with complete sex reversal (Huang et al. 1999). 46,XY ovotesticular DSD associated with a 12;17 translocation upstream of *SOX9* has also been identified by Refai et al. (2010). Recent genome-wide studies, using a CGH array approach, have identified further genomic rearrangements upstream of the *SOX9* gene in patients with varying severity of both 46,XX and 46,XY DSD. In 46,XY DSD patients, a number of deletions upstream of *SOX9* have been identified. Pop et al. (2004) identified a 1.5-Mb de novo deletion in a patient affected by the acampomelic form of campomelic dysplasia (ACD) and 46,XY DSD (complete female phenotype) and Lecoindre et al. (2009) identified an inherited 960 kb deletion 517 kb to 1.477 Mb upstream of *SOX9* in a family affected by ACD combined with 46, XY DSD gonadal dysgenesis in a 46,XY DSD patient and the ACD-affected mother. White et al. (2011) identified a 1.2-Mb deletion in a 46,XY DSD patient with gonadal dysgenesis but without skeletal malformations. Benko et al. (2011) identified a 240-kb deletion upstream of *SOX9* in two cousins affected by isolated 46,XY DSD (one with a complete female phenotype, the other one with ambiguous genitalia). In this familial case, the deletion was inherited by the mothers (who were sisters and both normal females).

In 46,XX DSD cases, a gain in copy number (duplications, as well as a triplication) upstream of *SOX9* has been described in a number of patients. Cox et al. (2011) presented an extremely rare familial case of 46,XX complete sex reversal, where two brothers and their parental uncle were diagnosed with 46,XX testicular DSD (*SRY* negative and without any other developmental or skeletal phenotypes). Genetic analysis revealed a 178-kb duplication 600 kb upstream of *SOX9*. Vetro and colleagues (2011) identified a 96-kb triplication approximately 500 kb upstream of *SOX9* in two 46,XX azoospermic brothers (both *SRY* negative) and Benko et al. (2011) identified three familial cases of isolated 46,XX DSD with duplications upstream of *SOX9* of varying size. It should be noted that XX humans and mice show the opposite phenotypes where there is a loss of copy number upstream of *SOX9*/*Sox9*. In humans, a gain of copy number results in female-to-male sex reversal, whereas the Odsex mouse model shows female-to-male sex reversal when

they lose one copy of the *Sox9* upstream region (see above). The 1.9-Mb upstream region of *SOX9* is a gene desert, which is extremely conserved in mammals. Sekido and Lovell-Badge defined a 3.2-kb testis-specific enhancer of *Sox9* (TES) in mouse (Sekido and Lovell-Badge 2008), which is located in this gene desert, but not affected by the above mentioned CNVs in DSD patients. Further studies in mouse helped to refine this testis-specific region further to 1.4 kb, which is referred to as Testis Enhancer Sequence core element (TESCO). Functional analysis showed that *SRY* and *NR5A1* were required to bind to this enhancer region in order to initiate *Sox9* up-regulation. TESCO seems to play an important role in the testis-specific expression of *Sox9* in mice. However, in human DSD patients, the range of deletions, duplications, and translocations described above (see references above and review by (Gordon et al. 2009)), suggests the involvement of multiple testis-specific regulatory elements that are not functionally conserved between human and mouse. Aligning all the CNVs that have currently been identified in DSD patients, results in a 78-kb minimal overlap region, which is located 517–595 kb upstream of the *SOX9* promoter (Benko et al. 2011). This region is a highly likely to contain one or several gonad specific regulatory elements, which, when affected by CNVs, lead to DSD of variable severity, depending on the number and nature of the regulatory elements being affected. However, another possible scenario may be that the changes, especially when occurring, e. g., in tandem, influence the genomic architecture needed to activate *SOX9* in the proper spacio-temporal manner essential for gonad development. A recent study screened 66 patients with 46,XY DSD gonadal dysgenesis for mutations in the human, TESCO-corresponding region, but failed to identify point mutations in this regulatory region (Georg et al. 2010).

Conditional knockout of *Sox9* leads to ovary development in XY mouse embryos (Chaboissier et al. 2004; Barrionuevo et al. 2006) and over-expression of *Sox9* in XX embryos results in the development of testis (Bishop et al. 2000; Vidal et al. 2001). The importance of *Sox9* is highlighted in that all reported cases of XY sex reversal show a disturbance in the expression level or function of *SOX9*. Therefore, it is surprising that *Sox9* expression does not seem important for the maintenance of Sertoli cell fate and testis cord integrity. XY embryos with null mutations of *Sox9* show normal

embryonic testis development after E13.5 and are initially fertile, but become infertile at the age of about 5 months (Barrionuevo et al. 2009). This demonstrates that SOX9 is dispensable at later stages of testis development, which may be due to redundancy of the related factors SOX8 and SOX10. The importance of *Sox9* in testis differentiation, along with the close evolutionary relationship and the expression patterns it shares with the two other *SoxE* family members, raises questions about the functional role of *Sox8* and *Sox10* during gonadogenesis.

