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DSDs: genetics, underlying pathologies and psychosexual differentiation

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Abstract

Mammalian sex determination is the unique process whereby a single organ, the bipotential gonad, undergoes a developmental switch that promotes its differentiation into either a testis or an ovary. Disruptions of this complex genetic process during human development can manifest as disorders of sex development (DSDs). Sex development can be divided into two distinct processes: sex determination, in which the bipotential gonads form either testes or ovaries, and sex differentiation, in which the fully formed testes or ovaries secrete local and hormonal factors to drive differentiation of internal and external genitals, as well as extragonadal tissues such as the brain. DSDs can arise from a number of genetic lesions, which manifest as a spectrum of gonadal (gonadal dysgenesis to ovotestis) and genital (mild hypospadias or clitoromegaly to ambiguous genitalia) phenotypes. The physical attributes and medical implications associated with DSDs confront families of affected newborns with decisions, such as gender of rearing or genital surgery, and additional concerns, such as uncertainty over the child's psychosexual development and personal wishes later in life. In this Review, we discuss the underlying genetics of human sex determination and focus on emerging data, genetic classification of DSDs and other considerations that surround gender development and identity in individuals with DSDs.

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Introduction

Sex development is a critical component of mammalian development that provides a robust mechanism for continued generation of genetic diversity within a species. In mammals, sex development occurs in two distinct and sequential stages: sex determination and sex differentiation.¹ Mammalian sex determination is dictated by the complement of sex chromosomes within an organism and refers to the mechanisms that lead to specification of either a male or female gonad from a single undifferentiated bipotential gonad.² The presence of a Y chromosome drives the bipotential gonad toward testis-specific differentiation and formation of a male-specific gonad, whereas the absence of a Y chromosome results in development of an ovary, which is the female-specific gonad. In the process of male sex determination, expression of *SRY*, which is located on the Y chromosome, initiates a cascade of gene expression within Sertoli cells that ultimately drives the morphological differentiation of the testis.^{3,4} In sex differentiation, secretion of endocrine and local factors, such as testosterone, dihydrotestosterone and anti-Müllerian hormone, by the testis results in the development of male internal and external genitalia (prostate, vas deferens, penis and scrotum) and a reciprocal regression of the Müllerian ducts, which are the precursors of female internal genital structures (Fallopian tubes, uterus and vagina).^{5,6} Disorders of sex development (DSDs) arise when this tightly regulated process is disrupted, which occurs primarily as a result of genetic mutations that interfere with either the development of the testes or ovaries or the actions of endocrine and local factors in extragonadal tissues.^{7,8}

This Review covers the current classifications and emerging knowledge of genetic mechanisms that contribute to sex and gender development among individuals with errors in sex determination that culminate in the development of DSDs. Current concepts of human sex determination and data from mouse models that have contributed to understanding of mammalian gonadal development are discussed. Aberrations in the process of sex differentiation (for example, defects in androgen biosynthesis) are also known to result in DSDs; however, these mechanisms have been reviewed elsewhere.^{9,10}

Classification of DSDs

In 2006, the Consensus Statement on Management of Intersex Disorders was drawn up by international experts and patient advocates under the auspices of the Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. The consensus conference was convened to review clinical management practices in DSDs given new advances in diagnosis, introduction of modified and new surgical techniques, studies of psychosocial factors pertinent to outcomes and challenges to clinical practices voiced by patient advocacy organizations. A key consensus recommendation involved the introduction of new terminology to replace confusing and potentially stigmatizing terms, including intersex, pseudohermaphroditism, hermaphroditism and sex reversal.^{7,8} Instead, the expert panel recommended that all variations of sex development should be incorporated under the superordinate term DSDs, which they defined as “congenital conditions in which development of chromosomal, gonadal, or anatomic sex is atypical”. Within this definition, DSDs can have a wide range of gonadal phenotypes, such as partial or complete gonadal

dysgenesis and ovotestis, and external genital phenotypes, such as hypospadias, clitoromegaly and ambiguous genitalia or fully masculinized or feminized genitalia that are discordant with karyotype or gonadal phenotypes. Using this inclusive definition, DSDs are estimated to occur in approximately one in 100 live births.^{8,11} However, the incidence of 46,XY gonadal dysgenesis, in which genetic mutations result in disruption of testis development, is estimated at less than one in 10,000 live births. Given the rarity of this condition, neither the distribution of genetic mutations within these patients nor the long-term consequences of the diagnostic and management challenges associated with 46,XY gonadal dysgenesis are well understood. To address this issue and other gaps in our understanding, large multicentre studies in both the USA^{12,13} and Europe^{11,14} are ongoing.

Despite rapid and broad acceptance of the consensus definition of DSDs by clinical and research communities, controversy remains regarding whether particular conditions (such as Turner and Klinefelter syndromes) and genital phenotypes (such as hypospadias) constitute DSDs and, therefore, require a multidisciplinary approach to clinical management. The continuing uncertainty regarding definitions of DSDs carries with it a number of potential negative consequences. Most important among these drawbacks is the reduced likelihood that a patient with a DSD will receive comprehensive and integrated care if seen outside of a multidisciplinary DSD clinic.^{7,8} For example, endocrine abnormalities in a patient who has descended testes but has a history of mild or severe hypospadias might remain undetected until puberty.^{15,16} Additionally, despite positive and objectively-assessed cosmetic results following surgery to repair hypospadias, patients are frequently less satisfied with sexual function and less likely to experience intimate relationships than unaffected individuals, highlighting the importance of behavioural and/or sexual health involvement in the model of care.¹⁷ Incorporation of a multidisciplinary healthcare team that includes psychological counselling at the time of diagnosis could contribute to identification and prevention of adverse effects consequent to the DSD. One barrier to providing multidisciplinary care to a large population of patients with DSDs is the perception of a lack of resources. Contrary to this misperception, increased numbers of patients can make the logistics of multidisciplinary clinics practical. With regard to behavioural health services only a small proportion of patients with a DSD and their families would be expected to require services beyond anticipatory guidance and even fewer patients and families would be expected to need additional targeted interventions, such as addressing pre-existing psychosocial stressors that include financial problems or family conflict. Finally, clinical treatment would be reserved for patients and/or their families who exhibit multiple risk factors for maintaining distress, such as chronic and marked anxiety, depressive symptoms or other individual or family-based psychosocial problems.¹⁸ Triaging of resources can be effectively accomplished using psychometrically robust psychosocial screening tools, such as the Psychosocial Assessment Tool and Child Behavior Checklist.¹⁹

