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Title:
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Date:
2016-12-01

Citation:
Croft, B., Ayers, K., Sinclair, A. & Ohnesorg, T. (2016). Review disorders of sex development: The evolving role of genomics in diagnosis and gene discovery. Birth Defects Research Part C Embryo Today Reviews, 108 (4), pp.337-350. https://doi.org/10.1002/bdrc.21148.

Persistent Link:
https://hdl.handle.net/11343/292190

Disorders of Sex Development: the evolving role of genomics in diagnosis and gene discovery

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version record. Please cite this article as doi:10.1002/bdrc.21148.

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Abstract

Disorders of Sex Development (DSDs) are a major paediatric concern and are estimated to occur in around 1.7% of all live births (Fausto-Sterling, 2000). They are often caused by the breakdown in the complex genetic mechanisms that underlie gonadal development and differentiation. Having a genetic diagnosis can be important for patients with a DSD: it can increase acceptance of a disorder often surrounded by stigma, alter clinical management and it can assist in reproductive planning. While Massively Parallel Sequencing (MPS) is advancing the genetic diagnosis of rare Mendelian disorders, it is not yet clear which MPS assay is best suited for the clinical diagnosis of DSD patients and to what extent other established methods are still relevant. To complicate matters, DSDs represent a wide spectrum of disorders caused by an array of different genetic changes, many of which are yet unknown. Here we discuss the different genetic lesions that are known to contribute to different DSDs, and review the utility of a range of MPS approaches for diagnosing DSD patients.

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Introduction

Disorders of sex development

Disorders of Sex Development (DSDs) are defined as congenital conditions where development of chromosomal, gonadal, or anatomical sex is atypical (Hughes et al., 2006). They include a wide spectrum of disorders, ranging from mild hypospadias to ambiguous genitalia and gonad abnormalities causing complete sex reversal. An overview of the types and definitions of DSD are listed in Table 1.

Molecular genetics of sex determination and gonadal development

The following is a brief summary of human development and differentiation, and covers only the main genes/proteins involved. For more details, see other reviews (Eggers et al., 2014; Knarston et al., 2016; Ohnesorg et al., 2014). The gonads initially form on the ventro-medial surface of the embryonic kidney, at about 5 weeks of gestation in humans. The development of these early gonads differs from other organs, as they have the potential to differentiate into two functionally different organs, testes or ovaries (meaning they are bipotential). Following this differentiation, hormones released by either testes or ovaries lead to the development of secondary sexual characteristics and eventually to a fully functional male or female.

Several genes have been shown to be crucial for the initial development of a bipotential gonad (Figure 1). In mouse studies, knock-out mutations of *Nr5a1* (also known as SF1, steroidogenic factor 1), *M33/Cbx2*, *Wt1*, and *Lhx9* cause either complete absence of gonadal structures or streak gonads that fail to develop further (Birk et al., 2000; Kreidberg et al., 1993; Luo et al., 1994). Whether the bipotential gonad differentiates into a testis or an ovary depends on the presence or absence of a Y chromosome, respectively. The Y chromosome carries the *SRY* gene, the master male sex-determining gene that is both necessary and sufficient to trigger testis development (Koopman et al., 1991; Sinclair et al., 1990). At about week 6 of gestation in humans, *SRY* becomes expressed and kick starts the male developmental pathway that causes the bipotential gonad to differentiate into a testis. SRY's main function (in the gonad) appears to be the upregulation of *SOX9*, a transcription factor essential for testis development. Like *SRY*, *SOX9* has been shown to be both necessary and sufficient for testis development, as *SOX9* null mutations in XY individuals lead to female development and duplications induce testis development in affected XX individuals (Foster et al., 1994; Huang et al., 1999). NR5A1 acts together with both SRY and SOX9 and

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enhances their activity on regulatory regions (promoters and enhancers), e.g., in the mouse, Nr5a1 and Sry or Sox9 upregulate and maintain *Sox9* expression (Sekido and Lovell-Badge, 2008), and Nr5a1 and Sox9 or *Wt1* activate the *Amh* (Anti-muellerian hormone) promoter *in vitro* (Arango et al., 1999). Other known important components of the early testis development pathway are the transcription factors GATA4 and its co-factor, FOG2, which were shown to activate *Sry*, *NR0B1* (also known as *DAX1*), and ATRX, as well as the growth factors DHH and FGF9, hormone AMH, and map kinase MAP3K1 (reviewed in Ohnesorg et al. (2014)).