Initially, *Sox8* was shown to be specifically expressed in the testis cords at E13.5. This and its ability to induce *Amh* (anti-Muellerian hormone) expression in vitro, suggested a role of this member of the SOX gene family member in male sex determination, testicular differentiation, or germ cell development (Schepers et al. 2002, 2003). However, the first studies of *Sox8* ablated mice failed to result in abnormal sexual development and resulted only in idiopathic weight loss and reduced bone density phenotypes (Sock et al. 2001). Closer analysis of *Sox8*  $-/-$  mice by O'Bryan et al. (2008) showed that *Sox8*  $-/-$  males rarely produced litters, while *Sox8*  $+/-$  males and *Sox8*  $-/-$  females appeared reproductively normal. This study shows an essential role for *Sox8* in the maintenance of male fertility beyond the first wave of spermatogenesis. Loss of *Sox8* resulted in progressive degeneration of the seminiferous epithelium through perturbed physical interactions between Sertoli cells and the developing germ cells. Double knockout studies of *Sox8* and *Sox9* showed that SOX8 reinforces SOX9's role in testis differentiation (Chaboissier et al. 2004; Barrionuevo et al. 2006). Double knockout testis showed mainly complete absence of testis cord formation (tissue-specific *Sox9*<sup>fllox/fllox</sup> testis showed a variable degree testis cord formation and abnormal coelomic vessel formation) (Chaboissier et al. 2004). These results, together with the above mentioned in vitro studies regarding SOX8's ability to bind and synergistically activate *Amh* expression alongside NR5A1, support the idea of redundancy for SOX8 and SOX9 in testis differentiation. It has been postulated that the interchangeable roles of SOX8 and SOX9 are due to their shared ancestry and high sequence, as well as structural homology (Koopman 2005).

In *Sox10*-null mice, no testis phenotype has been described. *Sox10*, over-expression studies in XX gonads showed that SOX10 is sufficient to induce testis differentiation (Polanco et al. 2010), suggesting that, although

it might not be necessary for testis formation, *Sox10* still can function as a testis-determining gene. Human 46, XX testicular DSD patients who are masculinized or incompletely feminized and who have a duplication of the region encompassing *SOX10*, amongst a number of other genes, have been described (Aleck et al. 1999; Cantu et al. 1981; Nicholl et al. 1994; Seeherunvong et al. 2004). The possibility that other genes within this duplicated region are responsible for this phenotype, either solely or in combination with *SOX10*, cannot be excluded. However, *SOX10* is a strong candidate for the causative gene within this region. It is interesting to note that *Sox9* is upregulated in *Sox10*-transgenic XX gonads. It would be worthwhile investigating whether overexpression of *Sox8* and or *Sox10* would be sufficient to rescue the *Sox9* knockout phenotype.

#### Desert hedgehog

Desert hedgehog (DHH) is a member of the hedgehog family of signaling molecules, which also includes sonic hedgehog (SHH) and indian hedgehog (IHH) (Ingham 1998). Of the three family members, *Dhh* is the only one that is expressed in somatic cell population of the developing XY mouse gonad from E11.5 and continues later in Sertoli cells. No expression can be detected in XX ovaries at any stage (Bitgood et al. 1996; Yao and Capel 2002; Beverdam and Koopman 2006). DHH binds to its receptor Patched 1 (PTCH1), which is expressed shortly after DHH. PTCH1 is bound to the membrane of Leydig and peritubular myoid cells, and its expression is up-regulated via DHH (Clark et al. 2000; Yao and Capel 2002). *Dhh* null mice show disrupted testis cord formation due to abnormal peritubular tissue (Clark et al. 2000; Pierucci-Alves et al. 2001). DHH seems to be necessary for the up-regulation of *Sf1* in Leydig cells (Yao et al. 2002). Several mutations of *DHH* have been described in patients affected by 46, XY partial or complete gonadal dysgenesis. The first case was described by Umehara et al. (2000). The patient was affected by 46,XY partial gonadal dysgenesis and minifascicular neuropathy. A homozygous missense mutation at the initiation codon in exon 1 (c.T2C; p.M1T) was identified in the patient. The father carried the same mutation in a heterozygous state, showing that the phenotype displays a recessive mode of inheritance. Further studies identified a homozygous substitution in exon 2 (c.T485C; p.L162P) in one patient with 46,XY complete gonadal dysgenesis (CGD)