The distinction and clarification of the terms DSD and intersex is important and necessary. The term DSD was introduced to emphasize underlying genetic and hormonal factors responsible for atypical somatic sex development. However, there are individuals with DSDs who have assumed the term intersex as an identity and reject the notion that the human body must be dichotomous. These individuals view the term DSD as a negative label that implies that atypical sex anatomy must be corrected with surgical or hormonal

interventions.^{20,21} Supporters of this position recognize that some interventions may be necessary in order to maintain physical health, such as removal of dysgenetic gonads in patients at high risk for malignancy, but call for a clear distinction between what is medically necessary versus what is elective or cosmetic.^{22–24}

Genetic pathways of sex determination

Testis

The identification of genes involved in sex determination was spurred on by the discovery and characterization of the testis-determining gene *SRY*, which is a Y-chromosome-linked gene that encodes a transcription factor. In humans, levels of *SRY* mRNA are upregulated in the urogenital ridge at 7 weeks after conception and drive the bipotential gonad towards testis formation in 46,XY individuals.^{25,26} After translation, the *SRY* protein translocates to the nucleus and binds to the enhancer regions of *SOX9*, to drive the differentiation and proliferation of Sertoli cells and testis tubule organization.^{27–29} Definitive evidence that *SRY* is the initiating factor in human male sex determination was provided by the discovery of missense mutations that disrupt the DNA-binding region of *SRY*, nonsense mutations, and deletions in the 5' or 3' regulatory regions of *SRY* that alter the timing or levels of *SRY* expression and lead to 46,XY gonadal dysgenesis.^{3,30–33}

The second major gene involved in male sex determination is the gene that encodes the *SRY*-related transcription factor *SOX9*. In humans, autosomal dominant mutations in *SOX9* cause campomelic dysplasia with a 46,XY DSD and external genitalia ranging from ambiguous genitals to a female phenotype.^{34,35} *SOX9* protein expression is required for testis determination and, in conjunction with *SRY* and the transcription factor *NR5A1*, the *SOX9* protein binds to its own promoter to perpetuate a positive feedback loop that maintains high levels of *SOX9* expression. In mouse models, *Sox9* mRNA expression is also maintained by activation of the *Fgf9*–*Fgfr2*^{36–38} and prostaglandin D2 (*PGD2*) signalling pathways.³⁹ Clinical syndromes associated with skeletal dysplasias have been described for mutations in *FGFR2*.⁴⁰ However, mutations that result in gonadal dysgenesis and DSDs in humans have not yet been identified in components of the *FGF9*–*FGFR2* or *PGD2* signalling pathways, which suggests that phenotypes associated with such mutations might result in embryonic lethality or have redundant functions in the genetic networks that drive sex determination.

A number of mutations in the genes encoding additional transcription factors that are involved in testis determination have been identified in human DSDs. *NR5A1* (also known as steroidogenic factor 1, or *SF-1*), encodes an orphan nuclear receptor, which is a major contributor to the development of the hypothalamic–pituitary–gonadal–adrenal axis.^{41–43} High expression of *Nr5a1* in the mouse bipotential gonad suggested a role for this gene in cell proliferation prior to the onset of testis determination and in the upregulation of both *Sry* and *Sox9* gene expression.⁴⁴ Initial reports highlighted the involvement of *NR5A1* mutations in gonadal and adrenal dysgenesis in 46,XY individuals with a female phenotype.⁴³ In pre-Sertoli cells, *NR5A1* synergizes with the transcription factor *GATA4* at the onset of testis determination and binds to the *SRY* promoter to upregulate *SRY* expression.⁴⁵ Further analysis of *NR5A1* in individuals with DSDs or with infertility issues has shown that

mutations in this gene are associated with a variable range of phenotypes from mild hypospadias to ambiguous genitalia^{46,47} as well as infertility in adulthood.^{48,49} Emerging studies have also demonstrated that 46,XY individuals with mutations in *NR5A1* who are phenotypically female may present with clitoromegaly that is secondary to elevated testosterone levels at the onset of puberty despite their dysgenetic gonads.⁵⁰

NROB1 (also known as *DAX1*) is located on the X chromosome (at p21.3) and like *NR5A1* encodes an orphan nuclear receptor that has a function in mammalian sex determination.⁵¹ Duplications encompassing *NROB1* in humans⁵² or transgenic overexpression of *NrOb1* in mice^{53,54} lead to dose-dependent XY gonadal dysgenesis and a female phenotype. As XY individuals have only one copy of this gene, a duplication that results in *NROB1* overexpression is sufficient to block testis determination. One of the molecular mechanisms identified in XY mice transgenic for *NrOb1* is through direct inhibition of Nr5a1-mediated transcription of *Sox9*.⁵⁴ In 46,XX female individuals, the two functioning copies of the *NROB1* gene are crucial to preventing testis formation. Loss-of-function mutations or deletions of *NROB1* lead to congenital adrenal hypoplasia and life-threatening adrenal failure that is associated with abnormalities in male genital development owing to decreased steroidogenesis.⁵⁵

The *GATA4* and *ZFPM2* (also known as *FOG2*) genes encode transcription factors that are critical for testis development. The discovery of a familial heterozygous missense mutation in *GATA4* that resulted in a Gly221Arg mutation and development of a 46,XY DSD, which was also associated with congenital heart disease, underscores the fundamental role of *GATA4* in both gonad and cardiac development.⁵⁶ Genetic associations between two unrelated patients with rare mutations in *ZFPM2* who also have 46,XY gonadal dysgenesis have recently been described, which further underscores the important role of *GATA4*–*ZFPM2* interactions in testis determination.⁵⁷ In mouse models, mutations that disrupt associations between *Gata4* and *Zfpm2* give rise to abnormal testis development.⁵⁸ In a porcine model, *GATA4* directly activated the *SRY* promoter; however, in humans and mice direct activation of *SRY* expression has only been observed when the *WT1* protein is coexpressed.⁵⁹ The studies in mice support the findings in patients with mutations in these genes and offer additional mechanistic insights into sex determination. Mutations in *Gata4* or *Zfpm2* resulted in decreased interactions between *Gata4* and *Zfpm2* proteins, which led to decreased ability of either gene (independently or when coexpressed) to activate transcription of target genes such as *Amh*, *Sry*, and *Sox9*.^{56,57,59}

Deletions that encompass the region surrounding the *DMRT1* and *DMRT2* genes, which are located on chromosome 9p, have been identified in multiple cases of 46,XY gonadal dysgenesis with ambiguous genitalia.^{60,61} Fine mapping of this region has narrowed the minimal region associated with 46,XY gonadal dysgenesis to a small 260 kb region upstream of the *DMRT1* and *DMRT2* genes.⁶⁰ In addition to its role in somatic cell determination within the gonads, a highly significant locus on chromosome 9p near *DMRT1* was identified in genome-wide association studies performed in patients with gonadal germ-cell tumours.⁶² The finding that *DMRT1* is associated with a propensity towards germ-cell tumours is consistent with the phenotype of *Dmrt1*-knockout mice, which have a profound

failure in postnatal maintenance of the germline^{63,64} and, on specific genetic backgrounds, also have increased susceptibility to gonadal germ-cell tumour formation.⁶⁵