In contrast, relatively little is known about ovarian development. Since Jost (1947) discovered in the 1940s that the removal of embryonic gonads of rabbits leads to a female phenotype (regardless of the chromosome constitution), the female pathway was long thought to be a passive, or "default" pathway that will be followed, unless overridden by the regulatory cascade triggered by SRY in males. However, recent research strongly suggests that both the female and male pathways actively suppress the opposing developmental program to ensure proper ovarian or testicular development and function. Mouse studies have shown that this battle of the sexes is not only important during embryonic development, but continues when fully functional differentiated gonads have formed in the adult (Matson et al., 2011; Uhlenhaut et al., 2009). The main components of the female pathway are the transcription factor FOXL2, one of the earliest markers of the developing ovary, and the WNT-signaling pathway with its activating components, WNT4 and R-Spondin1 (RSPO1) (Chassot et al., 2008; Cocquet et al., 2002). Homozygous *RSPO1* null mutations have been reported to cause 46,XX testicular DSD, while large duplications on chromosome 1 that contain both *WNT4* and *RSPO1*, can cause 46,XY gonadal dysgenesis (Jordan et al., 2001; Parma et al., 2006). Based on mouse studies, it has been suggested that over-activation of β-catenin by WNT4 and RSPO1 is able to override the testis pathway and trigger ovarian development (Chassot et al., 2008).

DSD gene discovery and screening for causative mutations

To date, between 30 and 50% of DSD patients receive a specific molecular diagnosis in a research setting (Munger and Capel, 2012); however, in the clinic this rate drops to 13% (Arboleda et al., 2013; Lux et al., 2009). Yet we know already of hundreds of different mutations and variants that are attributed to DSD in humans, while even more have been discovered in animal models. While copy number variation (CNVs) and small indels account for a significant proportion, overall, the most frequent type of mutation found in patients with DSD are single nucleotide variants (SNV),

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such as missense mutations, splice site changes, and premature stop codons. The rise of Massively Parallel Sequencing (MPS) approaches for DSD diagnosis will undoubtedly lead to a dramatic increase of implicated variants. The biggest challenge will be the validation of these.

The history of DSD genetic screening

Before the onset of fast and affordable sequencing tools, determining the cause of genetic disorders was laborious and time-consuming (Figure 2). Karyotyping was often used to detect changes in chromosome numbers or gross morphological changes of a chromosome that could be easily identified by eye. For example, the function of the Y chromosome was established using a series of complex mouse crosses followed by karyotyping (Welshons and Russell, 1959). For other complex disease traits, large scale linkage analysis was used, however, as most DSD patients are infertile, there was a scarcity of family data (Arboleda and Vilain, 2011), consequently a linkage based approach was not feasible for these conditions.

The discovery of *SRY* is a perfect example of the complex and laborious nature of genetics in the late 20th century. In order to locate the dominant testis-determining factor on the Y chromosome, the researchers used DNA from sex–reversed patients who carried XX chromosomes but formed testes and were male in appearance (46, XX testicular DSD) (Sinclair et al., 1990). Approximately 80% of these XX males had a small portion of the Y chromosome, including the putative testis-determining factor translocated onto one of their X-chromosomes. This small translocated Y region was then cloned and the coding region identified by using a series of Noah's Ark blots. These were a particular type of Southern blot containing DNA from both sexes of several mammals on a Southern blot, probed with different small fragments of human Y material to establish which fragment of the human Y chromosome was most conserved and likely to contain a gene. The Noah's Ark blots identified one small fragment of the human Y chromosome that was also present on the Y chromosome of several other mammals, indicating this was the most evolutionary conserved region and most likely to contain the coding region of a gene. This fragment of the Y was then subjected to Sanger sequencing (Heather and Chain, 2016; Sanger et al., 1977), which ultimately revealed the long sought after testis determining factor what we now know as the *SRY* gene (Sinclair et al., 1990).

Once the sequence of *SRY* was discovered, researchers were then able to screen 46,XY patients for mutations, often discovering point mutations and frame shifts within the gene. Still, the cost

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involved in using sequencing technology of the time often limited researchers to restriction digest analysis followed by confirmation via Sanger sequencing (Berta et al., 1990; Jäger et al., 1990).

While mutations in *SRY* are attributed to a significant proportion of DSD (10-15% 46,XY gonadal dysgenesis (Cameron and Sinclair, 1997), it became clear that many other gonad genes were yet to be identified to explain the vast array of 46,XY DSD. The discovery of *SOX9* a few years later was crucial for furthering our understanding of sex determination and DSD. This was achieved by utilizing the conserved high mobility group (HMG) DNA binding domain discovered in *SRY*, to locate other potential sex determining genes. By creating probes for the HMG box and hybridizing to the genome at low stringency, the *SOX* gene family was identified (Goodfellow and Lovell-Badge, 1993; Sinclair et al., 1990). *SOX9* was the first gene of the family that showed any sex related phenotypes. Once the sequence of *SOX9* was determined, researchers were again able to screen DSD patients and discovered that a proportion of *SOX9* mutations is also associated with a skeletal malformation syndrome, campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994).

Over the following decade, an increasing number of genes associated with DSD were discovered. Often informed from animal studies, such as the case with *NR5A1* which was discovered using florescent *in situ* hybridization (FISH) using a drosophila gene (Taketo et al., 1995). As with many other disorders, candidate DSD genes were often identified and analyzed in mice before being screened in humans. *NR5A1* was further analyzed in animal models of XY sex reversal before targeted Sanger sequencing of DSD patients (Achermann et al., 1999).