and a homozygous frame-shift deletion in exon 3 (c.1086delG), which results in a premature stop codon four codons after the deletion in two patients with 46,XY CGD (Canto et al. 2004). The later mutation has also been identified in two patients with 46,XY partial gonadal dysgenesis (PGD), but in a heterozygous state (Canto et al. 2005). Recently, Das et al. (2011) described two novel heterozygous mutations (c.271\_273delGAG; p.D60del and c.57\_60dupAGCC resulting in premature translational termination) in *DHH* in two patients affected by 46,XY CGD.

#### Nuclear receptor subfamily 0 group B, member 1

The dosage sensitive gene nuclear receptor subfamily 0 group B, member 1 (*NROB1*) (dosage sensitive sex reversal, adrenal hypoplasia critical region, X chromosome gene 1), encodes DAX1 on chromosome Xp21.2. Mutations and deletions of *NROB1* result in congenital adrenal hypoplasia (CAH), whereas duplications of *NROB1* in 46,XY individuals lead to gonadal dysgenesis and a female phenotype (Bardoni et al. 1994). CAH patients develop testis, but the testis cords appear to be disorganized and hypogonadotropic hypogonadism occurs in these patients. Duplications of *NROB1* have been described in a small number of other 46,XY gonadal dysgenesis cases. Two studies identified duplications of *NROB1* in patients with isolated 46,XY DSD complete gonadal dysgenesis (Barbaro et al. 2007; White et al. 2011) and a unique 257 kb deletion located 250 kb upstream of *NROB1* was reported in a 46,XY female with complete gonadal dysgenesis (Smyk et al. 2007). This deletion presumably affects regulatory elements of *NROB1* and thus results in dysregulation of its expression. As duplications of *NROB1* resulted in 46,XY male-to-female sex reversal, it led to the concept of *NROB1* as an “anti-testis” gene. However, XY *NrOb1* null mice also show sex reversal, which suggest a potential “pro-testis” role of *NrOb1*. Ludbrook and Harley (2004) propose a dosage-based mechanism that allows both a pro- and anti-testis function in an attempt to resolve these apparently contradictory roles of *NROB1* (Ludbrook and Harley 2004).

#### Doublesex and mab-3 related, transcription factor 1

Doublesex and mab-3 related, transcription factor 1 (*DMRT1*) is located at the tip of chromosome 9p in

human and encodes the transcription factor DMRT1 (doublesex and mab-3 related transcription factor 1). In some vertebrates, *DMRT1* is up-regulated in the developing genital ridge of XY embryos (Moniot et al. 2000; Smith et al. 1999) with continued expression confined to Sertoli cells. Interestingly, *DMRT1* represents one of the few sex determining genes that is shared between different species and phyla. Orthologous genes are found in a medaka species (*Dmy*) on the Y chromosome (Lutfalla et al. 2003) and in chicken (*Dmrt1*) on the Z sex chromosome (Nanda et al. 1999). Both, *Dmy* in medaka and *Dmrt1* in chicken have been shown to be testis-determining genes (Matsuda et al. 2002; Smith et al. 2009). Interestingly, in platypus, a single copy of *Dmrt1* is located on chromosome X5 (Grutzner et al. 2004) although its function, if any, in sex determination is unknown. All these genes encode a protein containing a DM domain as a DNA-binding motif (Raymond et al. 1998). In chicken and medaka, these genes are believed to initiate the molecular network leading to testis development (Smith et al. 2009; Matsuda et al. 2002; Nanda et al. 2002). In humans and mice, *DMRT1* is required for the postnatal maintenance of Sertoli and germ cells. Matson et al. (2011) have recently shown in mouse that *Dmrt1* and *Foxl2* form another regulatory network necessary for maintenance of the testis during adulthood. Loss of *Dmrt1* in mouse Sertoli cells leads to the upregulation of *Foxl2*, amongst other genes. This induces the reprogramming of Sertoli cells into granulosa cells. Subsequently theca cells form, oestrogen is produced and germ cells appear feminized.