Within testis differentiation, *DMRT1* is critical for the maintenance of Sertoli cell fate. Once the testis fate has been established, the phenotypes of the heterogeneous cells within the testis must be actively maintained. In mice, postnatal expression of *Dmrt1* simultaneously promotes testis-specific gene expression in Sertoli cells, through maintenance of high levels of *Sox9* expression, and represses ovary-specific granulosa cell differentiation. Loss of *Dmrt1* from Sertoli cells in the postnatal mouse testis results in transdifferentiation of these cells into granulosa cells.⁶⁶

An individual with multiexonic deletions in the *WWOX* gene on chromosome 16 presented with 46,XY gonadal dysgenesis (see Table 1),⁶⁷ which suggested that this gene was also involved in testis development. Pathological analysis of the gonads of this patient revealed immature testis and the presence of premalignant gonadal germ cells.⁶⁷ Given this phenotype it is possible that *WWOX* may function in the same developmental pathway as *DMRT1* to promote somatic cell differentiation of Sertoli cells and maintain germ cells. Additional studies in mice support this hypothesis, as XY mice homozygous for a hypomorphic *Wwox* allele have testicular atrophy and decreased fertility.⁶⁸

Ovary

Before the emergence of molecular and genomic tools in the past decade, ovarian sex determination was considered a passive default pathway that occurred in the absence of *SRY* expression and testis development. An equivalent gene to *SRY* has not yet been identified for ovarian differentiation. Furthermore, few morphological changes indicative of ovarian development have been identified that occur at the equivalent developmental time point as when an XY bipotential gonad begins to develop the organizational structure of the testis.

Despite the lack of visible morphological changes, gene expression patterns within XX somatic cells in the bipotential gonad have been shown to drive differentiation of granulosa cells and steroid-producing theca cells.⁶⁹ The primary signals for initiation of granulosa cell differentiation are unclear. Nevertheless, high mRNA levels of the signalling factors *WNT4* and *RSPO1* upregulate expression of and stabilize the transcription factor CTNNB1 (also known as β -catenin), which suppresses male-specific *SOX9* expression, maintains *WNT4* gene expression and promotes germ-cell proliferation (Figure 1).^{70–73}

Morphological differentiation of human ovaries occurs at week 7 of gestation, which is the developmental stage in which female germ cells enter the first steps of meiosis. Much of the knowledge we have of this developmental process has been gleaned from studies in animal models. In mice, meiosis in female germ cells is triggered by cell-extrinsic factors, such as retinoic acid and expression of *Stra8*.^{74,75} At this point, high levels of *Wnt4* signalling through either Frizzled or Lrp5–Lrp6 receptors drive both germ cell meiosis and differentiation of theca cells.⁷⁶ Similar to the developing testis, after the point of fetal ovarian determination, the ovarian phenotype and granulosa cell differentiation are actively maintained by expression of the *FoxL2* protein and the estrogen receptors α and β .^{77–79} Loss of *Foxl2* expression in mouse adult granulosa cells results in upregulation of *Sox9* and

transdifferentiation towards a Sertoli cell phenotype. Thus, like male sex determination, initial ovarian determination is dependent on Wnt4 and Rspo1 signalling, while maintenance of germ cells and the ovarian phenotype requires other proteins, such as FoxL2 and the estrogen receptors.

Failure of correct ovarian development in individuals with the 46,XX karyotype can result in one of two distinct states: gonadal dysgenesis or the presence of some testicular tissue within a 46,XX gonad. Gonadal dysgenesis has been suggested to have a distinct aetiology from that of a DSD; however, we believe that failure of ovarian development, particularly during the early stages, is similar to 46,XY gonadal dysgenesis. The primary difference between 46,XX and 46,XY gonadal dysgenesis is that, in the former case, the phenotype of the internal and external genitalia is congruent with the complement of sex chromosomes. By contrast, in individuals with the 46,XY karyotype, there is some degree of phenotypic variability of the external genitals that can range from hypospadias to a female phenotype and can include ambiguous genitalia.⁷

Gonadal dysgenesis associated with the 46,XX karyotype manifests clinically as primary ovarian insufficiency, which is defined as premature depletion of ovarian follicles and onset of menopause before 40 years of age.⁸⁰ This condition is represented by a range of phenotypes, from individuals who have not entered puberty by 15 years of age⁸¹ to those who experience early cessation of ovulation known as secondary amenorrhoea.⁸⁰ Although primary ovarian insufficiency can occur as a result of a variety of nongenetic aetiologies (for example, chemotherapy, auto-immunity and environmental factors), a genetic basis is thought to be the primary cause in 10–15% of all cases.^{82,83}

Female sex determination is intricately linked with the initiation of meiosis in germ cells.⁸⁴ The most frequent genetic cause of gonadal dysgenesis in a phenotypic female is 45,X Turner syndrome, which is estimated to occur in one in 2,000 live female births.⁸⁵ The follicle depletion observed among patients with 45,X Turner syndrome can result both from haploinsufficiency of critical genes on the X chromosome that escape X-inactivation and from incorrect pairing of the X chromosomes during meiosis.

Mutations or deletions that affect the expression of the autosomal gene *FOXL2* can lead to 46,XX gonadal dysgenesis with blepharophimosis, ptosis and epicanthus inversus syndrome (BPES) in humans and goats.^{86,87} BPES in humans is divided into two types. BPES type I is a sex-limited, autosomal dominant form with the full spectrum of disease. This severe phenotype results from truncating mutations that lead to haploinsufficiency of FOXL2 protein.⁸⁶ BPES type II is limited to the blepharophimosis phenotype, is present in both male and female individuals and has no gonadal phenotype. The subset of patients who have this limited form of the disease have small duplications within the *FOXL2* gene.⁸⁸ BPES types I and II can rarely occur together within a single family.⁸⁸ Autosomal dominant mutations in the nuclear orphan receptor *NR5A1*, described in detail above, can also result in premature ovarian failure in 46,XX individuals and account for <3% of all genetic cases of this condition.^{48,89,90}

In addition to *FOXL2*, ovarian determination is dependent on the presence of either functional *WNT4* or functional *RSPO1*.⁹¹ Mutations in *WNT4* and *RSPO1* (Tables 1 and 2, Figure 1) are discussed in detail in the section of this Review focused on signal transduction pathways. Genetic mutations in the *WNT4* gene can have a wide range of functional effects, including impaired lipid modification, defects in receptor signalling, and aggregate formation.^{92,93}

