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Review Article
Monogenic forms of DSD: An update

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Abstract

Background: DSD encompasses a wide range of pathologies that impact gonad formation, development and function in both 46,XX and 46,XY individuals. The majority of these conditions are considered to be monogenic, although the expression of the phenotype may be influenced by genetic modifiers. Although considered monogenic, establishing the genetic etiology in DSD has been difficult compared to other congenital disorders for a number of reasons including the absence of family cases for classical genetic association studies and the lack of evolutionary conservation of key genetic factors involved in gonad formation. In recent years, the widespread use of genomic sequencing technologies has resulted in multiple genes being identified and proposed as novel monogenic causes of 46,XX and/or 46,XY DSD.

Summary: In this review, we will focus on the main genomic findings of recent years, which consists of new candidate genes or loci for DSD as well as new reproductive phenotypes associated with genes that are well established to cause DSD. For each gene or loci, we summarise the data that is currently available in favor of or against a role for these genes in DSD or the contribution of genomic variants within well-established genes to a new reproductive phenotype.

Key messages: Based on this analysis we propose a series of recommendations that should aid the interpretation of genomic data and ultimately help to improve the accuracy and yield genetic diagnosis of DSD.

Introduction

Disorders/differences in Sex Development (DSD): are defined as congenital conditions with discordant development of chromosomal and gonadal/anatomical sex and cover a wide range of phenotypes that involve the endocrine and reproductive systems [1-3]. These pathologies are particularly challenging in terms of the clinical diagnosis, genetic etiology, patient management and predicting long-term outcomes. The diagnosis of DSD is made either during fetal life, at birth, in the first months of life or at puberty as these phenotypes can evolve throughout the lifetime of an individual [1-4]. Although a genetic cause is suspected in most cases of DSD, accurately identifying the causal variant has been historically challenging [5, 6]. The most problematic phenotypes are those associated with either very early gonad formation or development of the external genitalia with apparent normal gonad formation [5, 6]. The former are often due to errors in sex-determination (e.g. 46,XY gonadal dysgenesis or 46,XX ovotesticular DSD), a genetic program that unlike other developmental process is not well conserved in evolution even amongst mammals. For this reason, testing candidate genes from other model organisms is often not informative. Indeed, several genes, which when mutated in the human cause gonadal anomalies, do not cause a DSD phenotype in the mouse [5, 7, 8]. At the other end of the phenotypic spectrum are the mild phenotypes of hypospadias and/or cryptorchidism, where population studies indicate that a mixture of genetics and environment may be responsible for the majority of cases . [9-11].

Changes in sequencing technologies during the last decade has resulted in a rapid increase in the number of new genes proposed as candidates for monogenic forms of DSD [5, 6]. Unbiased sequencing approaches such as exome sequencing, where all the coding genes in the genome are sequenced, has thrown up many surprises, including those that would otherwise not have been considered to be involved in DSD, such as the RNA helicase *DHX37* [12, 13]. The other theme to emerge from large scale exome sequencing studies is the broadening of the range of phenotypes associated with what were considered to well-characterised DSD genes such as *WT1* and *NR5A1* [14-16]. Advances in our understanding of the genetic etiology of DSD will continue as whole genome sequencing rather than whole exome sequencing becomes more widely available. This will inevitably lead to an emphasis on the contribution of regulatory variants to DSD, which will present a new set of challenges to establish causality. In this review we will focus on genes that have recently been proposed to cause monogenic forms of DSD. We will assess the evidence in favor of, or against, the causality of these genes and provide a summary of the available data. A summary of these genes is listed in Tables 1-3. For many of these genes the phenotypic variability is considerable. This often includes individuals carrying the same amino acid change in a DSD gene [5, 17, 18]. In this review an important consideration is what we mean by the term monogenic. A monogenic phenotype is a phenotype produced by the effect of a single pair of genes or alleles. In this review the genes/variants that are described are considered to be monogenic causes of DSD (i.e. variants involving such genes have triggered DSD and DSD would not have occurred in the absence of these variants). However, the phenotypic variability may well be due to other genomic variants or environmental influences on the regulatory pathways required for gonad formation and/or function. There are efforts underway to define the range of genetic variants that influence the DSD phenotype for a given individual but caution is needed in the interpretation of these data. In the absence of any large scale, unbiased, whole genome-wide association studies, these proposed genetic modifiers remain unproven with no functional or statistical evidence to support a role in pathogenicity [19-21].

Main Text

46,XY DSD

The majority of new genes that have recently been identified to cause DSD are mainly associated with either syndromic or non-syndromic forms of 46,XY gonadal dysgenesis (GD) [5, 6]. The main reason being that GD is a very well-defined DSD phenotype that permits a detailed cohort analysis. This subgroup of XY DSD is also considered to be monogenic with little or no known environmental influences on the development of the phenotype. This contrasts with milder XY DSD phenotypes, which are considered multigenic with an environmental contribution, and where the genetic components have proven difficult to identify (9-11). Here, we will provide a background and update of the genes and variants involved that compliments the information in Tables 1-3. Overviews on the genetic aspects of other well-established DSD genes that are a common cause of 46,XY DSD have been published recently including the AR, AMH signalling and disorders of androgen synthesis [22-25].

CBX2

The human Chromobox homolog 2 (*CBX2*) gene, encodes a component of the polycomb (PcG) multiprotein complex. PcG proteins together with the counteracting trithorax (trxG) proteins control transcription throughout development via chromatin remodeling and/or histone modification. Disruption of Cbx2 in mice results in male-to-female gonadal sex reversal (26). These mice show a delay in appearance of genital ridges and formation of hypoplastic gonads in both the sexes. XY sex-reversal caused by loss of Cbx2 can be rescued by simultaneously deleting Wnt4 (27). Available data indicate that during testis development, Cbx2 stabilizes the testis fate by blocking the upregulation of genes in ovarian pathway (27). In the human, only a single case with 46,XY DSD has been reported to carry pathogenic variants in CBX2 (28). The patient was a phenotypic female with complete lack of testis determination. She had bilateral ovaries and carried the biallelic variants, p.P98L (paternally inherited) and p.R443P (maternally inherited). Both of these CBX2 variants are absent in the general population (https://gnomad.broadinstitute.org/) and functional analysis indicated that they affect the biological activity of the protein (Table 1). Pathogenic variants in CBX2 causing XY DSD are very rare as other studies have failed to identify any pathogenic variants in the gene (29-34).

An isoform of CBX2, termed CBX2.2, is a truncated form of the reference sequence CBX2.1. CBX2.2 has 211 amino acids, including only the chromodomain, compared with the reference CBX2.1 isoform which has 532 amino acids. CBX2.2 has been suggested to cause 46,XY DSD [35]. To support this, the authors identified two variants, a missense (p.Cys132Arg) variant and a complete loss-of-function (LOF) variant (p.Cys154fs). However, population genetics data indicate that the latter variant is carried by 1/125 African Americans (https://gnomad.broadinstitute.org/variant/17-77755771-CT-C?dataset=gnomad_r2_1), including several XY males who are homozygous for the variant. This variant is carried by 12% of North African individuals (unpublished data). Consequently, this variant cannot be pathologic. A further analysis of population genetics datasets indicates that the CBX2.2 isoform has currently 23 loss-of-function (LOF) variants (https://gnomad.broadinstitute.org/gene/ENSG00000173894?dataset=gnomad_r2_1). Several of these LOFs variants are present in the general populations at frequencies that are both inconsistent with CBX2.2 having a biological function and also exclude CBX2.2 as a cause of 46,XY DSD. The CBX2.2 isoform should therefore not be considered as cause of 46,XY DSD (Table 2).

DHH

The Hedgehog (Hh) signaling pathway is important for gonadal development in mice and humans [36]. Hh proteins, including Desert Hedgehog (DHH), are expressed as unprocessed preproproteins that undergo processing and auto-catalytic cleavage. Following cleavage, the N-terminal fragment of 19 kDa (HhN) retains all Hh signaling activity. DHH, a product of Sertoli cells in the fetal testis, regulates the specification and formation of androgen producing fetal Leydig cells [36]. Biallelic

variants of DHH have been described in patients with 46,XY gonadal dysgenesis with or without polyneuropathy. These patients are rare with less than 20 cases reported in the literature [37]. Why some patients present with both GD and polyneuropathy and others only with GD is unclear. However, functional studies suggest that variants which disrupt the N-terminal fragment HhN are associated with GD and polyneuropathy, whereas those variants that effect the auto-processing activity of DHH are associated only with GD [37].

One of the challenges created by large-scale exome sequencing studies of DSD cohorts is the interpretation of heterozygous variants of genes known to cause DSD in an autosomal recessive manner [30]. Heterozygous variants in DHH have been reported in 46,XY DSD and interpreted as variants of unknown or uncertain significance (VUS). Recently, functional analysis of these variants confirm that they are unlikely to be involved in the DSD phenotype and should therefore be considered likely benign [38].

DHX37

Two subgroups of DSD are the comparatively rare condition of 46,XY testicular regression syndrome (TRS) and anorchia. TRS is defined by a 46,XY chromosome complement, ambiguous or atypical genitalia, anomalies of sexual duct formation and absence of gonadal tissue on one or both sides [39-41]. Some boys with TRS are born with normal external genitalia but present with cryptorchidism. Boys with TRS are considered to have variable degrees of testicular determination with the loss of gonad tissue early in gestation and the families of some patients with TRS also include other children with complete or partial 46,XY gonadal dysgenesis or agonadism [42, 43]. Thus, both 46,XY gonadal dysgenesis and TRS are regarded as a continuum of phenotypes due to errors in testis determination and maintenance rather than distinct and unrelated DSD categories. The closely related phenotype of anorchia is defined by the absence of testicular tissue in a 46,XY phenotypic male that may be unilateral or bilateral. However, since male-typical differentiation of the genital tract and the development of the external genitalia is dependent on the production of anti-Müllerian hormone (AMH) and androgens, the testis must have been present at least up to the 16th week of gestation in these individuals.