*Dmrt1*-null mice develop normal gonads but show severely impaired testis development from postnatal day 2 resulting in dysgenic testes (Kim et al. 2007a; Raymond et al. 2000) and feminized germ cells (Matson et al. 2011). Similarly, human patients with *DMRT1* hemizyosity are associated with dysgenic testis and 46,XY DSD gonadal dysgenesis (Crocker et al. 1988; Raymond et al. 1998; Veitia et al. 1998). In humans, heterozygosity of *DMRT1* is associated with DSD. Deletions of chromosome 9p24 are associated with varying degrees of 46,XY DSD gonadal dysgenesis in about 70% of cases with this chromosomal rearrangement (Barbaro et al. 2009; Raymond et al. 1999; Calvari et al. 2000). This region contains: *DMRT1*, *DMRT2*, and *DMRT3* and other genes, such as *FOXD4* (Forkead box protein D4), *INSL4*, and *INSL6* (insulin-like 4, 6 protein). Mutations or rearrangements of *DMRT1* are the most likely cause of the 46,XY DSD phenotypes. However, in

humans, there have been no mutations identified within the *DMRT1* gene itself, yet.

### Fibroblast growth factor 9

*FGF9* encodes fibroblast growth factor 9 (FGF9), one of a number of growth factors that play a role in various developmental processes such as cell proliferation, cell survival, cell migration, and cell differentiation. *Fgf9* is expressed in the bi-potential gonad immediately after the expression of *Sry*. Mice null for *Fgf9* show male-to-female sex reversal along with impaired development of Sertoli cells. However, this is only evident on some genetic backgrounds, but not on others (Colvin et al. 2001; Schmahl et al. 2004). In the absence of *Fgf9*, *Sox9* expression is not maintained, Sertoli cells fail to differentiate, testis development is aborted and the resulting somatic cells express genes characteristic for ovarian development and the female pathway. There is data supporting the concept that SRY and NR5A1 initiate a positive feedback loop by up-regulating SOX9, which in turn up-regulates FGF9, further increasing *Sox9* expression (Kim et al. 2006). FGF9 mediates its molecular function through the membrane-bound receptor FGFR2 (fibroblast growth factor receptor 2). FGFR2 is integrated in the plasma membrane of progenitor Sertoli cells and is critical for Sertoli cell proliferation and differentiation in the developing testis. Although no mutations in *FGFR2* have yet been reported in human DSD patients (Kim et al. 2007b), due to its crucial role in Sertoli cell proliferation and differentiation, mutations in this gene could account for some unexplained cases of DSD.

### A new role for MAP kinases MAP3K1 and Map3k4 in gonad development

In a linkage study of a large family with DSD, a putative testis determining gene was mapped to a region on the long arm of chromosome 5 (Jawaheer et al. 2003). A follow-up study identified a mutation in the gene *MAP3K1*, which segregated with the phenotype in the family. Further mutations within the *MAP3K1* gene were identified within a second family and two out of eleven sporadic cases analyzed. The mutations found in the first family and in the two sporadic cases were shown to alter the phosphorylation of MAP3K1 downstream targets, such as p38 and ERK1/2, and to enhance the binding of RHOA to the MAP3K1 complex.

*Map3k1* is expressed in the mouse gonad prior to, and following, sex determination but *Map3k1* null mice do not show a gonadal phenotype. The spatiotemporal expression profile of *Map3k1*, together with the identification of mutations within the *MAP3K1* gene in cases of human 46,XY DSD strongly implicates the mitogen-activated protein kinase (MAPK) pathway in normal human and mouse sex determination (Pearlman et al. 2010). However, a mutation in another member of the MAPK family, *Map3k4*, was recently shown to be associated with XY sex reversal in mice, providing further evidence, that mutations in genes of the MAPK signaling pathways may be associated with DSD (Bogani et al. 2009; Bashamboo et al. 2010).

### Genes required for ovary development

While many genes have been identified as part of the testis developmental pathways, until recently little was known about the molecular mechanisms underlying ovarian development. Figure 1c gives an overview of the current knowledge of molecular mechanisms leading to ovarian development (see also Table 1 for a summary of the mouse and human phenotypes of genes that are described below).

#### Wingless-type MMTV integration site family, member 4

*WNT4* encodes member 4 of the wingless-type MMTV integration site family, a family of locally acting signaling molecules that are known to play a role in a range of developmental processes. *Wnt4* is expressed in the mesonephric mesenchyme and coelomic epithelium in mouse as early as E9.5. At E11, it is expressed in the mesonephros and the bi-potential gonad of both sexes but is then downregulated in the developing testis at around E11.5. The expression persists in the mesonephros as well as the developing ovary and the mesenchyme surrounding the Müllerian ducts (Stark et al. 1994; Vainio et al. 1999). WNT4 plays a role in Müllerian duct formation, oocyte development, and sex-specific patterning of the vasculature (Heikkila et al. 2005). Lack of *Wnt4* in the mouse results in partial female-to-male sex reversal, suggesting that WNT4 acts to positively regulate ovary differentiation. The gonads of XX embryos appear to be masculinized, round shaped, unencapsulated, and associated with a fat body but do not form testis cords or express Sertoli cell markers. These