Testicular or ovotesticular DSDs that are associated with the 46,XX genotype are rare and arise primarily as a result of ectopic expression of *SOX*-family genes that are related to the major testis determining gene, *SRY*, within the fetal bipotential gonad. Up to 90% of isolated 46,XX testicular DSDs involve a translocation of *SRY* to the X chromosome or to an autosome.⁹⁴ A portion of the remaining cases can be explained by rare duplications or deletions in the promoter and enhancer regions of *SOX* genes, including *SOX9*,^{95–98} *SOX3*^{99,100} and *SOX10*^{101,102} (Table 1). In such cases, testicular tissue develops and causes some degree of masculinization of both the internal and external genital structures. Individuals with rare syndromic 46,XX testicular or ovotesticular DSDs have been identified who harbour mutations in *RSPO1*.⁷¹

Pathways of sex determination

As discussed above, transcription factors have a key role in male sex determination; however, other factors and pathways have recently been recognized to play an important part in this process.

Signal transduction pathways

A number of studies have addressed the role of cellular proliferation in the bipotential gonad and in the period before the onset of sexually dimorphic changes in sex determination. At the initiation of male sex determination, the *WNT4* and *RSPO1* signalling pathways become sexually dimorphic, with downregulation of *WNT4* and *RSPO1* expression and upregulation of *SOX9* expression in the developing testis.^{70,71,73}

Mitogen-activated protein kinase (MAPK) signalling has is the dominant pathway in testis determination (Figure 1). Heterozygous missense mutations in *MAP3K1* have been described in six published cases of 46,XY DSDs.^{103,104} Additionally, functional studies in human cell lines suggest that gain-of-function mutations in *MAP3K1* shift the balance of sex determination from testis development (driven by *SOX9*–*FGF9* signalling) towards ovarian development (mediated by *WNT4* and β -catenin signalling).¹⁰⁵ Furthermore, studies in mice have demonstrated that phosphorylation of the transcription factor Gata4 is regulated through *MAP3K4* signalling. This pathway might also regulate *Sry* expression during fetal gonadal development via modulation of chromatin structures.¹⁰⁶

Mutations in another signalling pathway, the desert hedgehog (DHH) pathway, have also been described in a small subset of patients with 46,XY DSDs with complete gonadal dysgenesis, as well as in a subset of patients with minifascicular neuropathy.^{107,108} Additionally, autosomal recessive mutations in the hedgehog acyltransferase gene, *HHAT*, have been identified in a patient with a 46,XY DSD with gonadal dysgenesis and

chondroplasia.¹⁰⁹ Gonad-specific deletion of *Hhat* in mice resulted in abnormal testis tubule formation, decreased gonadal size, and testicular dysgenesis. High expression of *Sox9* and *Cyp11a1* (which encodes a steroidogenic enzyme marker of Leydig cells) in these mice showed that differentiation of both Sertoli and Leydig cells was affected; however, *Sry* expression remained normal, which indicated that these disruptions occur downstream of *Sry* expression.¹⁰⁹

In female sex determination, WNT4 and RSPO1 signalling via β -catenin is crucial for normal ovarian development. In humans, dominant missense mutations in *WNT4* have been associated with Müllerian aplasia and hyperandrogenism,^{92,93} a finding that is supported by the phenotypes of female *Wnt4*-deficient mice.⁷⁰ Rare recessive mutations in *WNT4* cause SERKAL syndrome, which is associated with multiple developmental anomalies in the kidneys, adrenal gland and lungs, as well as testicular DSDs with ambiguous genitalia in individuals with the 46,XX karyotype.¹¹⁰ Findings in *Wnt4*-knockout mice predated the human discoveries and demonstrated early loss of germ cells and increased expression male steroidogenesis enzymes, which leads to elevated androgen levels driving Wolffian duct formation.⁷⁰ Further studies have shown that *Wnt4* also functions to upregulate retinoic acid signals that both initiate the onset of meiosis in germs cells and maintain germ cells throughout adult life.^{74,75,111}

In conjunction with *WNT4*, *RSPO1* is upregulated in the developing human ovary, and missense mutations in this gene result in syndromic forms of 46,XX testicular and ovotesticular DSDs that are associated with palmoplantar hyperkeratosis and a predisposition for development of squamous cell carcinoma in the skin.^{71,73} Findings in mouse models suggest that *Rspo1* is required for ovarian determination in both normal XX individuals and in individuals with XY sex reversal.^{37,71} Thus, the interplay between signalling pathways and transcription factors in ovary determination is without clear hierarchy, unlike that which exists with *SRY* signalling in testis determination. What is clear in both ovary and testis determination is that multiple pathways are required to program the heterogeneous group of cells within the differentiating gonads.

Epigenetic pathways

The emergence of sequencing technologies has enabled dissection of epigenomes *in vivo* on a large scale and has brought heightened recognition of the importance of crosstalk between epigenetic and transcriptional factors in developmental processes. Epigenetic factors that influence the expression of *SRY* have a substantial role in sex determination.

Chromobox2 (*CBX2*) has been shown to modulate DNA histone marks, which alter the expression of downstream genes in other developmental processes. To date, a single case of an individual with compound heterozygote mutations in *CBX2* that result in Pro98Leu and Arg443Pro amino acid substitutions has been reported.¹¹² A follow-up study of 47 patients with 46,XY or 46,XX DSDs did not identify any pathogenic *CBX2* mutations, which indicates that *CBX2* is probably not a frequent cause of these disorders.¹¹³ The two *CBX2* mutations were placed independently into the gene that encodes the long-*CBX2* isoform and transfected into a variety of human cell lines. Introduction of each mutation independently disrupted DNA transactivation of downstream sex determination genes, such as *NR5A1*, and

transfection of the same cell with a gene that carried both mutations had a synergistic effect and further decreased activation of the sex determination genes.¹¹²

Mechanistic studies using a human epithelial cell line grown in culture suggest that CBX2 functions as a component of the Polycomb repressive complex, which silences transcription through binding to H3K27me3 histone tags (Figure 1)¹¹⁴ In addition to CBX2's known role as part of a polycomb repressive complex, emerging evidence in both *Drosophila melanogaster* and mouse models suggests that the long isoform of the protein may influence the transactivation of sex determination genes through its DNA-binding domain.^{115,116} Findings from mouse models of sex determination suggest that Cbx2 acts upstream of Sry to either directly or indirectly upregulate gene expression and suppress the ovarian determination pathway in XY mice.¹¹⁷ However, whether one or both of the known roles of CBX2 are important in human gonadal development has yet to be determined.