Current genomic analysis of DSD

After the publication of the first human genome in 2001, the potential of using sequencing arrays to discover and diagnose cases of DSD was evident to all (Venter et al., 2001). Researchers now had the ability to design primers for direct PCR sequencing, instead of spending significant amounts of time tediously cloning patient DNA.

These new technologies have facilitated the analysis of large cohorts of patients for causative variants in a relatively cost effective manner. Many variants in gonad determining genes were identified by focusing on single candidate genes (Table 2) and subsequent sequencing of those genes in appropriate DSD patients. Other gonad genes were first identified in mouse models and then sequencing revealed variants in these genes in DSD patients (De Baere et al., 2002). For

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example, a *Wnt4* null mouse showed absent Müllerian structures which, in turn, led to the sequencing of 46,XX DSD patients with similar phenotypes and eventually identified *WNT4* missense variants (Biason-Lauber et al., 2004). However, knowing a variant exists does not guarantee it is causative. This requires extensive functional analysis, in both *in vitro* and *in vivo* model systems, as well as multiple similar mutations in other DSD patients with the same phenotype.

Copy number analysis in DSD

Examining point mutations in patients can identify causative variants, however not all genetic variation in DSD is due to changes in the coding sequence of a gene. As with many other developmental disorders, sex determination and differentiation can be highly sensitive to gene dosage effects (Bashamboo et al., 2010). Studies of changes of gene copy number began with karyotype banding, but this could only detect very large changes in chromosome structure. Smaller duplications and deletions were more difficult to detect. Previously, fluorescent *in situ* hybridization (FISH) has been employed to detect smaller CNVs by binding specific probes to the chromosome. FISH approaches were used to show that a *SOX9* deletion was causative of campomelic dysplasia syndrome, in the absence of gonad related defects (Olney et al., 1999). While FISH can provide reliable data for large scale chromosomal rearrangements, the protocol itself is cumbersome and time consuming (Rudkin and Stollar, 1977), leading many researchers to move away from the technique in favor of genomic array based technologies.

Comparative Genomic Hybridization (CGH) arrays have been extensively utilized to detect small to large-scale genomic duplications and deletions. CGH arrays utilize small oligonucleotide probes that can bind to regions of interest, allowing fine mapping of chromosome rearrangements by comparing the copy number of the test sample to a control sequence. This approach in the context of DSD has the potential to identify CNV's that affect both known and novel gonad genes (Bashamboo et al., 2010).

The use of CGH arrays has been especially useful when examining variants related to *SOX9* and its regulation (Table 2), by discovering micro-duplications, whole gene duplication, and deletions related to campomelic dysplasia and DSD (Chen et al., 2012; Cox et al., 2011; Huang et al., 1999; Smyk et al., 2007b). CNV arrays have been essential for regulatory region analysis, which is usually missed by only focusing on the coding regions of a gene. In the case of *SOX9,* multiple large CNVs

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have been identified far upstream of the coding region. In these cases, both duplications and deletions of 46,XX and 46,XY DSD patients helped define the minimal overlap regulatory region upstream of SOX9, known as *RevSex* (Benko et al., 2011). CGH technologies have also identified many putative regulatory regions in genes such as: *SOX3, NR0B1, and GATA4* (Harrison et al., 2014; Smyk et al., 2007a; Sutton et al., 2011).

Over the years, the use of CGH arrays has been essential for screening large cohorts of DSD patients. One study screened 116 patients with both 46,XX and 46,XY DSD, detecting candidate CNVs in 21.5% of patients (Tannour-Louet et al., 2010). Another large cohort of 87 46,XY GD patients was screened with a diagnostic hit rate of 30% (Ledig et al., 2010), while a smaller screen of 46,XY DSD patients found CNVs in 23.8% of cases by jointly utilizing a CGH microarray, as well as Multiplex ligation-dependant Probe Amplification (MPLA) for validation (White et al., 2011). More recently these technologies have been combined with epigenomic studies using a retrospective analysis to create a high-density gene specific CNV and epigenomic maps from patients with DSD, in an effort to improve the diagnostic potential of CNV screening (Amarillo et al., 2016).

Targeted Gene Panels

The relative speed and ease of MPS technologies have allowed many groups to develop disease specific sequencing panels. Targeted gene panels have taken advantage of the vast range of MPS platforms, and having a reduced data set makes the bioinformatic challenges that much easier compared to WES/WGS. Whole exome and genome sequencing is currently economically prohibitive for very large patient cohorts. Consequently, a smaller and more specialized targeted gene panel can provide a cost-effective method for directly screening known and candidate DSD genes (Tuhan et al., 2016). Mutations associated with *MAMLD1* were first identified using this approach in a cohort of severe non-syndromic 46,XY DSD (Kalfa et al., 2012). Targeted sequencing can also be used to determine the frequency of known DSD mutations in diverse patient cohorts (Tantawy et al., 2014).