mice lack Müllerian ducts and retain Wolffian ducts, have degenerating oocytes, and produce testosterone (Vainio et al. 1999). A human patient resembling this *Wnt4* null mouse phenotype was shown to carry a mutation in *WNT4* (Biason-Laubier et al. 2004). Ectopic over-expression of *Wnt4* in mice resulted in disruption of testis-specific vasculature (the coelomic vessel still forms, but the structure and branching seem to be abnormal) and inhibition of testosterone synthesis in XY embryos (Jordan et al. 2003). As the coelomic vessel forms in XX embryos with *WNT4* over-expression, it suggests either, that *WNT4* is not the only factor required to suppress male-specific vasculature or that the testes express another factor that is able to compensate for *WNT4*-mediated repression. Yao et al. (2004) propose that *WNT4* regulates vascular boundaries and the maintenance of germ cell survival in the ovary via *follistatin* (*Fst*) as both *Wnt4* and *Fst* null ovaries develop the male-specific coelomic vessel and show a substantial apoptotic loss of germ cells at E16.5. *Wnt4*  $-/-$  ovaries show a loss of *Bmp2* and *Fst* expression between E12.5 and E14.5 and *Fst*  $-/-$  ovaries (Matzuk et al. 1995) have normal *Bmp2* and *Wnt4* expression within that time frame. These results suggest that *WNT4* acts upstream of (and directly or indirectly activates) *Bmp2* and *Fst* in the developing ovary (Yao et al. 2004). *Wnt4*  $-/-$  XY embryos do not show complete sex reversal, suggesting that inactivation of additional genes are required to disrupt the male-specific pathways and promote female ovarian development (Kim et al. 2006; Kim and Capel 2006).

Studies of in vitro cultured XX gonads showed that addition of exogenous FGF9 induces *Sox9* and blocks *Wnt4* expression. In *Wnt4*  $-/-$  XX gonads *SOX9* and FGF9 were found to be transiently up-regulated between E11.5 and E12, indicating that *WNT4* would usually act as repressor of the male pathway by suppressing *Sox9* up-regulation. However, no mechanism has been proposed to explain how this interaction occurs (Kim et al. 2006).

Human patients with duplications of chromosome 1p, including *WNT4*, show a broad range of gonadal anomalies including complete sex reversal (Jordan et al. 2001).

The Wnt morphogens are known to signal through binding to integral membrane-bound co-receptors LRP5 and LRP6 (low-density lipoprotein receptor-related protein 5 and 6). These co-receptors heterodimerize with the integral membrane receptor Frizzled and thus

transfer the signal into the cytoplasm of the cell. Within the cell, this leads to the dephosphorylation of cytoplasmic  $\beta$ -catenin (via the glycogen synthase kinase 3 beta/casein kinase 1 (GSK3- $\beta$ /CK-1) complex), which results in the stabilization and accumulation of  $\beta$ -catenin in the cytoplasm. At a certain threshold, dephosphorylated  $\beta$ -catenin enters the nucleus and interacts with the TCF/LEF/BCL9 (transcription factor/lymphoid enhancer-binding factor/B-cell CLL/lymphoma 9 protein) complex of transcription factors. This results in the transcriptional activation of downstream target genes. In the absence of Wnt signaling molecules,  $\beta$ -catenin is phosphorylated and subjected to proteosomal degradation, which results in the repression of target genes (Bernard and Harley 2007). It seems likely that *WNT4* uses this canonical  $\beta$ -catenin pathway in the developing gonad.