An advantage of using unbiased genetic screens, such as exome or genome sequencing, is that they often reveal unexpected genetic associations with the phenotype. The discovery by us and others of frequent pathogenic variants in the RNA helicase DHX37 in association with 46,XY gonadal dysgenesis or 46,XY testicular regression sequence is an excellent example [12, 13]. RNA helicases, including DHX37, play prominent roles in ribosome biogenesis in eukaryotic cells by the recruitment or dissociation of ribosomal proteins or other binding factors [44-47]. The ribosome performs the essential function of translating mRNA into proteins [48]. Eukaryotic ribosome assembly is characterized by the sequential modular assembly of pre-ribosomal complexes. The human ribosome consists of ribonucleoprotein complexes with a small 40S subunit (SSU) containing the 18S rRNA chain and 33 proteins (RPS) and a large 60S subunit (LSU) which has the 28S, 5S and 5.8S rRNA chains, and 47 proteins (RPL). Ribosome biogenesis is an intricate, complex and coordinated process that takes place initially in the nucleolus and later in the cytoplasm [48].

DHX37 is a member of the DEAH family of RNA-helicases, which share a similar protein core structure that consists of two flexibly linked RecA domains, within which are conserved sequence elements and conserved structural motifs. The two RecA-like domains of DHX37 use the conserved residues and motifs to both bind ATP (motifs I, II, III, Va, and VI) and target RNA sequences (motifs Ia, Ib, Ic, IV, IVa, and V; [49, 50]; Figure 1). This family of RNA helicases do not possess intrinsic substrate

specificity and function by interacting with a large number of cofactors [50]. Although much of our knowledge on ribosome biogenesis and the role of DHX37 comes from studies in yeast, the function of DHX37 in ribosome biogenesis appears conserved in the human since the lack of the helicase in HeLa cells results in a reduction of the ribosome 40S subunit [51]. The importance of DHX37 in this process is highlighted by the observation that in these cells the absence of DHX37 triggers a surveillance pathway that leads to degradation of pre-ribosomal particles [51].

Variants in DHX37 have been reported to cause 46,XY GD, TRS or Anorchia [12, 13, 32, 52]. A total of 36 individuals have been reported with 46,XY DSD associated with novel or very rare missense variants in DHX37 (Table 4 and Figure 1). Most variants are located within or immediately adjacent to highly conserved motifs within the RecA1 and RecA2 domains (Figure 1). Pathogenic variants in DHX37 are an important cause of 46,XY DSD, since 10-15% of all cases of non-syndromic 46,XY complete GD carry pathogenic DHX37 variants [12, 13, 32, 52]. This frequency is similar to the prevalence of pathogenic variants in the *SRY, MAP3K1* or *NR5A1* genes. DHX37 variants also account for approximately 20% of all cases of TRS [12, 13, 32, 52]. A striking feature of the variants causing DSD is that the affected amino acid residues fall within highly conserved functional motifs and the residues themselves are conserved through to yeast. Indeed, almost half of all cases are due to a single amino acid variant, p.R308Q and there is no obvious genotype-phenotype correlation (Table 2). The gonadal phenotypes of individuals carrying the p.R308Q variant range from 46,XY complete or partial gonadal dysgenesis raised as female (6 individuals), 46,XY DSD with atypical external genitalia raised as female (2 individuals) and 46,XY TRS with severe micropenis (with or without cryptorchidism and hypospadias) raised as male (9 individuals). Where the transmission of the variant p.R308Q can be established 5 are *de novo*, 2 are maternally inherited and one is paternally inherited. This is consistent with a sex-limited autosomal dominant mode of inheritance.

However, variants in DHX37 are also associated with other congenital anomalies with no apparent DSD [53, 54]. Compound heterozygous as well as *de novo* heterozygous missense variants in DHX37 cause a complex congenital developmental syndrome consisting of microcephaly, global developmental delay, seizures, facial dysmorphia, kidney and cardiac anomalies as well as cortical atrophy. This syndrome, which has been termed NEDBAVC (Neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies; OMIM 618731) has been described in 6 patients in association with homozygous missense variants (p.R487H and p.N419K), compound heterozygous missense variants (p.V731M/p.L467V and p.R93Q/p.E167A) or *de novo* missense variants (p.T1094M and p.D382G) [53-54]. Three of the affected children were 46,XY boys and three 46,XX girls. DSD was not reported in any of these 6 cases. Therefore, pathogenic missense variants in DHX37, even within the same RecA functional domain may generate two distinct, non-overlapping phenotypes. The DSD phenotype is limited to the formation and maintenance of Sertoli cells with no other reported developmental anomalies, whereas NEDBAVC is a complex syndromic form of developmental delay and/or intellectual disability with somatic anomalies but with apparent normal gonadal development. To date missense variants shared by both 46,XY DSD and NEDBAVC have not been reported.

Errors in the process of ribosome production, including defects in ribosomal proteins, rRNA processing or ribosome assembly factors, leads to the development of a highly specific group of pathologies affecting selective organs or cell types that are collectively termed ribosomopathies [55, 56]. The most studied ribosomopathies include Diamond–Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS) and Treacher Collins syndrome (TCS). DBA is autosomal dominant disorder which usually presents in early childhood as bone marrow failure [55, 57]. Patients may also display a series of distinct of congenital birth defects including skeletal abnormalities and cardiac and

genitourinary malformations, together with an increased cancer susceptibility. DSD that is caused by pathogenic variants in the *DHX37* gene constitute a new form of human ribosomopathy. Although the genetic causes of ribosomopathies have been known for over two decades, the mechanisms involved are poorly understood. Pathogenic variants are predicted to cause reduced ribosome assembly and these pathologies reflect tissue/organ specific needs for optimum protein production during development. This would impact on highly proliferative tissues such as haematopoiesis or skeletal development that require high protein synthesis. However, this does not adequately explain specific differences in the phenotypic presentation of these diseases. One possibility is that differing phenotypes reflect ribosome heterogeneity and functional specialization or that some of these factors have could have acquired additional biological roles other than ribosome biogenesis.

Evidence in support of specific biological functions for DHX37, independent of its role in ribosome biogenesis, are indicated by both zebrafish studies and human genome wide screens to identify factors that modulate human T cell function. Zebrafish, carrying a homozygous missense variant p.K489P in Dhx37 (Figure 1) [58], exhibit changes in a tactile-evoked escape response. Wild-type fish turn and then swim away, whereas Dhx37 mutant fish show an atypical dorsal bend, followed by swimming. This behavior strongly resembles zebrafish embryos treated with strychnine, which blocks glycine receptors. The glycine receptor is a pentameric receptor composed of alpha and beta subunits that mediates postsynaptic inhibition in the spinal cord and other regions of the central nervous system. The abnormal motor response in mutants may be caused by a deficit in glycinergic synaptic transmission [58]. This was confirmed by both decreased expression levels of GlyR alpha and beta subunits in mutants. RNA immunoprecipitation assays demonstrated that zebrafish Dhx37 physically interacts with GlyR alpha subunit transcripts. Remarkably, the mutant fish exhibited no changes in ribosome biogenesis suggesting a specific neuronal function. In the human, CD8 T cells play essential roles in anti-tumor immune responses. Recently, genome-wide CRISPR screens using CD8 T cells in a cancer immunotherapy setting has identified DHX37 as an important regulator of anti-tumor effects [59]. Tumor infiltrating lymphocytes lacking DHX37 have upregulated expression of specific genes in multiple immune response pathways [59]. This evidence suggests a link between ribosome biogenesis and tissue-specific gene expression profiles. How variants in DHX37 cause 46,XY DSD is unclear. This group of DSD patients is unique insofar that their underlying cause has no obvious link to known genetic pathways involved in human early testis formation (e.g. SRY/SOX9/NR5A1). This subgroup of 46,XY DSD may require a careful long-term clinical follow-up since patients with other forms of ribosomopathies have a 2.5- to 8.5-fold higher risk to develop cancer throughout their life, and for certain cancer types these risks can be up to 200-fold higher [60].

DMRT1

DMRT1 and its orthologues play essentials roles in sex-determination and differentiation in many animals [61]. In mice, Dmrt1 is not required for testis determination, however, its continuous expression in the adult testis is required to maintain organ identity, because forced attenuation of Dmrt1 expression in adult testis results in trans-differentiation of the testis to an ovary [62]. In the human, deletions of terminal chromosome 9p which includes several genes as well as *DMRT1*, are associated with monosomy 9p syndrome. This is characterized by intellectual disability together with a distinctive series of somatic anomalies and in approximately 70% of 46,XY individuals anomalies of testis development are seen that range from a completely female phenotype to a male phenotype with hypospadias and/or cryptorchidism. [63]. Pathogenic variants within the DMRT1 coding sequences are remarkably rare. Evidence to indicate that DMRT1 is a key player in human testis-determination came through the identification of a *de novo* missense variant (p.R111G) in the functionally important DM-DNA-binding domain in a patient with 46,XY complete GD [65]. There

were no other somatic anomalies in this healthy girl. *In vitro* studies indicated that lack of testis-determination seen in this patient is due to a combination of haploinsufficiency and dominant negative activity. The only other DMRT1 variant that has been reported to cause 46,XY CGD is a novel *de novo* p.R80S variant, which is also located with the DM-DNA-binding domain. This variant is predicted to disrupt the interaction between DMRT1 and the minor groove of the DNA. Pathogenic variants of DMRT1 have not been reported in other large-scale exome sequencing studies of DSD cohorts [e.g. 30, 34]. One reason why pathogenic variants are rare is that to cause XY DSD, they must be located within a well-characterized functional domain and show dominant negative activity. A similar hypothesis has been suggested for the rarity of *SOX8* coding variants associated with XY DSD (see below).

