CBX2 in DSD: The Quirky Kid on the Block

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Abstract
Sex development is an intricate and crucial process in all vertebrates that ensures the continued propagation of genetic diversity within a species, and ultimately their survival. Perturbations in this process can manifest as disorders/differences of sex development (DSD). Various transcriptional networks have been linked to development of the gonad into either male or female, which is actively driven by a set of genes that function in a juxtaposed manner and is maintained through the developmental stages to preserve the final sexual identity. One such identified gene is Chromobox homolog 2 (CBX2), an important ortholog of the Polycomb group (PcG) proteins, that functions as both chromatin modifier and highly dynamic transactivator. CBX2 was shown to be an essential factor for gonadal development in mammals, as genetic variants or loss-of-function of CBX2 can cause sex reversal in mice and humans. Here we will provide an overview of CBX2, its biological functions at molecular level, and the CBX2-dependent transcriptional landscape in gonadal development and DSD.

Introduction
The process of sexual development is directed by two unique and key stages: sex determination and sex differentiation. Whilst genetic sex determination occurs at the time of fertilization, the undifferentiated gonad develops and remains bipotential until approximately 6 weeks post-conception [Biason-Lauber and Chaboissier, 2015], from which point it drives the differentiation towards either a male- or female-specific gonad and subsequent formation of the testis or ovary, respectively [Wilhelm et al., 2007].

The identification and characterization of the Sex-determining Region Y (SRY) gene, located on the Y chromosome, and expressed in Sertoli cell precursors [Sinclair et al., 1990], revealed SRY as master regulator of male sex determination [Koopman et al., 1990]. SRY, together with the autosomal gene SRY-box9 (SOX9) which is upregulated shortly after SRY expression commences, promotes the differentiation and proliferation of Sertoli cells in testicular development [Sekido and Lovell-Badge, 2008; Hiramatsu et al., 2009]. Despite an equivalent dominant gene to SRY lacking for ovarian differentiation, in the absence of SRY expression critical factors acting in an antagonistic manner in this dynamic molecular pathway [Kim et al., 2006], such as Wing-
less Type MMTV integration site family member 4 (WNT4), Forkhead box L2 (FOXL2), and Root-plate specific Spondin1 (RSPO1), have been shown to suppress male-specific SOX9 and thus development of the testis [Vainio et al., 1999; Parma et al., 2006; Boulanger et al., 2014]. Disruption of this closely regulated process, which is predominantly due to either genetic variants that hinder gonadal tissue development or the actions of secreted hormonal and local factors, can lead to DSD in patients [Hughes et al., 2006].

In addition to the earlier known genes, mechanisms and pathways involved in developmental processes, genetic discoveries brought about by the advent of sequencing technologies, novel gene editing tools such as CRISPR/CAS9, coupled with the power of bioinformatics have unearthed a complex cascade of multiple gene networks, intracellular signaling and endocrine events that govern the conditions of sex determination and gonadal differentiation [Tilmann and Capel, 2002]. A relatively new player in the field is CBX2, found in a 46,XY DSD patient presenting with ovarian-like tissue at histology and a uterus, having compound heterozygous mutations resulting in Arg443Pro and Pro98Leu amino acid substitutions [Biason-Lauber et al., 2009].

Findings in mouse models of sex determination have placed the functional homologue of CBX2 (M33) as acting upstream of SRY in the sex development network, consistent with the expression of CBX2 at week 7 of gestation around the time of testis determination in humans, and reported M33 KO in XY mice with reduced expression of SRY and its direct target, SOX9. Concurrently, overexpression of SRY or SOX9 in M33 KO mice could rescue their sex reversal, although these mice had smaller gonads compared to wild-type mice [Ostrer et al., 2007; Katoh-Fukui et al., 2012]. Intriguingly, apart from CBX2 upregulating male-specific factors, it has also been shown to negatively regulate female-specific genes, such as FZD1 and FOXL2 [Eid et al., 2015].