Few groups have taken targeted screening to the next level as a tool to detect novel candidate DSD genes. One of the earliest studies focused on only 35 specific genes (screened using MPS) known to be causative for DSD. This preliminary analysis provided a diagnostic rate of 28% from two out of seven patients (Arboleda et al., 2013).; yet, more recently larger panels have been used. One group targeted 219 known and candidate DSD genes and was able to produce a specific

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diagnosis in 69% of 13 46,XY and 25% of eight 46,XX DSD cases screened (Dong et al., 2016). Our group has applied a gene panel of 64 diagnostic genes for DSD and 967 candidate genes to over 326 46,XY and 46,XX DSD patients, allowing a likely diagnostic rate of 43% (Eggers et al. 2016, *Genome Biology*, in press). These studies highlight the utility of targeted sequencing panels for the diagnosis of unsolved DSD cases.

Targeted gene panel sequencing can be a highly cost effective test, in terms of reagents, data handling, and curation. Customized targeting also allows sequencing of non-exonic regions; for example, non-coding regions, regulatory regions, and miRNAs can all be included on a panel. This offers a distinct advantage over WES. Finally, as only select genes or regions are sequenced, incidental findings are decreased, making consent processes and variant reporting simpler, compared to WES or WGS.

Despite the significant cost reductions of MPS, the majority of publications still used CGH arrays and Sanger Sequencing for diagnosis (Baetens et al., 2014), as targeted gene panels currently lack reliable methods for CNV analysis. This is a major issue for DSDs, in particular 46,XX DSD, because CNVs are thought to contribute to a large percentage of cases. Whether using a screen for diagnosis or research, a wider net will always allow for the possibility of discovering something novel, which is where whole exome (WES), and especially whole genome sequencing (WGS), becomes appealing.

Whole Exome Sequencing

We are currently at the cusp of a new wave of genomic technology. In recent years, the use of WES for DSD research has focused on small familial cases (Eggers et al., 2015; Werner et al., 2015). However, WES is beginning to be utilized in larger cohorts. Baxter et al. (2015) screened 47 46,XY DSD patients with a variety of DSD phenotypes. However, this study did not analyse the entire WES data generated, but rather focused their analysis on a subset of 64 known genes that had previously been associated with DSD in at least one human case. This analysis provided a diagnostic rate of 35% (Baxter et al., 2015). This study was a continuation from the research group's previous work with targeted sequencing array, which showed a similar diagnostic rate (28%) (Arboleda et al., 2013).

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A more recent study utilized WES to determine causes of 46,XX (ovo-)testicular DSD. Nine unsolved patients underwent WES and a diagnostic rate of 33% was achieved. However, this group also concentrated their analysis on known DSD genes, narrowing their focus to *NR5A1* as a key candidate for 46,XX DSD (Baetens et al., 2016). WES has also been used to validate the existence of potential genetic modifiers in patients, which could sensitize an individual to DSD (Bagheri-Fam et al., 2015). WES will allow for the discovery of novel genes related to DSD, however, the current trend to only analyze known DSD genes to ease the bioinformatic analysis only hampers the potential of WES for novel gene discovery.

Whole Genome Sequencing

To date, diagnostic screening concentrates on particular approaches which, due to their nature, will only detect specific genomic changes. For example, CNV arrays are often used in conjunction with WES or targeted panels (Figure 3). However, this is beginning to change as the technology for whole genome sequencing (WGS) approaches a point where it becomes cost effective. WGS can detect all variation in the genome, including non-coding regions and CNVs, although somewhat limited in detecting low coverage variants (Clark et al., 2011). In particular, WGS can improve detection of much smaller CNVs than traditional arrays (Tan et al., 2014), covering all possible genetic lesions in one test (Stavropoulos et al., 2016). However, the complex data analysis and cost of WGS is currently preventing large-scale uptake of the technology in a clinical setting. Few studies to date have utilized WGS as a diagnostic tool, although fields such as cancer genetics have begun using WGS to screen large populations to great effect (Foley et al., 2015). In the search for DSD variants one study investigating the cause of ectopic expression of *SOX3* leading to 46,XX sex reversal used WGS to confirm a result obtained using a CGH array (Haines et al., 2015). However, the rapid evolution of sequencing technologies means it is likely that WGS costs associated with producing and processing the data will continue to fall, such that it will eventually be suitable for use in a clinical setting.

Limitations of MPS technologies

As the amount of MPS data grows, it is becoming more apparent that conditions, such as DSD, are due to an array of genetic variations (Howrigan et al., 2011). Currently we like to think that a variant in a single gene causing a pathology affects a single pathway. However, many genetic variants associated with disease, when investigated individually, are not considered damaging on their own (Manolio et al., 2009; Munger and Capel, 2012). Yet, a single variant can cause multiple

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diseases, when those pathologies utilise the same pathway (Baxter and Vilain, 2013). This ever-increasing complexity only adds to the challenge of variant discovery and categorization, highlighting the need for better bioinformatic tools to holistically examine and call variants in these large genomic data sets (Zaghloul and Katsanis, 2010).