Copy number variations

The emergence of high-density single nucleotide polymorphism analyses to identify small and large copy number variations (CNVs) on a genome-wide scale has highlighted the importance of noncoding regions of the genomic DNA in the development of DSDs. A summary of duplications and deletions that have been implicated in DSDs is shown in Table 1.

Large and rare chromosomal duplications and deletions are associated with DSDs and these genomic regions are likely to harbour genes involved in sex determination. The most prominent chromosomal anomalies include 9p deletion⁶⁰ (the region that contains the known sex determination genes *DMRT1* and *DMRT2*), Xp duplication^{52,101} (the location of *NROB1*), 10q deletion^{118,60} (encompassing *FGFR2*) and 22q duplication^{60,102} (the location of *SOX10*).

There remain a number of rare DSD-associated CNVs in which a causative gene in sex determination has not yet been identified or in which the putative gene has not been well studied in the context of sex determination. Heterozygous deletions in the 13q33.2 chromosomal region result in a 46,XY male phenotype with ambiguous genitalia and growth restriction and, depending on the size of the deletion, multiple organ malformations. The critical region encompasses a 9.5 Mb region that contains over 20 genes, of which *EFNB2* is a potential candidate given that the phenotype of mice with *ephrin-b2* knocked out show defects in urorectal development.^{119,120}

Deletions on 19q12q13 have been described in several patients with 46,XY DSDs characterized by cryptorchidism, ambiguous genitalia, intrauterine growth restriction and other developmental anomalies;^{60,121} however, no genes have been associated with DSDs located in this region. The smallest region of overlap in these patients that is associated with a DSD phenotype has been difficult to pinpoint, as deletions in this region have a wide range of variable phenotypes with approximately 75% of affected individuals demonstrating a DSD phenotype.¹²¹

Small chromosomal deletions or duplications that result in either haploinsufficiency or overexpression of a single sex determination gene can also lead to development of a DSD. For example, deletions in *WWOX*⁶⁷ and duplications in *NROB1*⁵² can each lead to development of 46,XY DSDs. In addition, CNVs within noncoding regulatory regions of sex determining genes are associated with development of DSDs in individuals with 46,XY and 46,XX karyotypes.^{31,100,122} The exact mechanisms by which these noncoding CNVs function is incompletely understood; however, it has been postulated that they disrupt long-range repressor or activator elements or alter the balance between these elements, which are critical for correct spatiotemporal expression of nearby sex determination genes.¹⁰⁰ Several noncoding CNVs have been described for *SOX9*.^{96,98,101} Nevertheless, it is important to note that while 98% of the human genome is non-protein-coding, emerging evidence suggests that a large proportion of the non-protein-coding DNA is transcribed into functional RNA molecules, such as long non-coding RNA and microRNA species. These regions may harbour causative mutations in non-protein-coding genome that might function to regulate developmental gene expression.¹²³

Multigenic inheritance

The availability of next-generation sequencing technologies has provided the potential to identify concurrent mutations in genes that are associated with DSDs, which might contribute to pathogenesis in a cumulative fashion. One such example is the discovery of mutations in *AKRIC4* and *AKRIC2*, which are involved in regulating fetal testosterone synthesis. In two families with multiple affected members who presented with 46,XY DSDs and varying degrees of undervirilization and cryptorchidism, mutations in *AKRIC4* and *AKRIC2* were identified using Sanger sequencing of coding regions of genes within an overlapping linkage peak. Variable inheritance of splicing and missense mutations in *AKRIC4* and *AKRIC2*, respectively, were found to result in a dose-dependent 46,XY DSD with ambiguous genitalia.¹²⁴ The variable expression that is often observed in large families with multiple members affected by DSDs might be influenced by co-inheritance of other mutations in sex determination genes or modifier genes.

The shift from traditional viewpoints of Mendelian genetic inheritance as relates to diseases has been cultivated as the complexities of the human genome continue to be unravelled. Mutations within the same developmental pathways may be additive or multiplicative and can result in an increased mutational load and increased severity of the DSD phenotype. An example of a DSD with complex inheritance is Bardet–Biedl syndrome. This syndrome is a ciliopathy characterized by dysfunction in multiple systems and is associated with obesity, developmental delays, renal malformations and genital anomalies. The pattern of inheritance of Bardet–Biedl syndrome displays substantial locus heterogeneity and has been shown to map to at least 15 loci with rare instances of triallelic inheritance.^{125,126} Advances in next-generation sequencing will enable rapid genetic diagnosis in patients with DSDs that arise from multigenic inheritance, such as Bardet–Biedl syndrome or in cases with multiple defects within the same steroidogenic pathway.

Environmental factors

In addition to the genetic factors addressed in this Review, the contribution of environmental factors to gonadal and genital development, which range from intra-uterine environment and placental insufficiency^{127,128} to low doses of endocrine disruptors, such as industrial and agricultural chemicals,¹²⁹ cannot be discounted. Emerging evidence suggests that an association might exist between environmental factors and development of DSD-associated conditions such as cryptorchidism and hypospadias.^{129–131} However, precise understanding of the contributions of genetic, hormonal and environmental factors to genital development and their interactions with genes remains limited.

Genetic diagnosis

The genetic and clinical diagnosis of DSDs has become increasingly complex as a consequence of the rapid expansion of knowledge about the genetic mechanisms that cause DSDs over the past 20 years. Mutations in the same gene can result in a wide range of genital phenotypes among individuals with DSDs; conversely, similar genital and gonadal phenotypes in different individuals can be caused by mutations in any one of 20 or more genes.¹² When an individual presents with a suspected DSD, a minimal initial endocrinological and genetic evaluation is required to rule out life-threatening adrenal disorders, such as congenital adrenal hyperplasia.¹³² However, technological advances have provided the tantalizing possibility that next-generation sequencing might be used for the primary identification of genetic mutations associated with DSDs, either using a targeted sequencing panel or whole-exome sequencing with a focus on genes known to be associated with DSDs.^{132,133}

Given the limitations of current understanding of the human genome, it is imperative to evaluate the parental DNA alongside that of the affected child in order to narrow down the pathogenic genetic variant, particularly in the case of previously unidentified mutations. Many types of genetic mutations (missense, nonsense, small deletions, small insertions, deletions and duplications) and inheritance patterns can be ascertained using whole-exome sequencing, with the exception of deletions, duplications, or chromosomal inversions that are limited to the non-coding portion of the human genome.

Next-generation sequencing provides a rapid and unbiased approach to detection of genetic mutations compared with the approach of sequentially sequencing individual genes, which is both expensive and can delay diagnosis and initiation of appropriate treatments for individuals with DSDs. Next-generation sequencing can identify a genetic diagnosis in almost 40% of cases of DSDs, a number which is likely to be an underestimate, as only cases that are refractory to other diagnostic approaches, such as a thorough endocrine work-up and single-gene sequencing are currently sent for this type of analysis. Given the limitations of whole-exome sequencing mentioned above, the cases in which no causative mutation is identified should be further analysed by microarray or complete genomic hybridization for CNVs.