**The Molecular Function of CBX2**

The PcG family, of which CBX2 and its mouse homologue M33 are members of, are a class of highly conserved epigenetic regulators that form multimeric protein complexes, and function as modulators of chromatin remodelling through interaction with post-translational modifications (PTMs) in histone proteins [Schuettengruber et al., 2007; Muller and Verrijzer, 2009]. At the molecular level, two main types of PcG proteins assemble actively to form distinct major enzymatic complexes, called Polycomb repressive complex 1 (PRC1) and PRC2, and act together to silence their target genes, as first seen from their discovery as key transcriptional suppressors of Hox genes during *Drosophila* development [Lewis, 1978]. Several PcG proteins have now been widely implicated in all metazoans for their principal roles in various biological and cellular processes, including cell fate transitions, genomic imprinting, stem cell pluripotency, cancer, and sex differentiation [Bracken and Helin, 2009; Gieni and Hendzel, 2009; Biason-Lauber, 2010; Di Croce and Helin, 2013].

As an active ortholog of the mammalian PRC1 complex, CBX2 is a principal methyl reader that interprets the major gene repressive epigenetic mark histone 3 lysine trimethylation residue K27 (H3K27me3) [Gao et al., 2012], and is able to directly promote chromatin compaction in vitro [Tatavosian et al., 2019]. This function is predominantly mediated by the chromodomain at its N-terminus, comprising a stretch of positively charged amino residues [Muller, 1995; Kaustov et al., 2011]. Human CBX2 exists in two discrete isoforms, a 532-amino acid long transcript encoded by five exons (NP_005,180) that consists of the conserved chromodomain and Polycomb (Pc) box, named CBX2.1, and a shorter 211-amino acid isoform of four exons (NP_116,036) referred to as CBX2.2, which lacks the Pc box but still retains the chromodomain (shown in Fig. 1).

Despite this additional degree of complexity of alternative splicing in the CBX2 variants, the core function of gene silencing remains preserved by both isoforms, albeit with varying efficiency and through different mechanisms. The Pc box present in the long CBX2.1 isoform, but missing in the short CBX2.2 isoform, has been shown to recruit additional members of the PRC1 complex, such as polycomb group ring finger (PCGF), E3 ubiquitin-protein ligase (RING1), and polyhomeotic homolog (PHC2) [Bardos et al., 2000; Vandamme et al., 2011]. In Pc and mammalian subunits the conserved Pc box has been demonstrated as a necessary element for transcriptional suppression (shown in Fig. 2) [Bunker and Kingston, 1994]. In contrast, transcriptional reporter assay experiments revealed CBX2.2 is able to mediate gene silencing in a PRC1-independent manner, by self-assembling to form homopolymers and prohibits the recruitment of transcriptional components when bound to a promoter, but is less inhibitory due to the lack of interaction with the PRC1 members (shown in Fig. 2) [Volkel et al., 2012].
Given that CBX2 is a potent transcriptional regulator, it is involved in several important processes of development. As the only PcG gene known to uniformly bind chromatin during mitosis, CBX2 is a key mediator of cell cycle progression and cellular senescence [Zhen et al., 2014]. SiRNA knockdown of CBX2 elevates cyclin-dependent kinase inhibitor p21/CDKN1A, and interacts with a major cell-growth repressive pathway p53, both of which drive cell proliferation and programmed cell arrest [Sauvageau and Sauvageau, 2010; van den Boom et al., 2013]. Furthermore, through its active suppression of p21/CDKN1A, CBX2 was shown to have a dynamic function in stem cell identity and self-renewal, whilst synergistic association of CBX2 with the BCL6 co-repressor (BCOR) and Ring Finger Protein 2 (RNF2) proteins regulate early lineage commitment in human embryonic stem cells (ESCs) [van den Boom et al., 2013; Wang et al., 2018]. In CBX2 knockout (KO) mice germ cell viability is decreased and present with extensive chromosome aberrations throughout meiosis, and during tissue development CBX2 KO mice exhibit abnormalities in adrenal formation [Katoh-Fukui et al., 2005; Baumann and De La Fuente, 2011]. Additionally, genetic mutations in CBX2 play a critical role in sexual development, shown by the
fact that M33-deficient mice have male-to-female sex reversal. The M33 XY−/− mice were sterile and more than half of the SRY-positive animals presented phenotypically female (uterus, ovaries with follicles, and normal external genitalia) [Katoh-Fukui et al., 1998]. More specifically, the discovery of the human parallel of this murine model in the abovementioned 46,XY DSD patient who had normal female genitalia, ovarian-like tissue and uterus, highlighted CBX2 variants may lead to congenital disorders in humans [Biason-Lauber et al., 2009].