Limitations of sequencing analysis technology

Currently there is a plethora of companies and technologies providing options for library preparation, sequencing, and bioinformatics analysis. It is not surprising that it is challenging to decide which technology and pipeline is the best suited for diagnostics, especially when there can be little compatibility between these technologies. Several studies have shown that the concordance between up to five variant callers is surprisingly low, 57% (O'Rawe et al., 2013) and 70% (Cornish and Guda, 2015). Accuracy of variant calling also depends on the genomic region and read depth, in which only 77% of likely pathogenic SNVs are located in a genomic region of high sequencing confidence (Goldfeder et al., 2016). A lack of consensus between different alignment and variant calling pipelines is worrisome, casting doubt on the validity of any single call. It is suggested that using multiple sequencing analysis platforms, to average out the calls, could help with this issue (Humble et al., 2016). However more recent analysis of variant callers has shown greater concordance of ~92% (4 popular variant callers), indicating that the pipelines are increasing in accuracy over time (Hwang et al., 2015).

Future of genomics and DSD

While MPS promises to improve the genetic diagnostic rate of patients with DSD, discovery of novel genes that contribute to DSD will further increase this diagnostic power. The validation of a novel gene is a long process, often requiring two independent pathogenic variants in probands with similar phenotypes coupled with functional analysis. Given the rarity of many types of DSD (ie. 46,XY complete gonadal dysgenesis or 46,XX testicular DSD), analysis of large cohorts with MPS is therefore required and data sharing between groups becomes paramount. Furthermore, data sharing will also allow researchers to discount non-causative variants (thereby improving certainty in diagnosis).

While MPS technologies focus on the sequence of genes and regulatory regions, it is important to also consider variation in epigenetics when investigating DSD. DNA methylation is involved in various genetic processes, such as silencing, switching on, and stabilizing genes, as well as

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remodeling chromatin. Studies in mice have found that the 5'-flanking region of the *Sry* gene is hypomethylated at 11.5 dpc when *Sry* is highly expressed in the gonads, and hypermethylated earlier in 8.5-dpc embryos when this gene is silenced (Gierl et al., 2012; Nishino et al., 2004). Methylation may also play a role in human *SRY* regulation. A pericentric inversion of the Y-chromosome that results in increased methylation of the *SRY* promoter has been described in a 46,XY female patient (Gimelli et al., 2006). These studies highlight the need to screen for a broader set of genomic changes in humans. As epigenetic changes are tissue specific, this poses a difficulty in obtaining the affected tissue from DSD patients for analysis.

Another issue currently faced by DSD researchers is that screening of DSD defects in the affected tissue still remains difficult, and many screens rely on DNA from blood samples. This means that understanding how processes like RNA splicing and expression or epigenetic marks are affected is challenging. However, with the advances in pluripotent stem cell technology, differentiation of gonadal or urethral plate cells from patient fibroblasts in the future may allow us to assess the effects of genomic variants in a more representative cell type.

Mosaic mutations also remain an under-researched area in DSD. Genomic studies are beginning to highlight the importance of these, especially when parents who are low copy mosaic may pass on the affected allele on to children who are then affected with the disorder. Consequently, it can be difficult to predict the chance of recurrence in subsequent children. With the increased power of whole exome or whole genome sequencing, including longer reads with better coverage and depth, identification of low copy variants will become more and more prevalent. Although recent developments in single molecule sequencing may also solve this problem, as the technology evolves (Laszlo et al., 2014; Pushkarev et al., 2009).

Concluding statement

Since the discovery of *SRY* over 25 years ago, significant progress has been made in understanding gonad development and the genetics underlying DSD. However, significant gaps remain. The majority of human DSD cases still cannot be explained at the molecular level. While environmental factors might play a role, it is more than likely that other components of this complex genetic network await discovery. The dramatic reduction in sequencing costs leading to the rapid uptake of MPS for diagnosis will undoubtedly greatly assist in identifying novel DSD genes and many more causative variants. However, two current major bottlenecks need to be addressed. Firstly, better

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bioinformatic methods (handling/storing data, greater prediction for damaging/benign variants, the ability to interpret CNV in WES and targeted gene panels) and more importantly, higher throughput/rapid functional assays to confirm these variants. Developments in both these areas will help to improve the currently poor diagnostic rate of DSD patients and the clinical management of these difficult conditions.

Acknowledgments

B.C is supported by an Australian Postgraduate Award, Monash University, K.A, T.O and A.S are supported by a NHMRC program (grant number GNT1074258).