GATA4 and partner *ZFPM2* (*FOG2*)

GATA4 is a zinc finger transcription factor, characterized by presence of two conserved type IV zinc fingers domains (amino acids 217-241 and 271-295), that interacts with NR5A1 to regulate gene expression during testis-determination and differentiation [65]. The key role of Gata4 in testis development has been known for some time. XY mice lacking *Gata4* show partially descended small testis with irregular cords and are infertile [66]. Severe testicular dysgenesis is also observed in mice, which carry a p.Val217Gly mutation in the N-terminal zinc finger (ZF) domain of the protein. This variant abolishes the physical interaction of Gata4 with its cofactor Zfpm2 (Fog2) [66-68]. Pathogenic variants were first reported in GATA4 in association with only congenital heart disease (CHD), although a proportion of XY males carrying deletions of human 8p23.1 that includes the *GATA4* gene have hypospadias and bilateral cryptorchidism [69]. We identified a familial case of 46,XY DSD and CHD that affected both 46,XX and 46,XY individuals [70]. The family carried a heterozygous missense mutation (p.Gly221Arg) located immediately adjacent to the mouse p.Val217Gly mutation in the N-terminal ZF domain [70]. In functional studies the p.Gly221Arg variant failed to bind to DNA, did not transactivate AMH promoter and lacked the ability to bind to ZFPM2. Other pathogenic missense variants in GATA4 have been reported since this initial publication but they do appear to be very rare, are located within the N-terminal ZF and may not involve CHD [21, 71, 72 and unpublished] (Figure 2). In large scale exome sequencing studies other variants have been found in 46,XY DSD patients in sequences flanking the N-terminal zinc-finger. Although these were initially classified as VUS (30), functional studies indicate that these have wild-type or near wild-type biological activity and are therefore benign (72). Other missense variants, located in regions flanking the N-terminal ZF domain in have been proposed to cause isolated hypospadias and this needs to be confirmed by other studies (73).

ZFPM2 (FOG2) is a zinc-finger cofactor that modulates the activity of GATA4 by binding to the N-terminal ZF [74]. There is considerable evidence in the literature to support a role for ZFPM2 in testis-determination including the observation that XY *Zfpm2*$^{-/-}$ mice fail to develop testis [75]. Since ZFPM2 interacts with GATA4 through the GATA4 N-terminal ZF which harbors pathogenic variants, there is the possibility that variants of ZFPM2 may also cause 46,XY DSD. We identified a familial case of 46,XY gonadal dysgenesis, where a heterozygous missense variant (p.S402R) segregated with the phenotype [76]. This variant is absent from GnomAD and it abolishes the interaction of the ZFPM2 protein with GATA4. A second independent individual had a more complex *ZFPM2* genotype with a *de novo* missense variant (p.R260Q) located within the N-terminal ZF of the protein together with homozygosity for a rare missense variant p.M544I [76]. The ZFPM2 protein carrying these variants also showed altered biological activity. Pathogenic variants in ZFPM2 associated with DSD are also very rare. Although heterozygous variants *ZFPM2* have been reported in large scale exome sequencing studies, and classified as VUS, functional studies indicated that these variants are benign [30, 72].

HHAT

The Hedgehog family of secreted signaling proteins plays a fundamental role during embryonic development, including early testis formation, by acting as morphogens to form concentration gradients for long-range and short-range signaling [36]. Three Hedgehog proteins are expressed in vertebrates Sonic (Shh), Indian (Ihh), and Desert (Dhh). The latter is secreted by Sertoli cells and functions as a commitment factor by inducing the formation of the Leydig cell lineage [36]. As described earlier, biallelic variants in DHH are associated with 46, XY GD [37]. Hedgehog acyltransferase (HHAT) is an ER-resident multipass membrane protein consisting of 10 transmembrane domains and 2 re-entrant loops [77]. It is a member of the membrane bound-O-acyltransferase (MBOAT) family of enzymes that catalyze the attachment of specific fatty acids to secreted proteins [77]. The palmitoylation of a member of the Hedgehog family, sonic hedgehog is catalyzed by HHAT.

In a familial case of two sibs presenting with a complex phenotype including 46,XY DSD, exome sequencing identified a homozygous p.G287V missense variant in the MBOAT domain of HHAT [78]. One sib presented with chondrodysplasia, 46,XY GD, and multiple congenital anomalies (Nivelon-Nivelon-Mabille syndrome). The other sib was 46,XX with histologically normal ovaries, and presented with a similar complex somatic phenotype. The variant disrupts the ability of the HHAT protein to palmitoylate DHH [78]. *Hhat*$^{-/-}$ mice display severely impaired development of fetal Leydig cells, Sertoli cells and testis cords [78]. These data indicate that in the mouse HHAT is required for the initiation of Leydig cell formation. Three other families with homozygous HHAT variants have been reported [79, 80]. Two 46,XX female sisters who presented with microcephaly and cerebellar vermis hypoplasia carried a homozygous missense variant (p.L257P) in the MBOAT domain HHAT [79]. There was no evidence of short stature, chondrodysplasia or 46,XX gonadal dysgenesis in the sibs. [79]. A second consanguineous family presented with with multiple malformations in three pregnancies [80]. The proband presented with severe microphthalmia, microcephaly, skeletal dysplasia, facial dysmorphia and 46,XY GD. The other two pregnancies also had similar somatic anomalies but the karyotype is unknown. The proband carried a novel biallelic in-frame deletion (p.Thr122del) within the MOAT domain. A fourth family consisted of a girl with 46,XY GD and microcephaly, but with normal weight and height for her age and no evidence of other anomalies [80]. She carried a novel homozygous HHAT missense (p.N443K) variant, again located within the MBOAT domain. These data indicate that homozygous variants within the MBOAT domain of HHAT cause a very rare syndromic form of DSD in 46,XY individuals together with microcephaly as the common feature and variable somatic anomalies.

MAMLD1

The mastermind-like domain-containing 1(MAMLD1) gene, located on chromosome Xq28, is expressed together with NR5A1 in Sertoli and Leydig cells during early gonad formation [81]. The typical MAMLD1 phenotype is a 46,XY boy with hypospadias and cryptorchidism, bifid scrotum, and/or a micropenis. 46,XY complete GD has rarely been described [82, 83]. A single homozygous missense variant has been reported in association with 46,XX ovarian dysgenesis [84]. Most MAMLD1 variants that are classified as pathogenic are LOF variants (usually nonsense or frameshift variants). However, the interpretation of MAMLD1 missense variants associated with DSD has generated controversy. There are two elements to this controversy. The precise biological role of MAMLD1 in

male genital development is unclear. Mice lacking *Mamld1* show reduced expression of Leydig cell transcripts but otherwise have normal genitalia and are fertile [85]. Therefore, there is both an inability to model missense variants using the mouse model and, in the absence of a precisely known biological function, a functional relevant biological assay is not available. The other aspect to consider is the population genetics. Some of the missense variants proposed to cause XY DSD are actually common polymorphisms in the general population. For example the reported variants p.H322Q (p.H347Q) and p.V480A (p.V505A) carried by 6.5% and 17.8% of African/African Americans are polymorphisms (83). They are both considered benign by ClinVar (ClinVar accession IDs 712305, 804096). The recently published p.P334S variant, which is reported as likely pathogenic (86) is carried by 12% of Europeans and it is therefore unlikely to be pathogenic. Further controversy concerning the contribution of MAMLD1 variants to DSD arises in familial cases, where the MAMLD1 variant does not always segregate with the phenotype (82). To understand the contribution of MAMLD1 to DSD, there is a need to distinguish between those individuals carrying rare or novel LOF variants and those individuals who are carrying common polymorphisms that in all likelihood do not contribute to the phenotype. Functional analysis of potentially pathogenic missense variants has shown little difference between the wild-type and proteins carrying missense variants but this is difficult to interpret in the absence of a known biological activity of MAMLD1 (87). Population genetics data supports a role for MAMLD1 variants causing DSD. In the general population the *MAMLD1* gene is only partially tolerant to LOF variants (https://gnomad.broadinstitute.org/gene/ENSG00000013619?dataset=gnomad_r2_1) with a small number (n=14) of LOF alleles reported in >68,000 alleles sequenced. A recent study and review of the literature by Li et al., 2020, indicated that approximately one third of MAMLD1 variants that cause XY DSD are LOF (86). This enrichment of MAMLD1 LOF variants in DSD cohorts compared with the general population is consistent with the hypothesis that variants which severely disrupt or abolish biological function are indeed responsible for DSD. However, in the absence of relevant and robust functional assays the contribution of rare or novel missense variants to DSD remains to be determined and these are likely to be continued to be classified as VUS in genomic studies.