**CBX2 in DSD**

The earlier described work of Katoh-Fukui et al. suggested CBX2 regulates SRY expression indirectly during testis determination, through action on other SRY-interacting genes, and may affect gonadal size. Interestingly, in males of two XO/XO species of spiny rat (Tokudaia) with SRY totally absent, additional copies of CBX2 in these species were suggested to repress female-specific factors, thereby inducing the undifferentiated gonad to develop testis, and thus putatively placing CBX2 as part of a novel sex-determining mechanism independent of SRY [Kuroiwa et al., 2011]. Moreover, work in Drosophila and mice hinted at the potential additional function of M33/CBX2 as transactivator by its activation of steroidogenic factor 1 (SF1/NR5A1) expression, apart from the known role of CBX2 as chromatin modifier [Milne et al., 1999; Katoh-Fukui et al., 2005]. This was supported by the finding that mutations in CBX2.1 disrupted DNA transactivation of downstream target genes critical for sex determination in humans, such as SF1/NR5A1 [Biason-Lauber et al., 2009]. Additionally, another study reported two cases of gonadal abnormalities linked to CBX2 variants. The first patient was a female having 46,XY complete gonadal dysgenesis, whilst the other patient a 46,XX individual with body dysmorphic disorder, uterine hypoplasia and lacking ovaries [Merel et al., 2019, unpublished data].

Several studies have made use of recent advances in technology, such as DNA adenine methyltransferase identification (DamID), integrated with next-generation sequencing (NGS) with subsequent Gene Ontology (GO) enrichment analysis, and RNA sequencing (RNA-Seq), to identify CBX2-dependent transcriptional targets, so as to better elucidate the position of CBX2 in the developmental network [Eid et al., 2015; Bouazzi et al., 2019; Sproll et al., 2019]. The initial groundwork for this was laid by a study that provided genome-wide evidence of SOX9 as a direct binding target of CBX2 in human cells, and in addition, found a number of male-related genes (MAMLD1, SOX3, INSL3, and FGF2) with known association to DSD [Baxter and Vilain, 2013] being upregulated by CBX2, along with many novel genes, including TEX10, EXO1, TBX2, and TSPYL4 [Eid et al., 2015]. TEX10 has been identified as transcriptional target of SRY, and TSPYL4 associated with dysgenesis of the testis [Puffenberger et al., 2004; Bhandari et al., 2012]. TBX2 is essential during mesoderm lineage commitment, while EXO1 KO mice presented sterile and is necessary for meiosis [Wei et al., 2003; Douglas et al., 2012]. These findings underline the essential role of CBX2 in male sex development.