Using a next-generation-sequencing diagnostic approach, after one or more genetic mutations are identified an astute clinician may pursue functional validation of the predicted

effects of the identified variant(s) *in vivo* using endocrinological and/or imaging approaches. For example, in cases in which a mutation in *NR5A1* is identified as causative, one should perform adrenal function tests such as measurement of serum cortisol and adrenocorticotrophic hormone levels. A mutation in *GATA4* would prompt imaging of the heart to rule out the possibility of an associated congenital heart defect.

From the perspective of both clinicians and researchers, correct genetic classification is essential to best assess the pathophysiology and treatment outcomes in patients with DSDs. Use of terminology and classifications should be consistent and careful and a genetic diagnosis should be obtained for all patients with DSDs. Currently, only a small fraction of individuals with a DSD receive a genetic diagnosis,¹³² but patients and their families embrace such a diagnosis for the purposes of fully understanding and planning for the potential health-related and psychological issues that might be associated with an individual's mutation(s). The particular genetic mutation(s) that underlie a specific DSD diagnosis can influence health-related considerations include management of complications such as cancer within the gonad and in other organ systems (for example Wilms tumours, skin tumours and adrenal tumours), whose risk might be increased; the potential need for hormone replacement therapy; and family planning considerations, both for the affected individual and for their parents.

Psychosexual differentiation

The terms sex and gender are often used interchangeably in studies with human patients; however, there are important distinctions between them. According to recommendations of an Institute of Medicine report, the term sex should be used to refer to classifications of male or female individuals according to physical attributes that are determined by an individual's karyotype, specifically the reproductive organs and their functions. By contrast, the term gender is defined by the individual's self-representation and identity as either a male or a female person, as well as by society-specific expectations regarding the appropriateness of attributes, activities, or behaviours for boys and men or girls and women.¹³⁴

Psychosexual differentiation involves the developmental unfolding of gender identity, gender role and sexual orientation. Gender identity refers to a person's identification of self as a girl (or woman), a boy (or man) or a mixture of both. Gender role refers to behaviours that differ in frequency or level between male individuals and female individuals in culture and time (such as toy play or maternal interest). Sexual orientation refers to sexual arousal to persons of the same sex (homosexual), opposite sex (heterosexual) or both sexes (bisexual), and is expressed in behaviour, fantasies and attractions.¹³⁵ The consensus statement advises that gender identity, gender-typical behaviour, and sexual orientation should all be viewed as separate components. As such, if an individual with a DSD exhibits atypical gender role behaviour this is not an indication of having been reared in the wrong gender.⁷

The issue of gender identity in DSDs has been reviewed elsewhere.^{136–138} Gender identity has been suggested to generally follow gender of rearing,^{136,137} with the exception of syndromes that result from errors in biosynthesis of androgens, such as deficiencies of 5- α -reductase-2 and 17 β -hydroxysteroid dehydrogenase-3.^{138–140} In these conditions, the

masculinizing puberty that results from endogenous testosterone production is frequently associated with a shift in gender identity among individuals reared as girls.¹³⁸ Unfortunately, the details provided in published case series are inadequate to test competing explanations for the observed change in gender identity in these individuals, such as the role of atypical genital appearance, masculinized gender behaviour, a contra-sexual puberty and cultural pressure that favours living as a man.¹⁴¹

Other challenging considerations from the perspective of gender assignment are those associated with non-hormonal DSDs, such as cloacal exstrophy and penile agenesis. A report of self-initiated gender change in a cohort of patients with 46,XY cloacal exstrophy who were reared as girls¹⁴² has been associated with a shift in gender assignment recommendations by paediatric urologists, from recommendations of rearing as a girl to recommendations of rearing as a boy for these patients.^{143,144} In a six-year follow-up survey of US paediatric urologists, 79% recommended gender assignment as a boy for patients with cloacal exstrophy, with 97% identifying 'brain imprinting' by prenatal androgens as an important factor in their decision. Nonetheless, a review of literature on gender identity stability in female-reared individuals with a 46,XY karyotype who have nonhormonal genital defects concluded that evidence is lacking to infer that gender identity is fully determined by prenatal androgen exposure.¹⁴⁵

DSDs have been studied to test hormonal hypotheses of gender development that extend beyond gender identity. In contrast to the broadly accepted notions that gender is determined by both biological and social environmental factors,¹⁴⁶ associations between DSDs and gender-atypical behavioural development have often been interpreted as directly resulting from prenatal androgen exposure.^{147,148}

Prenatal androgens have been shown to exert a masculinizing effect on the development of behaviours that exhibit gender-related variability (for example, childrens' toy and play preferences) and sex differences in neurocognitive function.^{149,150} As noted, less certainty exists regarding the role of early androgens in shaping gender identity and this is also the case for sexual orientation. The possibility that early androgen exposure shapes sexual orientation has been investigated, in particular among women with classic congenital adrenal hyperplasia, a population in which increased rates of non-heterosexual orientation have been reported.^{149,151,152} The reason for this observed correlation remains to be determined but could be attributed to a number of factors, such as a direct influence of androgens on sexual differentiation of the brain, or influences of early androgens on gender role expression. Additionally, the effects of having a chronic medical condition and its management on physical appearance and body image could also be involved, and the possibility that the correlation results from a combination of these and possibly other factors cannot be ruled out.¹⁵²

Finally, animal experiments have demonstrated that early exposure to sex hormones during steroid-sensitive periods of brain development has long-lasting effects on neurocognitive function and sex-dimorphic behaviours.¹⁵³ However, few studies have addressed the possibility of sex-hormone-independent effects on brain structure and function.^{154,155} Genetically modified mice have been used to separate gonadal phenotypes from

chromosome complement. This separation is accomplished by moving *Sry*, which determines testes development, from the Y chromosome to an autosome. This paradigm has enabled investigators to consider whether observed sex differences in brain and behaviour are consequent to hormone exposure, genetic factors or both.^{154,155} Little evidence exists to support a role for genetic factors acting independently of sex hormones in shaping aspects of human psychosexual differentiation, such as gender identity, gender role or sexual orientation. Nonetheless, studies in mice have demonstrated sex differences in physiology and behaviour with similarities in humans that are unaccounted for by differential hormone exposure.¹⁵⁵ Examples include autoimmune diseases such as multiple sclerosis, which are more common in female individuals,¹⁵⁶ and more rapid escalation of substance abuse to the point of addiction in women than in men.¹⁵⁷

Conclusions

The classification of patients with DSDs has been revolutionized by use of multidisciplinary approaches and large research networks that seek to better understand the pathophysiology of DSDs. By providing a consistent framework for diagnosis, clinicians can begin to identify patients with similar phenotypes and better understand the medical, social, and psychological factors that contribute to the overall well-being of patients with DSDs. Given the rarity of individuals with DSDs, multicenter studies are essential to identifying the methods that will lead to consistent diagnosis and optimal medical care for these people.