MAP3K1

The mitogen-activated protein kinases (MAPKs) are activated through an evolutionarily conserved three-component signal transduction cascade, composed of a mitogen-activated protein kinase kinase kinase 1 (MAP3K1), a MAP2K and a MAPK. In the human, pathogenic variants in *MAP3K1* are an established cause of 46,XY DSD (88). Precisely how *MAP3K1* variants cause a failure of testis-determination is unclear. Pathogenic variants are usually heterozygous and for the most part are not LOF. They are either missense or splice-site variants or in-frame deletions. A disruptive variant, such as nonsense or frameshift mutation has not been reported, perhaps due to the fact that more severe variants of protein function may be embryonic lethal. Available data suggest that the missense variants associated with 46,XY DSD may be subtle gain-of-function variants that result in the increased phosphorylation of the downstream MAPK targets (88, 89). Patients carrying MAP3K1 variants show no other apparent phenotypic anomalies other than 46,XY GD (88). In our experience about 10% of 46,XY GD cases harbour rare or novel variants in the *MAP3K1* gene that could potentially contribute to the phenotype. However, the *MAP3K1* transcript is large (>7 kb) spanning 20 exons and there are over 600 rare (MAF<0.001) or novel LOF or missense variants reported in the general population (https://gnomad.broadinstitute.org/gene/ENSG00000095015?dataset=gnomad_r2_1). This makes the pathogenic interpretation of a missense variant carried by an individual with DSD difficult, although there is some evidence to suggest that pathogenic variants cluster in specific functional domains of the protein (90). However, in the absence of a robust and simple functional assay, the

clinical interpretation of *MAP3K1* variants associated with 46,XY DSD will remain a challenge and most will continue to be classified as VUS.

MYRF

Myelin regulatory factor (MYRF) is a large membrane-associated homo-trimeric protein that self-cleaves to release an N-terminal immunoglobulin-type Ndt80 domain for DNA-binding domain (91-93). The protein contains an intramolecular chaperone domain for trimerization and auto-proteolysis in the central portion and a transmembrane-domain in its carboxyterminal part that anchors the protein to the membrane of the endoplasmic reticulum (ER). Upon auto-cleavage, the MYRF N-terminal homo-trimer is released from the ER membrane and enters the nucleus to function as a homo-trimer transcription factor (91-93).

In the murine central nervous system, MYRF is specifically expressed by oligodendrocytes. MYRF was considered a myelin-specific transcription factor since conditional knockout of *Myrf* in oligodendrocyte precursors leads to widespread dysmyelination and severe neurological anomalies (94). However, despite its name, MYRF is a pleiotropic transcription factor that is widely expressed during embryonic development including in the gonads. A broad range of phenotypes, including 46,XY and 46,XX DSD are associated with pathogenic variants in the gene. Deleterious or LOF and often *de novo* variants in MYRF are associated with congenital diaphragmatic hernia (CDH), cardiac anomalies including Scimitar syndrome, urogenital anomalies, and an encephalopathy syndrome (95-99). The genitourinary anomalies in XY individuals include ambiguous external genitalia, hypospadias, horseshoe kidney, chordee or cryptorchidism. A total of 14 46,XY individuals with syndromic phenotypes have been described to date, with 12 of them presenting with urogenital anomalies (96-99). A further two cases of 46,XY boys have been described with only urogenital anomalies and no other somatic anomalies (96). These two boys had micropenis, hypospadias, small testis and cryptorchidism with low levels of testosterone and AMH (96). Five affected 46,XX individuals have been described. Of these one was reported to have no internal genital organs with a blind-ended vagina. A pair of 46,XX monozygotic twins were also reported who presented with small or absent ovaries and Mullerian duct aplasia with no other somatic anomalies (96). These data indicate that variants in the *MYRF* gene can be considered as cause of either syndromic or non-syndromic 46,XY or 46,XX DSD. There is no apparent relationship between the genotype and the phenotype, however only a small number of cases have been reported to date and this may evolve over time.

PPP2R3C

The protein phosphatase 2A (PP2A) is one of the four protein phosphatases in eukaryotic cells that is responsible for the dephosphorylation of serine and threonine residues in proteins. PP2A forms several holoenzyme complexes (100). The core enzyme consists of a catalytic C subunit (PP2Ac) and a regulatory A subunit that is associated with a regulatory B subunit. The regulatory B subunit can be classified as a member of the B, B' or B'' families (100). The gene *PPP2R3C* encodes the B''gamma subunit of PP2A (101, 102). A single report has been published describing biallelic variants in *PPP2R3C* with a syndromic form of 46,XY DSD (103). Four affected girls from unrelated families presented with 46,XY complete GD, a typical facial dysmorphia, low birth weight, myopathy, rod and cone dystrophy, anal atresia, omphalocele, sensorineural hearing loss, dry and scaly skin, skeletal abnormalities, renal agenesis and neuromotor delay. Each girl carried carried biallelic variants in the PPP2R3C gene, establishing this as a new recessive form of syndromic 46,XY GD (103). Many of the syndromic features are consistent with impaired chondrogenesis and consistent with this hypothesis data suggest that these variants may alter the phosphorylation of SOX9 which is required for both

testis-determination and chondrogenesis. Phosphorylation of SOX9 results in enhanced DNA-binding activity and translocation of the protein to the nucleus (104). Male and female heterozygous carriers of these variants exhibit various degrees of infertility (103). Carrier men have teratozoospermia, whilst some carrier females were reported to have oligomenorrhea or premature menopause (103).

SOX gene family variants and DSD: SRY, SOX8 and SOX9

15% of 46,XY CGD patients carry variants involving Y-linked testis-determining gene SRY. The majority of pathogenic variants are hemizygous missense variants clustered within the DNA-binding HMG-domain (105), although rare deletions upstream and downstream of the gene as well as variants in the minimal promoter region have been reported (105-108). Pathogenic variants in the *SOX9* gene are associated with Campomelic dysplasia (CD) and testicular dysgenesis of variable degree is observed in 75% of affected XY individuals (109). In rare occasions patients with pathogenic variants may present with gonadal anomalies but not CD. Missense variants have been reported in undervirilized men with unpalpable testis and either hypospadias or micropenis (110). In recent years there has been renewed interest in *SOX9*, with data indicating that rearrangements involving the *SOX9* locus are relatively common causes of both non-syndromic 46,XY or 46,XX DSD. These structural changes, involving multiple regions both upstream and downstream of *SOX9,* may disrupt the appropriate developmental timing of *SOX9* expression. The structural changes include duplications, deletions, translocations and inversions, and explain about 10% of all patients with either 46,XY GD or *SRY*-negative 46,XX (ovo)testicular DSD. A comparison of common overlapping rearrangements in human DSD individuals has defined the key regulatory elements required for the control of *SOX9* expression in the developing gonad. The first regulatory element to be defined was termed *TESCO* (111). This 1.4 kb region is located 13 kb upstream of *Sox9* and can positively regulate *Sox9* expression by binding of key sex-determining factors including NR5A1, SRY and SOX9 itself and it can repress *Sox9* expression through the binding of Foxl2 (111). However, deletion of the *Tesco* enhancer in mice does not cause male-to-female sex-reversal and rearrangements of human *TESCO* has not been reported in DSD (112). The analysis of individuals with either XY or XX DSD has identified other key regulatory elements of SOX9. Located approximately 600kb upstream from SOX9 the *RevSex* element is duplicated in 46,XX (ovo)testicular DSD and deleted in individuals with 46,XY GD (113-116). The minimal region associated with 46,XX-SRY negative DSD has been narrowed down to a 40.7-41.9kb element, which contains two predicted enhancer motifs (116). Further analysis of 46,XX DSD individuals has narrowed the *RevSex* region to a 24 kb minimal region that contains a core enhancer motif termed eSR-B (117). An immediately adjacent and non-overlapping second region that when deleted is associated with 46,XY GD is termed XYSR (118). Further analysis of patients has refined XYSR to a minimum critical region of 5.2 kb (119). Within this region a core enhancer element, termed eSR-A has been identified. Deletion of the minimum region including eSR-A cause XY GD, whereas duplications of this region cause XX DSD (119). A bioinformatic screen of human sequences upstream of *SOX9* locus identified a third potential enhancer element of 1259 bp located immediately upstream of *TESCO*, termed eALDI that may regulate *SOX9* expression although to date no patients have been identified with rearrangements or other variants involving the aALDI enhancer (119).

Recent data from murine studies indicates another *Sox* family gene member, *Sox8*, is involved together with Sox9 and other transcription factors in testis development as well as in the

maintenance of Sertoli cell identity (120-122). SOX8 is co-expressed with NR5A1 and SOX9 in the early stages of human testis-determination in Sertoli cells and Leydig cells as well as in Sertoli and Leydig cells in adult men (123). SOX8 variants associated with 46,XY DSD are rare. Three individuals with 46,XY DSD and rearrangements at the *SOX8* locus have been described (123, 124). A pericentric inversion and a complex rearrangement of *SOX8* are associated with 46,XY non-syndromic and syndromic GD respectively (123). A further case of 46, XY GD, skeletal and cardiac anomalies and developmental delay had a 560 kb duplication located approximately 18 kb upstream of *SOX8* (124). Variants in the *SOX8* coding sequences, all located in regions flanking the HMG-box, are associated with both male and female infertility. A single case of 46,XY GD has been described with a pathogenic missense variant located within the HMG-box (123). This mutant protein displays dominant negative activity over the wild-type SOX8 and SOX9 proteins. This may explain the severity of the phenotype compared with other SOX8 variants causing infertility. However, we cannot rule out the possibility of genetic redundancy between SOX8 and SOX9 function. Thus, it is likely that pathogenic missense variants in SOX8 will be rare (32).