### R-spondin 1

R-spondin 1 (*RSPO1*) encodes a secreted factor that can stabilize  $\beta$ -catenin as part of the canonical Wnt-signaling pathway and is expressed at high levels in mouse, as well as human gonads around the critical time of gonad development (mouse: (Parma et al. 2006; Chassot et al. 2008a; Smith et al. 2008), human: (Tomaselli et al. 2011)). Its role in ovarian development was first implicated when a homozygous single nucleotide insertion within the *RSPO1* coding sequence was identified in four 46,XX testicular DSD patients from a consanguineous family. In addition, a homozygous exonic deletion was identified in an unrelated sporadic case of 46,XX testicular DSD (Parma et al. 2006). This study was the first to show that inactivation of a single gene can cause female-to-male sex reversal. A subsequent study identified a homozygous splice-site mutation in a 46,XX ovotesticular DSD patient (Tomaselli et al. 2008). In vivo studies on ovotesticular tissue from this patient showed no changes in *CTNNB1* ( $\beta$ -catenin) mRNA levels, but  $\beta$ -catenin protein levels were decreased. In addition, *WNT4* mRNA expression was significantly decreased compared to normal ovaries. These results suggest that *RSPO1* acts either upstream of *WNT4* or synergistically with *WNT4* during early ovarian development (Tomaselli et al. 2011). The conserved expression pattern of *RSPO1* in other vertebrates suggests that it is a critical gene in ovarian development. XX *Rspo1* knockout mice have masculinised gonads, but do not show complete sex reversal (Tomizuka et al. 2008). This phenotype is similar to *Wnt4* null mice, and provides further evidence, that these

genes act within the same pathway to activate  $\beta$ -catenin. *Wnt4*, *Fst*, and *Bmp2* were down-regulated in *Rspo1*  $-/-$  XX ovaries at E13.5, suggesting that RSPO1 acts as a positive regulator of *Wnt4* (Yao et al. 2004; Tomizuka et al. 2008; Chassot et al. 2008b; Trautmann et al. 2008). Chassot et al. (2008b) showed that in the absence of *Rspo1*, the canonical Wnt-signaling pathway was not activated, and there was no increase in *Wnt4* expression. The phenotype of *Rspo1* null ovaries could be rescued by stabilized  $\beta$ -catenin expression in ovarian somatic cells, suggesting that  $\beta$ -catenin acts downstream of *Rspo1* to block testis development in XX gonads (Chassot et al. 2008b).

In 46,XX female individuals, both WNT4 and RSPO1 are known to promote ovarian development and repress testis development. In 46,XY DSD gonadal dysgenesis females with a duplication of chromosome 1p, that includes both *WNT4* and *RSPO1*, testis development is suppressed and the ovarian pathway dominates. WNT4 as well as RSPO1 perform their actions in the somatic cell population of the ovary via  $\beta$ -catenin and the canonical Wnt-signaling pathway.

Conditional knockout of  $\beta$ -catenin in ovarian somatic cells (the putative precursors of granulosa cells) leads to ovarian defects similar to those of *Rspo1* and *Wnt4* null mice (Manuylov et al. 2008). Ectopic overexpression of a stabilized form of  $\beta$ -catenin in mouse somatic cells, leads to the disruption of testis development in XY embryos and causes male-to-female sex reversal (Maatouk et al. 2008). This suggests that  $\beta$ -catenin is a key ovarian and anti-testis signaling molecule. As a consequence, 46,XY DSD gonadal dysgenesis female patients, without any apparent mutations in known testis-determining genes, could be the result of stabilized  $\beta$ -catenin which then undermines the role of *SRY* in the developing testis by inhibiting its critical downstream target *SOX9* (Maatouk et al. 2008).

#### Forkhead box L2

Forkhead box L2 (*FOXL2*) encodes the forkhead transcription factor 2, which is a member of the winged helix/forkhead transcription factors (Hannenhalli and Kaestner 2009). A role for *FOXL2* in ovarian development was first suggested with the identification of a deletion near *FOXL2* in the polled/intersex syndrome (PIS) goat (Pailhoux et al. 2001). In humans, mutations in *FOXL2* are associated with blepharophimosis/ptosis/

epicanthus inversus syndrome (BPES), which is characterized by eyelid defects with or without ovarian dysfunction, such as premature ovarian failure (POF) (Crisponi et al. 2001). In addition, BPES patients have been shown to carry a 7.4-kb deletion of a *cis*-regulatory region, which is thought to interrupt the *FOXL2* promoter (D'Haene et al. 2009).

*Foxl2*  $-/-$  mice develop normal testis during embryogenesis but show masculinization of the supporting cells in the postnatal gonad (Ottolenghi et al. 2005). *Foxl2* null and *Wnt4/Foxl2* double knockout ovaries have reduced *Fst* and *Bmp2* expression at E13.5. In *Wnt4*  $-/-$  ovaries, the expression of *Fst* is back to wild-type levels at birth (postnatal day 0; P0), whereas the expression of *Fst* remains decreased in both, the *Foxl2*  $-/-$  and *Wnt4*  $-/-$ , *Foxl2*  $-/-$  double knockout ovaries. *Bmp2* expression remains significantly reduced in all three mouse strains. In vitro studies show that FOXL2 and BMP2 up-regulate *Fst* expression. Exogenous BMP2 increased *Fst* expression in ex vivo studies on fetal mouse gonads, but this was counteracted by the BMP antagonist Noggin (Kashimada et al. 2011). Together this data suggest that FOXL2 cooperates with BMP2 to ensure correct expression of *Fst* in the developing ovary, whereas WNT4 is necessary for the initiation, but not the maintenance of *Fst* expression.