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Figure Legends

Figure 1: The genes and pathways necessary to drive development of either a testis or an ovary from the Bipotential gonad. The genes specific to the development of the Bipotential gonad have all been identified in specific mouse knock out strains in which no gonadal development occurs, EMX2, LHX9, CBX2, NR5A1, AND WT1(-KTS). Downstream activation of SRY in a XY gonad drives the formation of a mature testis beginning at 6 weeks in the developing human embryo and at 11.5 dpc in the mouse. SRY, along with NR5A1, activates SOX9 which drives the expression of AMH, as well as the expression of FGF9/FGFR2. This in turn creates two positive feedback loops sustaining the male specific factors SOX9 and FGF9, leading to the activation of downstream genes, such as DMRT1, which maintains the differentiation of the testis. In an XX gonad, absence of SRY leads to the upregulation of WNT4, FOXL2, GATA4, and RSPO1, which are required for ovary development. FOXL2 is also required to maintain and mature the ovary. The male and female pathways antagonize each other through the presence of SOX9, FGF9, and DMRT1 in the male and WNT4, RSPO1 and FOXL2 in the female pathway (red lines). Due to the complex nature of sex determination, only the key sex determining genes discussed in the text are present in this figure.

Figure 2: Historical timeline of key genomic technologies for discovering variants in DSD
Timeline depicting the changes in popular genomic technologies used for the discovery of variants related to DSD from 1959 to 2016. Highlighting key historical time points, such as the discovery of SRY and the advent of next generation sequencing.

Figure 3: Overview of the multiple approaches for genetic testing available to research DSD candidates. Initiated by identification of abnormal phenotype in a patient and followed by hormonal and physical analysis in parallel with genomic screening, with the initial analysis for translocations of SRY, attributing to 90% of 46,XX testicular DSD. This flow chart compares the choices for either a broad approach involving WGS and WES or a targeted gene panel approach, as well as the genomic outcomes of each pathway and the point of feedback to the patients.

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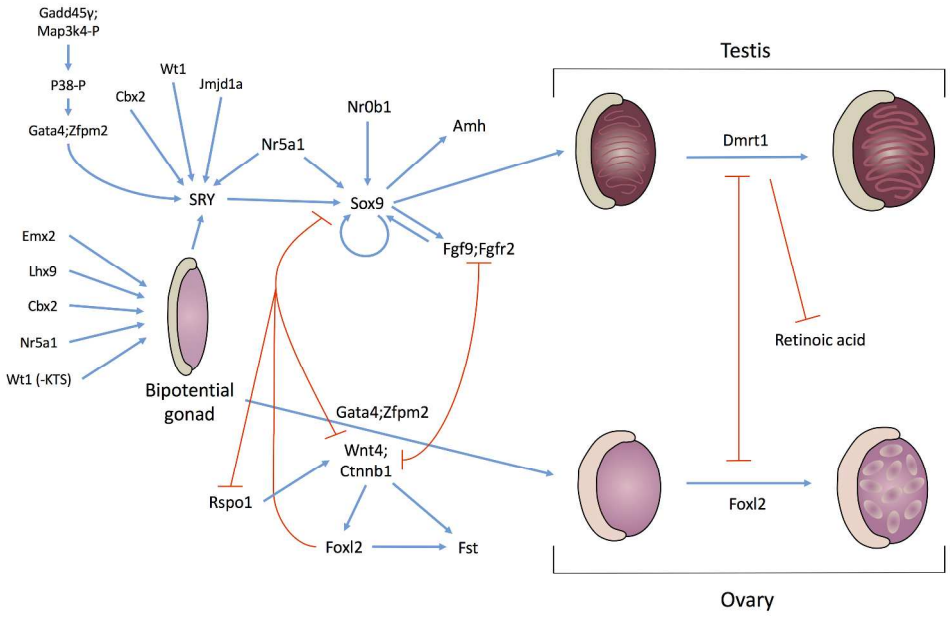

The genes and pathways necessary to drive development of either a testis or an ovary from the Bipotential gonad.

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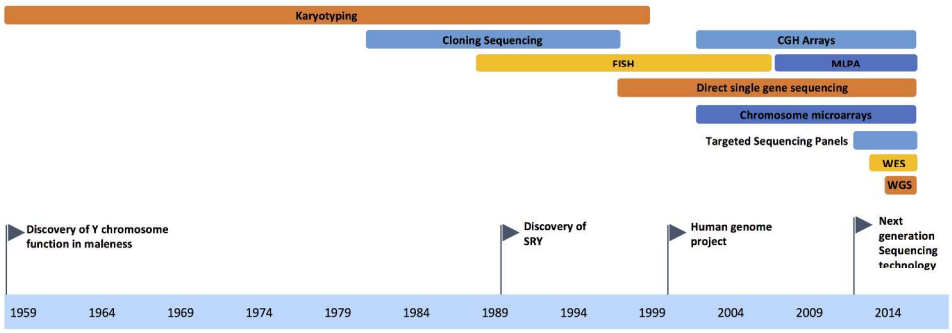