ZNRF3

In mammalian testis-determination Sry initiates a positive feedback loop between Sox9 and Fgf9, which results in up-regulation of Fgf9 and repression of the ovarian factor Wnt4 (125). Canonical WNT/β-catenin signals are required for normal ovarian development and variants in either WNT4 or Rspondin-1 (RSPO1), which are required for the stabilization of β-catenin, can result in syndromic forms of 46,XX DSD or virilization in 46,XX individuals (125-128). The transmembrane E3 ubiquitin ligase ZNRF3 functions to inhibit WNT signaling by targeting Frizzled receptor for degradation by ubiquitination and increased membrane turnover. R-spondins function to promote WNT signaling by binding to and sequestering the negative regular ZNRF3 (129, 130). In exome sequencing studies of 46,XY DSD cohort, we identified two novel ZNRF3 variants and two known variants in five individuals (131). No other DSD-associated variants were present in these patients. Two 46,XY females with GD carried novel *ZNRF3* variants (c.2767+5G >A and p.Ser554Asn), two sisters with a milder phenotype of 46,XY DSD carried a rare missense variant (p.Arg768Gly) and a boy with perineal hypospadias, intrascrotal testis carried a different, rare missense variant (p.Arg621Ser). The genomic data was suggestive for a role of ZNRF3 in the pathogenesis of 46,XY DSD but they were not conclusive. However, using *in vitro* cellular assays and zebrafish model we demonstrated that the two missense variants (p.Ser554Asn and p.Arg768Gly) disrupted the ability of ZNRF3 to inhibit canonical WNT signals as compared to wild-type ZNRF3. Mice carrying only one copy of the *Znrf3* gene on the B6.Y^{AKR} background showed widely different degrees of sex-reversal consistent with the human DSD phenotypes. Together these data indicate that ZNRF3 variants may contribute to a wide spectrum of DSD phenotypes. Since there has been only a single published study, the prevalence of ZNRF3 variants causing DSD remains to be established (32).

Monogenetic associations with 46,XY DSD requiring further genetic or experimental evidence

ESR2

Estrogens control development and cell differentiation by binding and activating their nuclear receptors, estrogen receptor α (ESR1) and β (ESR2). Mice lacking *Esr2* exhibit both male and female infertility with no evidence of DSD (132-134). Consistent with these observation, a heterozygous missense variant (p.K314R) in ERS2 was identified in a 46,XX 16.5 year-old-girl who presented with primary amenorrhea (135). Clinical evaluation revealed streak gonads, absent puberty, no breast development, infantile uterus and osteoporosis. This variant is absent from public databases and it is

predicted to impair the interaction of ESR2 with nuclear coactivator 1 (NCoA1). Functional studies showed that the variant significantly impaired ESR2 signaling and exhibited dominant negative activity over the wild type (135). A second case describing an ESR2 missense variant in association with 46,XX primary amenorrhea has been described although it is unclear if the phenotype was due to ovarian dysgenesis (136). The combination of animal models, functional studies and the location of the protein change suggest that pathogenic variants in ESR2 are a cause of ovarian dysgenesis, with the caveat that only a single patient has been described to date (Table 2).

The evidence to support a role for ESR2 variants contributing to 46,XY DSD is inconclusive. Monoallelic and biallelic variants in *ESR2* have been reported in patients with syndromic and non-syndromic 46, XY DSD (137). The phenotypes of these individuals are varied. One case presented with a complex developmental phenotype including absence of uterus, fallopian tubes, gonads, and vagina, anal atresia, rectovestibular fistula and ocular anomalies as well as facial dymorphism. A heterozygous 3bp deletion resulting in the loss of a single amino acid p.Asn181del within the DNA-binding domain was found by exome sequencing. This variant has an allelic frequency of 3:2000 46,XY men of South Asian origin (https://gnomad.broadinstitute.org/variant/14-64735621-CATT-C?dataset=gnomad_r2_1). Although rare, this allelic frequency in healthy 46,XY men excludes this variant as the cause of the phenotype. The second case was a XY female with clitoromegaly, urogenital sinus and absent uterus. Since FSH and LH levels were within normal range rather than elevated it suggests that the individual may have had androgen insensitivity. Information on the gonads or gonadal hormones was unavailable. This girl carried a rare heterozygous missense variant p.G84V. No somatic anomalies were observed. The third case diagnosed with 46,XY complete GD, carried a rare heterozygous p.L426R missense variant. Establishing an association between these ESR2 variants with 46,XY DSD is challenging since each of clinical the phenotypes are distinct, the functional analysis of the mutant proteins did not show a statistically significant change compared to the activity of the wild-type protein and ESR2 expression could not be detected in the gonad of an 8-week-old male human embryo (137). Therefore, the association of ESR2 variants with 46,XY DSD remains to be established.

FGFR2

FGFR2 plays an essential role in osteoblast differentiation and proliferation, and is required for normal skeleton development, embryonic patterning, trophoblast function, lung morphogenesis, and skin development (138). In XY mice the signaling pathway involving Fgf9 and its receptor Fgfr2, is required to repress pro-ovary Wnt4 signaling in order to promote testis development (139). Mice lacking *Fgfr2* in the early developing XY gonad show a failure of testis-determination (139). Although this establishes that FGFR2 is involved in testis formation in mice, there is a lack of convincing evidence to indicate that *FGFR2* variants in the human cause 46,XY DSD. A single 46,XY individual with GD and craniosynostosis has been reported to carry a heterozygous p.Cys342Ser variant (140). However, heterozygous pathogenic variants in *FGFR2* gene are associated with range of phenotypes including Crouzon syndrome, Pfeiffer syndrome and Apert syndrome, which share as a common feature craniosynostosis (141-144). DSD has not been reported in affected 46,XY individuals with these syndromes. The amino acid substitution, p.Cys342Ser, was previously reported to cause Crouzon or Pfeiffer syndromes and was carried by 46,XY males with no evidence for DSD (141, 144). This suggest that the gonadal dysgenesis seen in the patient carrying the FGFR2 variant is caused by an independent pathogenic variant involving a DSD gene elsewhere in the genome.

HMGCS2

Variants the gene 3-hydroxy-3-methylglutaryl coenzyme A synthase 2 (HMGCS2), encoding a metabolic enzyme in the liver important for energy production from fatty acids, have been proposed to cause human DSD (145). The expression of *Hmgcs2* in the developing gonad of the mouse is consistent with a role in early testis and ovary development, but mice that lack *Hmgcs2* have normally developed gonads even on a sensitized genetic background (145). In a screen of 46,XY DSD patients with gonadal dysgenesis, two patients were identified with a heterozygous deletion and a predicted deleterious heterozygous missense variant (p.Arg501Pro) in *HMGCS2* respectively (145). However, autosomal recessive variants, including LOF variants, in *HMGCS2* are a well-established cause of HMG-CoA synthase-2 deficiency (146) with no evidence of DSD. Heterozygous carriers are healthy. Data from the gnomAD database indicates that *HMCS2* is not a conserved gene in human populations (pLI =0) unlike other genes known to be involved in testis determination, which are intolerant to variation (e.g. *DMRT1, SOX9, NR5A1*). These data do not provide compelling evidence in favor of causality of HMGCS2 variants in human DSD.

LHX9

LHX9 is a member of the LIM homeobox gene family that contain a homeodomain and 2 cysteine-rich LIM zinc-finger domains. Variants in the LHX9 gene are an excellent candidate to cause DSD, since both XY and XX mice lacking *Lhx9* are female and do not have gonads (147). A single *de novo* and novel missense variant p.Q316R located within the DNA-binding homeodomain of the protein was identified in a 46,XY girl who presented with normal facial features, bilateral distal thumb hypoplasia with small nails and mild hypoplasia on the fifth fingernails bilaterally, absent left great toe and hypoplastic right great toe with absent distal phalanx (148). Although functional studies have not been performed, this is a good candidate variant for 46,XY DSD as it impacts a highly conserved amino acid residue in a well-characterised functional domain and it is predicted to disrupt the biological function. One or more other independent cases of DSD with variants in LHX9 would establish this as a rare cause of 46,XY DSD.

STARD8

Rho-GTPases are important molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells. *STARD8* is a Rho-GAPase that maps to chromosome Xq13. The protein consists of a sterile alpha motif (SAM), GAP and START (steroidogenic acute regulatory protein [StAR]-related lipid transfer) domain (149, 150). A missense variant in *STARD8* has been proposed to cause 46,XY GD in a familial case of DSD (151). Two sisters with 46,XY GD were found to carry a hemizygous missense variant (p.Ser993Asn; rs201005000, also termed p.Ser913Asn in a shorter isoform), inherited from their heterozygous mother. Gonadal tissue of one of the sisters contained Leydig cells overloaded with cholesterol droplets, i.e., structures previously identified in 46,XY DSD patients carrying variants in the *STAR* gene encoding a related START domain family member. The p.Ser993Asn variant, which *in silico* predictions indicate as benign, is present in Bulgarian populations with a relatively high allelic frequency (1/500 alleles). Although STARD8 is a promising candidate, the evidence for a role of pathogenic variants in *STARD8* causing DSD remains inconclusive.

WWOX

The human *WWOX* gene, located at a common fragile site *FRA16D* on chromosome 16q23.3–24.1, encodes a tumor suppressor WW domain-containing oxidoreductase, WWOX (152). The relationship between variants in the *WWOX* gene with DSD was first proposed by the identification of a boy with 46,XY DSD who carried a maternally inherited deletion removing exons 6-8 of the *WWOX* gene (153).