**Opposite Sides of the Same Coin**

On the other hand, Eid et al. also identified female-specific factors negatively regulated by CBX2, namely FZD1, PBX1, and FOXL2. A report showed FZD1 played a role in female mouse fertility, with FZD1 KO mice being sterile or sub-fertile, and a Wingless Type MMTV integration site family (WNT) member that is substantially expressed in mural and cumulus granulosa cells during preovulation is translated by FZD1, in a similar fashion to WNT4 [Lapointe et al., 2012]. WNT4 is widely known as an important pro-female factor in development, and connected to DSD in humans, with WNT4 duplication (extra copies) linked with male-to-female sex reversal, while WNT4 LOF induces SERKAL syndrome in women [Vainio et al., 1999; Jordan et al., 2001; Biason-Lauber, 2012]. Recently, it was shown in XY mouse gonads that CBX2 directly represses LEF1, a downstream target of WNT4, and ovary-determining transcription factor of β-catenin that stays bivalent in Sertoli cells [Behrens et al., 1996; Garcia-Moreno et al., 2019]. PBX1 has a function in female urogenital differentiation, and mice lacking PBX1 caused a loss of Müllerian ducts [Schnabel et al., 2003]. In humans, CBX2 is known to interact with PBX1, with a missense mutation in PBX1 identified in a patient with 46,XY gonadal dysgenesis and radiocubital synostosis shown to result in abrogated CBX2 interaction [Egozenou et al., 2019]. In ovarian development and maintenance, FOXL2 is regarded as the female equivalent of SOX9 [Veitia, 2010]. Interestingly, in mice a XX CBX2 female presented with small ovaries and infertility [Baumann and De La Fuente, 2011]. Furthermore, a study that employed single cell RNA-Seq from human fetal gonads revealed CBX2 was expressed from week 9 to week 24 post-fertilization in both male and female gonads [Li et al., 2017].
Taken together, in the sex development cascade, \( \text{CBX2} \) seems to actively repress the antagonistic female pathway in ovarian determination and maintenance, whilst strengthening the idea that \( \text{CBX2} \) is a pro-male factor (shown in Fig. 3).

The intriguing characteristic of alternatively-spliced \( \text{CBX2} \) variants gives rise to many nuances of its role during human sex development and DSD. Herein, a possible function of the shorter isoform \( \text{CBX2.2} \) was elucidated in further studies. In one study, whole exome sequencing (WES) identified novel \( \text{CBX2.2} \) variants in two unrelated 46,XY patients displaying complete gonadal dysgenesis. Transactivation experiments demonstrated that mutated \( \text{CBX2.2} \) could not adequately bind to and regulate the expression of transcription factors important for sex development, of note \( \text{EMX2} \) and \( \text{HOXA13} \) [Sproll et al., 2018]. \( \text{EMX2} \) XY KO mice show gonadal agenesis and carry defects of the coelomic epithelium, which generates Sertoli cells [Miyamoto et al., 1997]. In 46,XY patients, ablation of \( \text{EMX2} \) precipitates a spectrum of DSD from hypospadias to gonadal dysgenesis [Piard et al., 2014]. \( \text{HOXA13} \) has been implicated in male sex differentiation, by \( \text{HOXA13} \) variants in mice having reduced expression of androgen receptor in the testis, and causes malformations of the genitourinary regions marked by hypospadias in males [Morgan et al., 2003; Scott et al., 2005]. This data indicates \( \text{CBX2.2} \) may have a distinct role in human sex development, especially when considering the differences of gonadal phenotype in LOF of \( \text{CBX2.1} \), which causes vestigial gonadal development, as described earlier, and LOF of \( \text{CBX2.2} \) that may result in full 46,XY gonadal dysgenesis.

It should be noted there is a higher frequency of LOF variants in \( \text{CBX2.2} \) present in human population databases (e.g., gnomAD) compared to the rare \( \text{CBX2.2} \) variants (MAF <0.01) causing DSD phenotypes.

In this regard, it might simply be the case that \( \text{CBX2.2} \) may have no biological function and does not contribute to DSD. Alternatively, the authors also postulate this may be due to the varying efficiency and different mechanism through which \( \text{CBX2.2} \) mediates gene silencing relative to isoform 2.1, as alluded to before, and furthermore in the location of these rare variants with subsequent consequences in protein folding. This phenomenon is very well known for drug metabolizing enzymes (e.g., \( \text{NAT2} \), \( \text{CYPD6} \), \( \text{CYP2C19} \)). These are polymorphic genes with...
many SNPs and high frequency of variant alleles in the general population, and yet only a small number of variants lead to consequences in the enzyme function [Tomalik-Scharte et al., 2008]. To this end future studies on CBX2.2 variants that may be pathogenic are required to shed further light on this topic.