Historical timeline of key genomic technologies for discovering variants in DSD

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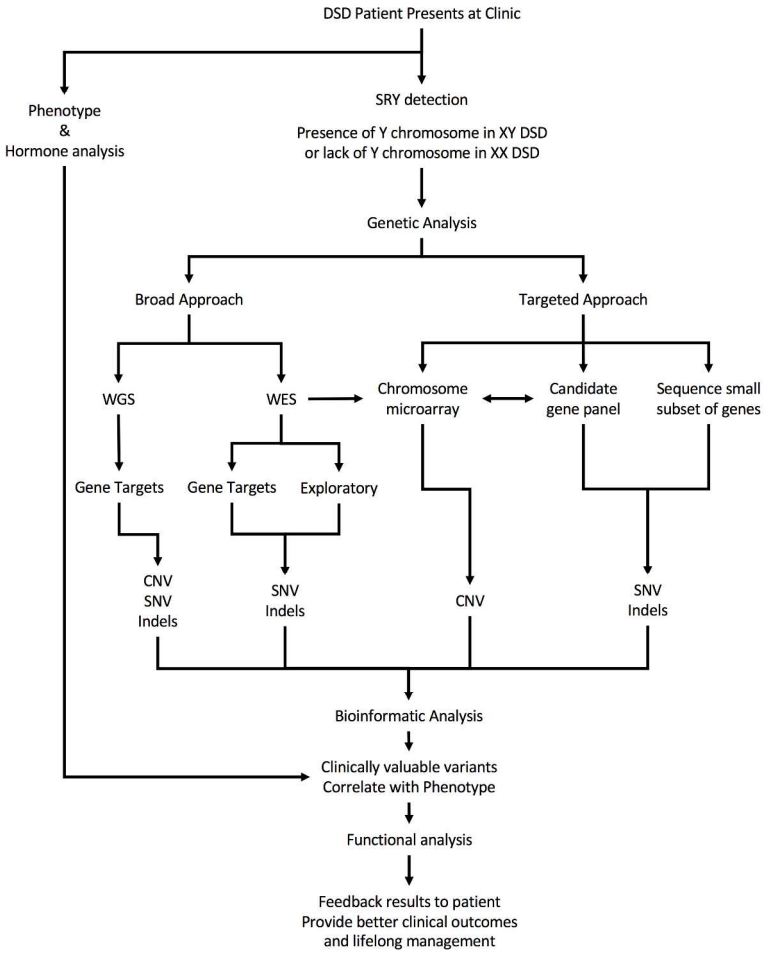

Overview of the multiple approaches for genetic testing available to research DSD candidates.

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DSD condition	Definition
46,XY DSD	Undervirilization of an XY male
46,XX DSD	Presence of male external genitalia ranging from normal to ambiguous with testosterone deficiency
Ovotesticular DSD	Presence of both testicular and ovarian tissue in a XX karyotype.
46,XX Testicular DSD	Presence of external male genitalia (ranging from normal to ambiguous) testicular tissue and absence of Müllerian structures.
46,XY Complete Gonadal Dysgenesis (GD)	Presence of external and internal female genitalia due to anomalies in gonadal development

Table 1. Definitions of DSD conditions

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Table 1: Variation in genes involved in 46,XY and 46,XX DSD in humans and the technique in which they were discovered

Gene	Kinds of mutation	Type of DSD	Screening Technique	Publications
CBX2	SNV	46,XY and 46,XX DSD	Targeted sequencing	(Norling et al., 2013)
	Hemizygous SNV	46,XY DSD	Targeted Sequencing	(Biason-Lauber et al., 2009)
DHH	SNV	46,XY GD DSD	Whole Exome sequencing	(Werner et al., 2015)
DMRT1	Gene deletion	46,XY GD DSD	CGH Microarray	(Tannour-Louet et al., 2010)
	Gene deletion	46,XY GD DSD	CGH Array	(Igarashi et al., 2013; Ledig et al., 2010)
DMRT2	Deletion along with DMRT1	46,XY DSD	FISH	(Raymond et al., 1999)
FOXL2	Related to premature ovarian insufficiency (POI), screened POI and XX males found only silent variation	46,XX DSD	Single gene sequencing	(De Baere et al., 2002)
GATA4	Loss of function missense mutation	46,XY DSD	Single gene sequencing	(Lourenco et al., 2011)
	Microdeletion upstream of GATA4	46,XY DSD	Targeted gene panel	(Harrison et al., 2014)
FGF9	Gene Duplication	46,XX DSD	CGH Array	(Chiang et al., 2013)
NR0B1	Duplication of the region containing NR0B1	46,XY GD DSD	CGH Array	(Ledig et al., 2010)
	Duplication	46,XY GD DSD	MLPA	(Barbaro et al., 2008)
	Upstream deletion 250kb	46,XY DSD	CGH Array	(Smyk et al., 2007)
NR5A1	Heterozygous missense mutation Mosaic discovered in the father at 17% penetrance	46,XY DSD	Single gene sequencing	(Tuhan et al., 2016)
	Premature stop codon and intron donor splice site mutation	46,XY GD DSD	Single gene sequencing	(Fabbri et al., 2016)
	Partial gene deletion	46,XY GD DSD	MLPA	(Barbaro et al., 2011)
	Deletion including the NR5A1 gene	46,XY DSD	CGH Array	(Brandt et al., 2013; Harrison et al., 2014; van Silfhout et al., 2009)
	Missense mutation activating MAMLD1	46,XX ovotesticular DSD	Whole Exome Sequencing	(Baetens et al., 2016)
MAP3K1	SNV leading to splice site acceptor	46,XY DSD	Linkage analysis Single gene sequencing	(Pearlman et al., 2010)