Although *WWOX* continues to be used in targeted panel and exome analysis of DSD individuals (30-32), there is now a large body of work to indicate that variants in *WWOX* are not associated with DSD. Homozygous or compound heterozygous variants are associated with autosomal recessive spinocerebellar ataxia-12 (SCAR12) (154) and early infantile epileptic encephalopathy-28 (EIEE28; also known as WOREE syndrome) (155). These data are supported by animal models (154, 156). Other families have been published with biallelic variants causing these syndromic phenotypes (157-160). In these families there is no evidence of DSD in either affected individuals or carriers of deleterious variants. Variants in WWOX should therefore not be considered a cause of human DSD.

Monogenic causes of 46,XX DSD

The most common form of 46,XX DSD is congenital adrenal hypoplasia. Excellent overviews of monogenic causes of adrenal insufficiency have been recently published (161, 162). World-wide 21-hydroxylase deficiency (21-OHD, CYP21A2) is the most common cause of autosomal recessive CAH with a variable incidence of 1:10,000–1:20,000 depending on degree of consanguinity within the population (161). Other rare autosomal recessive forms of CAH include 11 beta-hydroxylase deficiency (*CYP11B1*), 3 beta-hydroxysteroid dehydrogenase deficiency (HSD3B2) and 17 alpha-hydroxylase deficiency (*CYP17A1*) and P450 oxidoreductase deficiency caused by biallelic variants in the gene *POR* (161).

46,XX DSD due to errors in sex-determination i.e. the formation of testis tissue in a 46,XX gonad presents as either ovotesticular or testicular DSD. These phenotypes are usually caused by the presence of the testis-determining gene *SRY* on one of the X chromosomes. Very rare cases are due to variants in the WNT signaling pathway. Only 4 families have been identified with a recessive form of syndromic 46,XX testicular/ovotesticular DSD due to variants in the *RSPO1* gene (163 and references therein). Variants in WNT4 associated with 46,XX DSD are even rarer. Heterozygous LOF variants involving WNT4 have been described in in three 46,XX patients with mild virilization but with an apparent lack of testis tissue [164]. A single family has been described with a homozygous missense variant in WNT4 associated with a complex syndrome of renal agenesis, adrenal hypoplasia, and pulmonary and cardiac abnormalities. Testicular tissue was present in the affected 46,XX individuals who exhibited various degrees of virilization [164]. Here we will discuss some of the more recent and surprising causes of 46,XX DSD that have been identified through exome sequencing (Table 3).

WT1

The mammalian Wilm's tumour 1 (WT1) gene encodes for a transcription factor with over 30 potential isoforms that are generated by a number of mechanisms including alternative transcription start sites, alternative translation start sites, splicing and RNA editing. WT1 has a complex biology and as a result has a role in a myriad of developmental processes including but not limited to, homeostasis and disease of tissues arising from the intermediate and lateral plate mesoderm, mesenchymal epithelial plasticity and fundamental aspects of transcription and epigenetic regulation (165 and references therein). Point mutations in *WT1* are a well established cause of two rare forms of 46,XY DSD; Frasier syndrome (a splice site variant) and Denys-Drash syndrome (DDS; variants in exon 8 or 9). 46,XY individuals with DDS have ambiguous or female external genitalia with normal or undescended testes or gonadal dysgenesis with mesangial sclerosis of the glomerulus and may develop Wilms' tumour (166). Frasier syndrome is characterized by focal nodular glomerulosclerosis and 46, XY DSD with streak gonads and feminized to female external genitalia (167). Another, less

frequent syndrome, and one that overlaps with DDS, is Meacham syndrome, which is characterized by CDH, ambiguous genitalia and complex congenital heart defects but no renal abnormalities in 46,XY individuals (168). Until recently variants in the WT1 gene were considered to have a mild effect on ovarian development. 15 WT1 variants have been reported in 46,XX girls that are associated with either apparently normal functioning ovaries, premature ovarian failure (POF) or streak ovaries (169, 170).

Recently, in a series of 78 children presenting with *SRY*-negative 46,XX OTDSD/TDSD, we identified 7 families with recurrent missense and frameshift variants impacting the 4th ZF of WT1 (14). This is one of the most common causes of *SRY*-negative 46,XX (ovo)testicular DSD. *In-vitro* transient transactivation assays demonstrated that the WT1 protein with mutated 4th ZF shows aberrant biological activity as compared to the wild type protein. Remarkably, when introduced into a human granulosa cell line, the variant results in the upregulation of endogenous Sertoli-specific transcripts. Mutating the 4th ZF of Wt1 results in masculinization of the gonad in XX mice (14). These variants may induce testis formation through the ability of the mutated, but not the wild-type protein, to physically interact with the key pro-ovarian and anti-testis factor ß-CATENIN. This inappropriate interaction is predicted to result in the direct or indirect inactivation of pro-testis signaling pathway(s). OTDSD/TDSD has been reported in associated with WT1 ZF4 variants in other studies (171, 172). The question of whether variants in WT1, which cause 46,XX DSD, are also associated with somatic anomalies as well as the an increased tumour risk is unclear. Of the 7 original cases of 46,XX DSD none were reported to have renal disease nor tumour development although one individual had a diaphragmatic hernia (14). However, 6 of the 7 affected individuals were young children and they will require long-term monitoring. The 46,XX DSD girl with atypical genitalia reported by Gnomes and colleagues (171, 172), was diagnosed with proteinuria at 14 years of age indicating that there is a risk of renal disease developing in within this subgroup of 46,XX DSD.

NR2F2

The chicken ovalbumin upstream promoter–transcription factor type II (COUP-TFII also termed NR2F2) is a member of the steroid/thyroid nuclear receptor superfamily and is structurally related to the orphan nuclear receptor NR5A1 (173). Globally, murine Coup-tf2 is highly abundant at E14-E15 in the mesenchymal compartment of the developing organs and declines after the completion of organogenesis (173). The absence of NR2F2 in terminally differentiated epithelium suggests that NR2F2 plays a major role in the mesenchymal-epithelial transition. In mice, Coup-Tf2 is involved in the development of multiple organs and tissues by modulating the expression of downstream targets to promote cellular differentiation, proliferation, migration, survival, and intercellular communication (173, 174). Homozygous *Nr2f2* null mice die at embryonic day 10 due to its requirement for angiogenesis and heart development (175). *Nr2f2* also plays essential roles in cell differentiation and organogenesis of the stomach, uterus, diaphragm, limbs, skeletal muscle. There is emerging evidence over the last few years for an important role for NR2F2 in Leydig cell formation. In XY male mice NR2F2 is essential for the differentiation and function of foetal and adult Leydig cells (176-178). Inactivation of *Nr2f2* during prepubertal stages of male sexual development results in infertility, hypogonadism, and a block in spermatogenesis due to a failure of progenitor Leydig cells to mature (179). Murine *Nr2f2*$^{+/-}$ XX females show a wide range of reproductive anomalies including reduced fecundity, irregular estrus cycles, delayed puberty, retarded postnatal growth, and reduced levels of steroidogenic enzymes, but virilization and testis development has not been reported (179). XX mice lacking *Nr2f2* have both Müllerian and Wolffian ducts in the mesonephros (180). The ovaries

of these mice do not produce androgens, but an androgen-independent activation of the p-ERK pathway in the Wolffian duct epithelium was observed that lead to the presence of Wolffian duct tissue (180).

In the human heterozygous, and usually *de novo,* variants have been reported in *NR2F2* associated with CHD and/or CDH (181-183). In a screen of 79 individuals with 46,XX *SRY*-negative testicular or ovotesticular DSD, we identified three children with near identical heterozygous frameshift variants in at the N-terminal of NR2F2 (8). These are predicted to be complete LOF because the frameshift variants are located at the N-terminal region of the protein. In two of three children the variant was *de novo*. All three children presented with remarkably similar phenotypes. Each child presented with CHD, one child with congenital diaphragmatic hernia CDH, and two children with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES). The role of NR2F2 in human ovarian development is unknown. Functional studies on the mutant protein were not performed as the protein-truncating variant occurs at the N-terminus of the protein and the truncated protein does not contain any known functional domains. The contribution (if any) of NR2F2 variants to 46,XY DSD remains to be established.

NR5A1

NR5A1, also known as steroidogenic factor-1 (SF-1) is an orphan nuclear receptor transcription factor that plays a key role in many aspects of reproductive development and function (184). In 46,XY individuals the phenotypes associated with NR5A1 variants include a wide range of DSD conditions with or usually without adrenal insufficiency, including testicular dysgenesis with or without Müllerian structures, anomalies of androgen production, hypospadias, progressive androgenisation at puberty, and male infertility with normal genital development (185). In 46,XX individuals pathogenic variants are associated with primary ovarian insufficiency and early menopause (185). Recently the range of phenotypes associated with variant in NR5A1 has been extended to include 46,XX DSD. Amino acid variants of a specific arginine residue, p.Arg92, located in the highly conserved “A-box”, which is required for appropriate DNA-binding, are associated with 46,XX ovotesticular DSD or testicular DSD (16, 184-188). To date, this phenotype has not been observed with missense variants elsewhere in the NR5A1 protein in 46,XX individuals. More than twenty 46,XX (ovo) testicular DSD cases with changes in the p.Arg92 residue have been reported establishing the causality of p.Arg92. The phenotype is highly variable and the variant can be transmitted by a normal fertile mother. Changes in the Arg92 variant result in an absence of DNA-binding by NR5A1 (16). How this change in biological activity results in testis formation in 46,XX individuals is unclear although it has been proposed that the pathogenic variant may abolish the ability of NR5A1 to repress the pro-ovarian pathway of WNT4/□□Catenin (16, 188).