Despite the focus on the identification of genetic mutations that result in disruptions to the processes of sex determination and sex differentiation, genetics alone cannot completely explain the full range of health or psychological issues that might be experienced by an individual with a DSD. However, mutations identified in critical developmental genes have provided much needed insight into pathophysiological mechanisms of DSDs as well as a means of classification, which can be used to follow the long-term effects of genetic mutations and treatment outcomes in these individuals.

Emerging technological advances have transformed the ability to identify mutations and CNVs in individuals with DSDs; however, the interpretation and validation of identified mutations that are the direct cause of the observed phenotypes remains challenging. As sequencing technologies advance and our appreciation of the degree of variation within the human genome continues to expand, the distinction between causative mutations versus normal genetic variation will increase. The complexity of the human genome is evident in multiple layers of regulation, including at the level of DNA, messenger and long non-coding RNA transcripts, post-transcriptional and post-translational regulation, as well as within non-protein-coding genomic regions and the epigenome. Thus, while a large proportion of patients with DSDs have been diagnosed with a genetic lesion within known genes, much of the remaining variation that contributes DSDs have yet to be fully explored. Studying embryonic gonads in humans is ethically and technically very difficult, as these investigations require using tissues appropriate to the stage in development being examined. For example, fetal gonads of around 7 weeks of gestation would be needed to assess the epigenetic status, levels of various RNA species and mechanisms of post-translational regulation if sex determination were to be investigated.

Our understanding of the mechanisms within sex determination processes is heavily dependent on knowledge of the human patients with DSDs and studies in mouse models, which can be manipulated to determine the effects of individual genetic mutations. Understanding the multiple layers of genetic regulation that govern gonadal development and how these layers interact using next-generation sequencing tools coupled with careful studies in both animal models and in affected individuals might unlock the key to fully appreciating and treating the health needs of people with DSDs.

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Key points

- Disorders of sex development (DSDs) are defined as congenital conditions in which development of chromosomal, gonadal, or anatomic sex is atypical
- Mutations in genes that encode transcription factors, signalling components and epigenetic modifiers that are involved in sex determination can result in 46,XX and 46,XY DSDs
- At 6–8 weeks post-conception in human fetal development, upregulated expression of *SRY* in the bipotential gonad promotes testis determination, whereas activation of *WNT4* and *RSPO1* signalling promotes ovary determination
- Gonadal phenotypes in patients with DSDs range from gonadal dysgenesis (in which the gonads are fibrous streak gonads) to varying degrees of ovotestis (in which both ovary and testicular tissue are present)
- The complexity and interrelatedness of factors that contribute to the aetiology and the medical and psychological outcomes of DSDs demand a multidisciplinary team approach to health care
- In contrast to gender differences in activities and interests, associations between prenatal exposure to androgens and development of gender identity or sexual orientation are unclear

Review criteria

A search for articles published between 1991 and 2014 and focusing on disorders of sex development was performed in PubMed and MEDLINE. The search terms used were “sex determination”, “disorders of sex development”, “intersex”, “quality of life”, “gender”, “gender identity”, “gender role”, “sexual differentiation” and “psychosexual differentiation”. Specific search terms for genes were also used (“*SRY*”, “*SOX9*”, “*WT1*”, “*NR5A1*”, “*NR0B1*”, “*WNT4*”, “*RSPO1*”, “*FOXL2*”, “*DMRT1*”, “*MAP3K1*”, and “*CBX2*”). All articles identified were English-language full-text papers and abstracts.