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	Missense SNV	46,XY DSD	Single gene sequencing	(Das et al., 2013)
RSPO1	Homozygous splice site donor variation	46,XX DSD	Single gene sequencing	(Tomaselli et al., 2008)
SOX3	Rearrangements and deletions of *SOX3* upstream regulatory region	46,XX DSD	CGH Microarray	(Sutton et al., 2011)
	SOX3 duplication	46,XX DSD	CGH Array	(Moalem et al., 2012; Vetro et al., 2014)
SOX10	Inverted Duplication of the long arm of Chromosome 22	46,XX DSD	Karyotype	(Aleck et al., 1999)
	Partial duplication of chromosome arm 22q	46,XX DSD	Microsatellite analysis	(Seeherunvong et al., 2004)
SOX9	SNV causing Campomelic dysplasia (CD) leads to autosomal sex reversal in 75% of 46,XY cases	46,XY DSD	Single gene sequencing	(Cameron et al., 1996)
	Premature stop codon	46,XY DSD and CD	Cloning Sequencing	(Foster et al., 1994; Wagner et al., 1994)
	CNVs upstream of SOX9 ~600kb, comprising of duplications/triplications in XX DSD and deletions in XY DSD cases with a minimum overlap of ~41kb. Eluding to a testis specific regulatory region for *SOX9*, referred to as RevSex.	46,XY GD DSD	CGH Array	(Ledig et al., 2010)
		46,XY GD DSD	Microarray MLPA	(Sutton et al., 2011; White et al., 2011)
		46,XX DSD	Microarray	(Hyon et al., 2015)
		46,XX and 46,XY DSD	CGH Array	(Benko et al., 2011)
		46,XX DSD	CGH Array	(Vetro et al., 2011; Vetro et al., 2014)
	Duplication of a second upstream region of *SOX9* upstream of RevSex, referred to as XYSR.	46,XX and 46,XY DSD	MLPA	(Kim et al., 2015)
	Gene duplication	46,XX DSD	FISH	(Huang et al., 1999)
SRY	Frame shift mutation in unaffected father, with two affected offspring	46,XY GD DSD	Single gene sequencing	(Isidor et al., 2009)
	Mosaic sex chromosome SNV Within the HMG domain	XY, XX, (XX&XY) (X, XX,XY)	MPS	(Hersmus et al., 2012a)
	Mosaic gain and loss of Y chromosome causing mixed GD	45,X/ 46,XY DSD	CGH	(Tannour-Louet et al., 2010)
	SNV	46,XY DSD	Single gene sequencing	(Helszer et al., 2013)
	Post zygotic SNV in SRY leading to a premature stop codon	46,XY DSD	Cloning sequencing	(Braun et al., 1993)
WNT4	LOF missense mutation leading too absent Mullerian structures	46,XX Female DSD	Single gene sequencing	(Biason-Lauber et al., 2004)
WT1	Splice site mutation, with a SRY missense mutation	46,XY DSD	Single gene sequencing	(Hersmus et al., 2012b)

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	Gene deletion	46,XY GD DSD	FISH CGH Array	(Finken et al., 2015; Le Caignec et al., 2007)
	Frasier syndrome including XX females with Intron splice site mutations	46,XY DSD	Single gene sequencing	(Barbaux et al., 1997; Demmer et al., 1999)
ZFPM2	Non-synonymous heterozygous point mutation	46,XY GD DSD	Whole Exome sequencing	(Bashamboo et al., 2014)
	De novo balanced translocation truncating ZFPM2	46,XY DSD	FISH	(Finelli et al., 2007)
FGFR2	Deletions encompassing the gene	46,XY DSD	CGH microarray	(Tannour-Louet et al., 2010)
MAMLD1	Premature stop codon	46,XY DSD	Single gene sequencing	(Camats et al., 2015)
	Point mutations causing premature stop codons and splice site mutations Patients often have 2 SNV occurring together	46,XY DSD hypospadias	Single gene sequencing	(Igarashi et al., 2015; Kalfa et al., 2012; Ogata et al., 2009)

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