Conclusions

The widespread availability of exome and genomic sequencing is revolutionizing our understanding of the genetic causes of rare congenital disorders, including DSD. However, the generation of large genomic datasets also raises questions concerning the interpretation of the data. The first point to consider is the interpretation of variants in genes that are well-established as a cause of DSD. Aside from *in silico* tools that can provide information on the effects of a variant on protein function, the interpretation of these variants can be aided by simply determining their allelic frequencies in publicly available population genetics datasets. Using population genetics data is a very powerful tool to exclude the possibility that a gene or a specific variant is responsible for DSD. There is a need to consider the wealth of human population genetics data that is now available for different populations worldwide, which often includes the karyotype of the individual carrying each variant. This data can be rapidly used to exclude either a gene or specific variants as causal (e.g. *ESR2,*

MAMLD1). Databases such as GnomAD also offer tools that determine if a variant is likely to be pathogenic based on observed population genetics data. Determining the ancestry of the DSD family therefore, is essential for the interpretation of the population genomic datasets. In parallel with this, for genes that are established to cause DSD such as *MAP3K1* and *MAMLD1*, there is a need for simple, robust and biologically relevant functional assays to determine variant pathogenicity. In the absence of functional data the contribution of missense variants in genes such as *MAP3K1* and *MAMLD1* will continue to be classified as VUS. A good example of where functional studies can aid interpretation of pathogenicity are missense variants reported in GATA4, where variants located within the N-terminal ZF are pathogenic and those outside are not pathogenic (21, 70-73). Variant interpretation will be improved if variants that are identified in routine diagnostic screening are made available publicly through databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), which reports the relationships between human variations and phenotypes, together with supporting evidence. As more DSD variants are added to such publicly available databases, not only will the accuracy in the interpretation of pathogenicity of such variants improve but also genotype-phenotype relationships may be established.

The second point to consider is growing number of new genes which are reported to cause DSD. For some genes their contribution to DSD is questionable. This can be due to either the absence of supporting evidence or that available data indicates that the gene is unlikely to cause DSD (e.g. population genetics data, gene known to cause other phenotypes but not DSD, functional data shows no difference from wild-type protein). For some new genes, such as *LHX9*, causality may be established if other independent DSD cases with potentially pathogenic variants are reported with supporting functional studies. Sufficient affected individuals with the same or similar phenotype carrying potentially pathogenic variants may allow a statistical comparison with appropriate ancestry-matched control groups.

Statements:

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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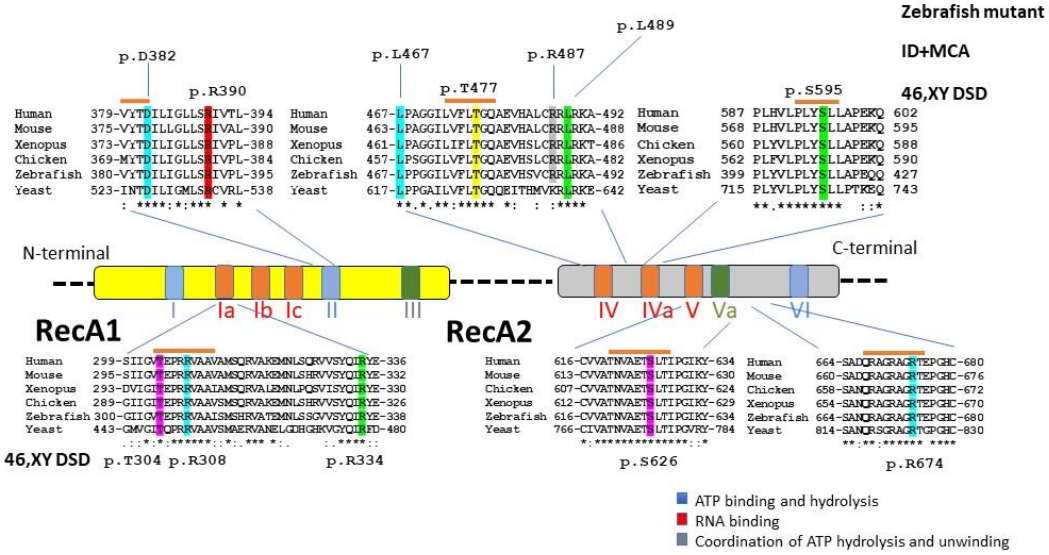
Figure Legends

Fig. 1. Schematic representation of the RecA1 and RecA2 domains in DHX37, indicating the position and evolutionary conservation of residues that are associated with either 46,XY DSD, the NEDBAVC syndrome of intellectual deficiency (ID) and multiple congenital anomalies (MCA), or the zebrafish tactile-evoked escape response mutant. The function of the motifs within each RecA domain is colour-coded

Fig. 2. Schematic representation of GATA4 showing the main functional domains and in detail the amino acid composition of the N-terminal ZF showing the position of variants established to cause 46,XY DSD. The valine residue that is mutated in mice which show male-to-female sex-reversal in highlighted in blue. Other variants associated with 46,XY DSD are indicated. TAD transcriptional activation domain, NLS nuclear localisation signal.



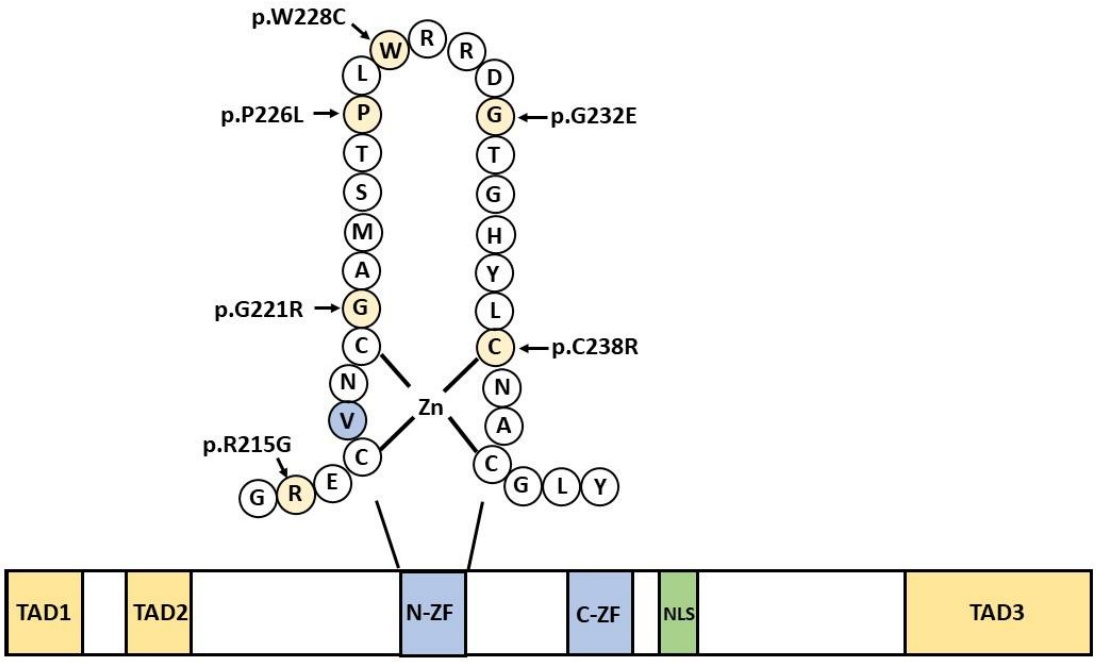


Table 1: Monogenic associations with 46,XY DSD with supporting evidence for a role in DSD.

Gene	Function	Chr. position	Human Pathologies		Mode of transmission	Comments (references)
			46,XY	46,XX		
Chromobox 2 isoform 1 - CBX2.1	Component of Polycomb group multiprotein PRC1-like complex involved in transcriptional repression	17q25.3	Girl with bilateral ovaries	?	Autosomal recessive	A single 46,XY patient reported (28)
Desert Hedgehog Signaling Molecule - DHH	Signaling molecule that plays an important role in regulating morphogenesis	12q13.12	GD with/without minifascicular neuropathy	?	Autosomal recessive	Phenotypic variability may be due to the position of the variant in the protein (37)
DEAH-Box Helicase 37 DHX37	ATP-binding RNA helicase	12q24.31	GD, TRS, anorchia, NEDBAVC syndrome	No reproductive phenotype reported, NEDBAVC syndrome	Autosomal dominant, sex-limited	Relatively common cause of XY DSD but mechanism involved is unknown (12, 13).
Doublesex And Mab-3 Related Transcription Factor 1 DMRT1	Transcription factor	9p24.3	GD, possible male infertility	?	Autosomal dominant	Well-established role in testis formation but coding variants are a rare cause of XY GD (32, 64)
GATA Binding Protein 4 GATA4	Transcriptional activator that binds to the consensus sequence 5'-AGATAG-3	8p23.1	Congenital heart disease with/without GD. Isolated GD	Congenital heart disease	Autosomal dominant	Functional studies indicate pathogenic variants are limited to zinc-finger DNA-binding motifs (21, 70-72)
Hedgehog Acyltransferase HHAT	Catalyzes N-terminal palmitoylation of HH proteins	1q32.2	Nivelon-Nivelon-Mabille syndrome. Complex congenital phenotype with progressive microcephaly, vermis hypoplasia, and skeletal dysplasia. XY individuals have GD.	Nivelon-Nivelon-Mabille syndrome. No apparent DSD phenotype.	Autosomal recessive	Rare cause of syndromic 46,XY DSD (78-80)
Mastermind Like Domain Containing 1 MAMLD1	Transcriptional co-activator	Xq28	Hypospadias with/without cryptorchidism	?	X-linked recessive	LOF variants are an established cause of XY DSD. Causality of missense variants is difficult to establish (82-87)
Mitogen-Activated Protein Kinase Kinase Kinase 1 MAP3K1	Component of a protein kinase signal transduction cascade	5q11.2	GD	?	Autosomal dominant, sex-limited	An established and relatively common cause of DSD but pathogenicity of missense variants difficult to establish, hence usually classified as VUS (88, 89).
Myelin Regulatory Factor MYRF	Transcription factor	11q12.2	Cardiac-urogenital syndrome Encephalitis/encephalopathy, mild, with reversible myelin vacuolization. Variable DSD phenotypes	Cardiac-urogenital syndrome Encephalitis/encephalopathy, mild, with reversible myelin vacuolization. DSD	Autosomal dominant	Emerging important contributor to XY and XX DSD with a wide range of phenotypes. Includes both syndromic and non-syndromic forms of DSD (95-99).
Protein Phosphatase 2 Regulatory Subunit B''Gamma PPP2R3C	Regulatory subunit of the serine/threonine phosphatase, protein phosphatase 2	14q13.2	GD, dysmorphic facies, retinal dystrophy, and myopathy. Teratozoospermia in heterozygous XY carriers	Possible POI in heterozygous carriers	Autosomal recessive	Independent families establish this is cause of a rare form of syndromic XY DSD (103).
SRY-Box Transcription Factor 8 - SOX8	Transcription factor	16p13.3	GD, spermatogenic failure	POI	Autosomal dominant	Highly variable phenotype. Variants may contribute to male and female infertility. Very rare cause of 46,XY DSD (123).