*Foxl2*  $-/-$ , *Wnt4*  $-/-$  double knockout XX animals show testis differentiation and the development of male germ cells (Ottolenghi et al. 2007). Surprisingly, conditional knockout of *Foxl2* in the adult mouse ovary results in *trans*-differentiation into testis (Uhlenhaut et al. 2009). The ovarian structures become more testis-like, with tubular features and Sertoli-like cells. Gene expression was investigated using in situ hybridization and showed that some male-specific genes were up-regulated, including *Sox9*. These results indicate that *Foxl2* has a role in maintaining the ovary even during adulthood, presumably by suppressing the pro-testis action of *Sox9/Fgf9*. There is experimental evidence that this role is carried out together with ESR1/ESR2 (estrogen receptor 1 and 2) by binding to sequences within the TESCO region and thus controlling *Sox9* expression (Bagheri-Fam et al. 2010). This study may be clinically relevant in humans, as somatic mutations in *FOXL2* could be the underlying cause of ovarian dysfunction in adult females.

As previously mentioned (see *DMRT1* section), Matson et al. (2011) have recently shown that loss of *Dmrt1* in mouse leads to the upregulation of *Foxl2*,



theca cell formation, estrogen production, and eventually feminized germ cells.

### Identifying novel genes in the sex determining pathway

We are currently still unable to determine the causative mutation in a relatively high percentage of DSD patients. This together with the recent discovery of several new genes in patients with previously unexplained DSD lend support to the idea that both, the genes involved in gonad development, as well as the etiology of DSD, are still incompletely understood. Mutations in regulatory elements of known DSD/gonad development genes, as well as novel gonad-determining genes, are likely to be responsible for a significant percentage of yet unexplained DSD cases.

#### Gene expression profiling, using microarrays

Over the years, several strategies were used to identify novel genes involved in gonad development. One approach was the analysis of differentially expressed genes using microarrays. Several studies performed these types of analyses using mouse (Beverdam and Koopman 2006) and human (Houmard et al. 2009) gonadal tissues. While the lists of genes that show sexually dimorphic expression patterns identified many of the known gonad genes (although rarely *SRY*) this approach has not lead to the discovery of any novel genes involved in sex determination and the etiology of DSD.

#### CNV arrays—genomic rearrangements associated with DSD

As previously mentioned, CNV arrays have been used over the last couple of years to screen for and identify copy number variations in patients affected by DSD. Throughout this review, we mentioned a number of cases where this approach lead to the identification of the causative mutation in DSD patients. Nevertheless, there are still various genomic rearrangements associated with human DSD phenotypes that have been reported but where no causative gene could be identified.

A duplication of 1p has been shown to be associated with 46,XY DSD gonadal dysgenesis, although the

gene(s) causing the DSD phenotype are still unknown (Wieacker and Volleth 2007). Several deletions of terminal 10q have been associated with 46,XY DSD gonadal dysgenesis together with other somatic anomalies. The gene responsible for this DSD phenotype is still unknown, but *EMX2* has been suggested to be a likely candidate (Ogata et al. 2000). Deletions and duplications of chromosome 22q11.2 have been identified in three cases of 46,XX testicular DSD (which were *SRY*-negative) (Aleck et al. 1999; Erickson et al. 2003; Seeherunvong et al. 2004). The causative gene(s) haven't been identified, but this region contains the *MAPK1* gene, which appears to play an important role in proper testis development. Deletions on chromosomes 11p13 and 9p24 were identified using CGH arrays and were found to be associated with syndromic forms of 46,XY DSD (Le Caignec et al. 2007; Vinci et al. 2007). Other CGH arrays revealed a novel recurrent 15q24 microdeletion syndrome with the deletions being between 1.7 and 3.9 Mb in size. The syndrome is characterized, amongst other features, by genital anomalies in male, including micropenis and hypospadias (Sharp et al. 2007; Andrieux et al. 2009), but the causative genes have not been identified.

#### *SRY*-related HMG-box gene 3—an unexpected role in 46,XX DSD

By far, the most successful approach to the identification and functional analysis of gonad genes used a combination of human DSD patient studies and mouse models. The recently published example of *SOX3* highlights this once more.