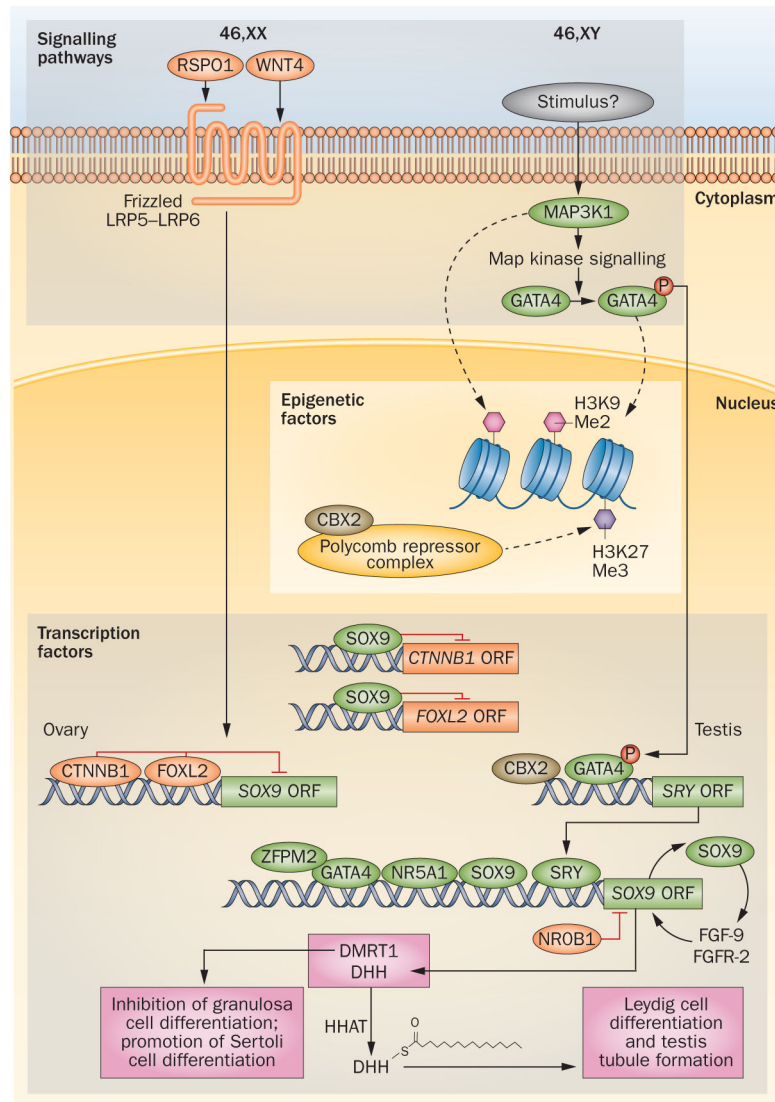


Figure 1. Genetic pathophysiology of human sex determination. Within the developing gonad, regulation of gene transcription occurs through cellular signalling pathways (WNT4–RSP01 in ovary determination, Map-kinase in testis determination) that activate genes through alteration of chromatin structures and modulation of epigenetic factors or by direct activation of transcriptional networks. In 46,XX individuals, WNT4 and RSP01 act through Frizzled or LRP5–LRP6 receptors to activate β -catenin (*CTNNB1*) transcription. β -catenin and FOXL2 promote expression of ovary-specific genes while inhibiting the expression of testis factors such as *SOX9*. In 46,XY individuals, Map-kinase signalling through MAP3K1 may alter chromatin conformation indirectly through histone modifications (dotted arrow). Map-kinase signalling also increases phosphorylation of transcription factors such as GATA4, which is thought to alter chromatin (dotted arrow) upstream of *SRY*, and was shown to directly bind to *SRY* promoter (solid arrow) to activate transcription. Within the nucleus, transcription factors GATA4 and ZFPM2 bind and transactivate *SRY* and *SOX9*.

Other important factors are CBX2 that has been shown to directly bind the *SRY* promoter and that, in conjunction with the NR5A1 protein, binds to the *SOX9* promoter. The SRY protein can then turn on downstream genes such as *SOX9*, which initiates the testis gene expression network and represses ovarian-specific genes such as *RSPO1* and β -catenin. Ovary-promoting transcription factors are noted in orange and testis-promoting factors are noted in green. Abbreviations: ORF, open reading frame; P, phosphate.

Table 1

Copy number variations in 46,XX testicular or ovotesticular DSDs and 46,XY gonadal dysgenesis

Chromosomal region	Duplication or deletion	Sex determination gene(s)	DSD phenotype(s)	Other phenotypes	Reference
9p	Heterozygous deletion	<i>DMRT1, DMRT2</i>	Complete gonadal dysgenesis to ambiguous genitalia	None	Tamour-Louet <i>et al.</i> (2010) ⁶⁰
10q25q26	Heterozygous deletion	Possibly <i>FGFR2</i>	46,XY gonadal dysgenesis, ambiguous genitalia with bilateral cryptorchidism	IUGR, congenital heart disease, mental retardation, cranial dysmorphism	Tamour-Louet <i>et al.</i> (2010) ⁶⁰ Irving <i>et al.</i> (2003) ¹¹⁸ Chung <i>et al.</i> (1998) ¹⁵⁸ Courtens <i>et al.</i> (2006) ¹⁵⁹
22q11.2	Duplication	Possibly <i>SOX10</i>	46,XX gonadal dysgenesis with ambiguous genitalia	Developmental delay, mental retardation, Wilms tumour, tetralogy of Fallot	Tamour-Louet <i>et al.</i> (2010) ⁶⁰
Xp21	Duplication	<i>NROB1</i>	46,XY gonadal dysgenesis	None	Barbaro <i>et al.</i> (2007) ⁵² White <i>et al.</i> (2011) ¹⁰¹
1p36.33	Heterozygous deletion	Possibly <i>WNT4</i>	46,XX cloacal exstrophy, prominent labioscrotal folds, no apparent genital tubercle	Midline defect, imperforate anus, left foot anomaly	Tamour-Louet <i>et al.</i> (2010) ⁶⁰ Battaglia <i>et al.</i> (2008) ¹⁶⁰
8p23.1	Heterozygous deletion	3' to <i>GATA4</i>	46,XY with complete gonadal dysgenesis, ambiguous genitalia	Adrenal hypoplasia congenita	White <i>et al.</i> (2011) ¹⁰¹
Xq27.1	Deletion or duplication	5' or 3' to <i>SOX3</i> or including <i>SOX3</i>	46,XX testicular DSD with male phenotype	Developmental delay, microcephaly, gender dysphoria, short stature	Moalem <i>et al.</i> (2012) ⁹⁹ Sutton <i>et al.</i> (2011) ¹⁰⁰
16q23.1	Multi-exon deletion	<i>WWOX</i>	46,XY gonadal dysgenesis with ambiguous genitalia, immature testis	None	White <i>et al.</i> (2012) ⁶⁷
17q24.3	Deletions or duplication	5' to <i>SOX9</i> , sometimes including <i>SOX9</i>	46,XY gonadal dysgenesis with female phenotype or ambiguous genitalia or 46,XX DSD with male phenotype	Skeletal anomalies in a subset of patients with a translocation involving upstream regions of the <i>SOX9</i> gene	Huang <i>et al.</i> (2011) ⁹⁵ Cox <i>et al.</i> (2011) ⁹⁶ Xiao <i>et al.</i> (2013) ⁹⁷ Benko <i>et al.</i> (2011) ⁹⁸
1p35-p31	Duplication	Possibly <i>WNT4</i> and <i>RSPO1</i>	46,XY DSD with female phenotype	Microcephaly, developmental delay, IUGR, dysmorphic features	Jordan <i>et al.</i> (2001) ¹⁶¹
19q12	Heterozygous deletion	Unknown	46,XY DSD with ambiguous genitalia and hypospadias	IUGR, ectrodactyly, developmental delay, feeding problems	Tamour-Louet <i>et al.</i> (2010) ⁶⁰ Chowdhury <i>et al.</i> (2014) ¹²¹
13q33.2	Heterozygous deletion	Possibly <i>EFNB2</i>	46,XY with ambiguous genitalia	Mental retardation, brain malformations eye malformations, gastrointestinal tract malformations, IUGR	Andresen <i>et al.</i> (2010) ¹¹⁹

Abbreviations: DSD, disorder of sex development; IUGR, intrauterine growth retardation.

Table 2

Genetic mutations associated with disorders of sex development

Gene involved in sex determination	Genomic region of mutation		Inheritance
	Noncoding	ORF	
<i>MAP3K1</i>	No	Yes	Autosomal dominant, gain-of-function
<i>WNT4</i>	No	Yes	Autosomal recessive
<i>RSPO1</i>	No	Yes	Homozygous recessive
<i>CBX2</i>	No	Yes	Autosomal recessive
<i>SRY</i>	Yes	Yes	Sex-linked recessive
<i>SOX9</i>	Yes	Yes	Autosomal dominant
<i>NR5A1 (SF-1)</i>	Yes	Yes	Autosomal dominant
<i>GATA4</i>	Yes	Yes	Autosomal dominant
<i>ZFPM2 (FOG2)</i>	No	Yes	Autosomal dominant
<i>NROB1</i>	Yes	Yes	Sex-linked recessive
<i>FOXL2</i>	Yes	Yes	Autosomal dominant
<i>DMRT1</i>	Yes	Yes	Autosomal dominant

Abbreviation: ORF, open reading frame.

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