SRY-Box Transcription Factor 9 - SOX9	Transcription factor	17q24.3	Campomelic dysplasia with/without GD. Isolated GD.	Campomelic dysplasia. Isolated testicular/ovotesticular DSD	Autosomal dominant	Variants in regulatory elements have emerged as an important cause of non-syndromic 46,XY GD and 46,XX (OVO)TDSD (119).
Zinc Finger Protein, FOG Family Member 2 - ZFPM2	Transcriptional cofactor that acts via the formation of a heterodimer with transcription factors of the GATA family	8q22.3	Congenital heart defects, diaphragmatic hernia without DSD. 46,XY complete or partial GD with no evidence for cardiac anomalies	Congenital heart defects, diaphragmatic hernia without DSD	Autosomal dominant	Rare cause of XY GD. Genetic data is supported by functional studies. (76).
Zinc and Ring Finger 3 - ZNRF3	E3 ubiquitin-protein ligase that acts as a negative regulator of the Wnt signaling pathway	22q12.1	GD or undervirilised male	?	Autosomal dominant	Small number of cases reported. Genetic data supported by functional studies. Variable phenotype (131).

A colour coding system of classification of genes proposed to be associated with DSD is used. Green indicates a definite role in DSD, Amber, some limited but inconclusive evidence and Red, no evidence to support a role in DSD.
*Homozygous variant. The father, who presented with unilateral anorchia, is considered an obligate carrier although he has not been tested for the variant.
WD, Wolffian Derivatives; GD gonadal dysgenesis; CGD complete gonadal dysgenesis; PGD partial gonadal dysgenesis; TRS testicular regression syndrome

Table 2. Monogenic associations with 46,XY DSD requiring further genetic or experimental evidence to establish causality

Gene	Function	Chr. position	Human Pathologies		Mode of transmission	Comments (references)
			46,XY	46,XX		
Chromobox 2 isoform 2 - CBX2.2	CBX2 isoform 1 is a component of Polycomb PRC1-like complex. Unlikely that isoform 2 has a biological function	17q25.3	2 patients reported with microphallus, perineal hypospadias, one with bilateral cryptorchidism	unlikely	Autosomal dominant	Population genetics data indicates that variants in the CBX2.2 isoform do not cause DSD (28, 35)
Estrogen Receptor 2 ESR2	Nuclear hormone receptor	14q23.3	Proposed to cause XY DSD. Highly variable phenotype	Ovarian dysgenesis	Autosomal dominant	Absence of conclusive genetic and functional evidence to support a role in XY DSD (137)
Fibroblast Growth Factor Receptor 2 FGFR2	Tyrosine-protein kinase cell-surface receptor for fibroblast growth factors	10q26.13	46,XY and 46,XX -Antley-Bixler syndrome without genital anomalies or disordered steroidogenesis; Apert syndrome; Beare-Stevenson cutis gyrata syndrome Craniofacial-skeletal-dermatologic dysplasia; Craniosynostosis, nonspecific; Crouzon syndrome; Jackson-Weiss syndrome; LADD syndrome; Pfeiffer syndrome; Saethre-Chotzen syndrome; Scaphocephaly and Axenfeld-Rieger anomaly. Proposed to cause XY DSD.		Autosomal dominant	Genetic evidence in favour of a role in XY DSD is lacking. A single XY DSD patient has been reported but the proposed pathogenic variant was reported elsewhere in males with no DSD. Variants in FGFR2 cause a wide range of craniosynostosis syndromes with no evidence of DSD (140-144).
3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 HMGCS2	Mitochondrial enzyme that catalyzes the first reaction of ketogenesis	1p12	HMG-CoA synthase-2 deficiency. Proposed as a candidate for XY DSD.	HMG-CoA synthase-2 deficiency	Autosomal recessive	Lack of conclusive genetic evidence as a cause of XY DSD. Biallelic variants cause HMG-CoA synthase-2 deficiency with no DSD (145, 146)
LIM Homeobox 9 LHX9	Transcription factor	1q31.3	GD with limb anomalies	?	Autosomal dominant	A single case described with no functional studies (148)
StAR Related Lipid Transfer Domain Containing 8 - STARD8	Rho GTPase activating protein	Xq13.1	Proposed as a cause of GD	?	X-linked recessive	A single family of two affected sibs described. Population genetics does not support a role for the reported variant in DSD (151)
WW Domain Containing Oxidoreductase - WWOX	Member of the short-chain dehydrogenases/reductases protein family	16q23.1	Developmental and epileptic encephalopathy; Spinocerebellar ataxia. Proposed to cause XY DSD	Developmental and epileptic encephalopathy; Spinocerebellar ataxia	Autosomal recessive	WWOX is now established as a cause of autosomal recessive congenital disorders with no evidence for DSD (154, 155).

The colour coding system of classification of genes is used (see Table 1). WD, Wolffian Derivatives; GD gonadal dysgenesis; CGD complete gonadal dysgenesis; PGD partial gonadal dysgenesis; TRS testicular regression syndrome

Table 3. Monogenic associations with 46,XX DSD with supporting evidence for a role in DSD

Gene	Function	Chr. position	Human Pathologies		Mode of transmission	Comments (references)
			46,XY	46,XX		
Myelin Regulatory Factor MYRF	Transcription factor	11q12.2	Cardiac-urogenital syndrome Encephalitis/encephalopathy, mild, with reversible myelin vacuolization. Variable DSD phenotypes	Cardiac-urogenital syndrome Encephalitis/encephalopathy, mild, with reversible myelin vacuolization. DSD	Autosomal dominant	Emerging important contributor to XY and XX DSD with a wide range of phenotypes. Includes both syndromic and non-syndromic forms of DSD (95-99).
Nuclear Receptor Subfamily 2 Group F Member 2 NR2F2	Transcription factor	15q26.2	Congenital heart defects.	Ovotesticular DSD with congenital heart defects and variable somatic anomalies including blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) and congenital diaphragmatic hernia	Autosomal dominant	Rare cause of a novel syndromic form of 46,XX DSD. Phenotype(s) associated with variants in XY individuals unknown (8)
Nuclear Receptor Subfamily 5 Group A Member 1 NR5A1	Transcription factor	9q33.3	Adrenocortical insufficiency, gonadal dysgenesis, undervirilised male, spermatogenic failure, asplenia, polysplenia	Adrenocortical insufficiency, POI, ovotesticular and testicular DSD	Autosomal dominant	Accurate phenotype-genotype correlations lacking. Circumstantial evidence to suggest other gene variants influence the variability of the phenotype (185)
WT1 Transcription Factor - WT1	Transcription factor	11p13	Denys-Drash syndrome; Frasier syndrome; Meacham syndrome, Nephrotic syndrome; Wilms tumor	Nephrotic syndrome; Wilms tumor; ovotesticular/testicular DSD	Autosomal dominant	Variants impacting the 4th ZF are emerging as a relatively common cause of 46,XX (OVO)TDSD (14).

The colour classification of genes is (see Table 1). WD, Wolffian Derivatives

Table 4. Summary of DSD phenotypes associated with published DHX37 pathogenic variants

DHX37 Variant (No. patients)	XY DSD Phenotypes
p.T304 (3)	Female, GD + WD
p.R308 (15)	Female, GD; Female, CGD+WD; Female, PGD; Female 46,XY DSD, virilised external genitalia; Male, TRS, micropenis, hypospadias+bilateral cryptorchidism; Male, TRS, micropenis, bilateral cryptorchidism
p.R334 (2)	Female, GD + WD; Male, TRS, micropenis, bilateral cryptorchidism.
p.R390 (1)	Female, GD
p.T477 (2)	Female, GD; Male, TRS micropenis, bilateral cryptorchidism*; Male, unilateral anorchia
p.S595 (2)	Sibs – female, GD+WD; Male TRS, micropenis
p.S626 (1)	Male, TRS, micropenis, bilateral cryptorchidism
p.R674 (9)	Female CDG; Female gonadal dysgenesis + WD; Male PGD+left testis; Male TRS, micropenis
p.G1030 (1)	Male, TRS, micropenis