*SOX3* is another member of the *SOX* gene family of transcription factors. *SOX3* shows near sequence identity to *SRY* so it has been proposed that the Y-linked *SRY* evolved from the X-linked *SOX3* gene (Foster et al. 1992; Graves 2001). This process occurred as the mammalian sex chromosomes evolved and differentiated. *SOX3* is not expressed in the developing gonads in either sex, and loss-of-function mutations in *SOX3* do not affect sex determination in humans or mice. However, a transgenic mouse line over-expressing *Sox3* showed ectopic expression of this gene in the bi-potential gonad. XX embryos showed female-to-male sex reversal, and further analyses suggest that *SOX3* induced testis differentiation by up-regulating *Sox9* expression in these animals through a similar mechanism to *SRY* (Sutton et al. 2011). The same

study identified three 46,XX testicular DSD patients with genomic rearrangements: two duplications including *SOX3* and one deletion upstream of *SOX3*, in the putative *SOX3* regulatory region. It was hypothesized that these rearrangements caused ectopic expression of *SOX3* in the embryonic gonad, where it was able to substitute for the absence of *SRY* and drive testis development. Together, these findings suggest that *SOX3* and *SRY* are functionally interchangeable in testis determination and lend further support to the hypothesis that *SRY* evolved from *SOX3*.

### Conclusion and future directions of DSD research

Since the discovery of *SRY* in 1990, we have significantly increased our knowledge of genes and gene networks involved in gonad development and the etiology of DSD. Nevertheless, still unexplained DSD cases, as well as gaps in the network of testis and ovary development show us that there is still much to understand.

With the development of more powerful algorithms, comparative genomic hybridization (CGH)/SNP chip arrays have the potential to detect subtle rearrangements of genes involved in gonad determination and differentiation. These two arrays-based methods allow the detection of rearrangements in putative regulatory elements, which, if mutated, may cause DSD. The use of microarrays to detect CNVs has had some success in identifying causative mutations in unexplained cases of DSD. However, this technique is limited to the detection of large rearrangements and may miss smaller genomic rearrangements or point mutations. Familial cases of both 46,XY DSD and 46,XX DSD often show a broad range of phenotypes in affected individuals within the same family, including instances, where the underlying genetic mutation has been identified (Temel et al. 2007; Lourenco et al. 2009). This phenotypic variability may be explained by additional mutations in other genes up and downstream of the target gene, which may interact or influence its activity. This has been shown in a familial case of hypogonadotropic hypogonadism, where a compound heterozygous *GNRHR* (gonadotropin-releasing hormone receptor) and a heterozygous *FGFR1* mutation were identified (Pitteloud et al. 2007). In addition, almost all cases of non-syndromic ovotesticular DSD remain unexplained although there have been considerable genetic analyses of candidate genes (Temel et al. 2007; McElreavey et al. 1992).

Although mutations in the androgen receptor (*AR*) gene are the most common genetic cause of 46,XY DSD, pathogenic mutations in 46,XY under-androgenized patients who are suspected to have mutations in this gene are only found in about 50% of affected individuals (Audi et al. 2010). The analyses of presumably multifactorial DSD phenotypes, such as hypospadias or cryptorchidism, have rarely identified the genetic cause, although epidemiological studies give clear evidence of a major genetic component in these conditions (Fukami et al. 2006; Schnack et al. 2008; Kohler et al. 2009). Next generation sequencing (NGS) or massively parallel sequencing approaches (MPS), such as whole exome, single chromosome, or targeted sequencing of sets of candidate genes (together referred to as targeted sequencing from this point), as well as whole genome sequencing, should provide substantially higher resolution for mutation detection and should assist in resolving unexplained cases of DSD. Whole genome sequencing, which combines the advantages of all other methods, is a powerful tool to detect the range of genomic mutations, including SNPs and all size ranges of genomic rearrangements and CNVs, in coding as well as non-coding and putative regulatory regions, in a non-biased manner. Currently, this method is still costly and requires immense bioinformatic analysis. However, as the cost of whole genome sequencing continues to decrease and as bioinformatic analysis improves, this will eventually become the method of choice for many researchers. In the interim, targeted sequencing approaches and whole exome sequencing offer a great opportunity to gain new insights into key genes required for gonad development with a lower level of bioinformatic complexity.

Despite the differences between human and mouse and the improvements in the techniques and bioinformatics, comparative genomics will still play a key role. Given the increasing resolution of the new techniques and the increasing power of bioinformatics, we are likely to identify an increasing number of new genes, which are mutated or rearranged in DSD patients. Comparative genetics and mouse models will be essential for establishing the biological function of mutations that do not lie within known DSD genes and crucial in verifying novel genes with a role in gonad development.

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