Later, the CBX2.1 and CBX2.2 transcriptional landscape was further expanded through protein/DNA interaction studies of CBX2 binding partners, coupled with in vitro functional analysis of downstream gene targets in human pre-granulosa (KGN) and Sertoli-like cells (NT2/D1), adding to the repertoire of CBX2-regulated factors.

| Gene symbol | Function in development and/or possible role in DSD |
|-------------|-----------------------------------------------------|
| CBX2.1 targets | |
| DKK1 | Suppresses WNT mediated β-catenin signaling during testis formation. [Combes et al., 2011] |
| CYP19A1 | Transforms androstenedione to estrone and testosterone to estradiol; male-pattern hair growth in female outer genitalia. [Ishikawa et al., 2006] |
| DMRT1 | Blocks female reprogramming in the postnatal mammalian testis; 46,XY abnormal testis development. [Chen and Heckert, 2001; Matson et al., 2011] |
| PITX2 | Regulator of the WNT/β-catenin pathway. [Basu and Roy, 2013] |
| ESR1 | Shown to participate with FOXL2 to repress SOX9 in the ovary; ESR1 deficiency causes delayed puberty in women. [Uhlenhaut et al., 2009; Quaynor et al., 2013] |
| ESR2 | ESR2 variants lead to 46,XY and 46,XX gonadal dysgenesis. [Lang-Muritano et al., 2018] |
| ALX4 | ALX4 dysfunction induces hypogonadism and undescended testis in male patients. [Kayserili et al., 2009] |
| GH1 | Critical growth factor during embryonic development; GH1 deficiency can result in penis and testicular growth retardation. [Laron and Sarel, 1970; Sanders and Harvey, 2004] |
| DUSP6 | Implicated in Kallmann syndrome; regulatory component of cell division in Leydig cells. [Miraoui et al., 2013; Mori Queiroz Garcia et al., 2013] |
| MTM1 | Ablation affects myotubular myopathy and deformed genitalia in human males. [Hu et al., 1996] |
| TBX3 | TBX3 variants are linked to genital aberrations typical in Ulnar-Mammary syndrome. [Tanteles et al., 2017] |
| ERBB4 | Epidermal growth factor involved in fertility and testis development. [Naillat et al., 2014] |
| NTF3 | Expressed in Sertoli cells and required for seminiferous cord formation in the embryonic testis. [Cupp et al., 2003] |
| NR2F2 | Negatively regulates NR5A1 and represses steroidogenesis in human adrenals. [Shibata et al., 2001] |
| NRG1 | Involved in granulosa cell differentiation and regulation of ovulatory events; in mice NRG1 KO triggers testicular hypoplasia. [Cooke et al., 2017; Grigoleit et al., 2018] |
| BMP2 | Highly expressed in the developing follicle; acts with FOXL2 to regulate follistatin during ovarian development. [Kashimada et al., 2011; Demiray et al., 2017] |
| NTRK1 | Cross-functional gonadal factor involved in ovarian follicle assembly and formation of seminiferous cords. [Cupp et al., 2002; Kerr et al., 2009] |
| PTGER2 | Mediator in ovulation and formation of the corpus luteum; causes anovulation in PTGER2 deficient mice. [Hizaki et al., 1999; Kim et al., 2014] |
| FSHR | Key regulator of follicle development and steroidogenesis; inactivating mutations of FSHR result in hypergonadotropic ovarian failure. [Aittomaki et al., 1995] |
| CBX2.2 targets | |
| BMP15 | Estradiol and progesterone production; causes ovarian failure. [Di Pasquale et al., 2004; Prapa et al., 2015] |
| TEX14 | Male-specific factor involved in spermatogenesis. [Greenbaum et al., 2006] |
| FZD5 | Implicated in activation of the β-catenin pathway through being a modulator of WNT signaling. [Carmon and Loose, 2008] |
The selected genes and their respective role during development or association with DSD is summarized in Table 1. Additionally, an overview of the influence of CBX2 on its direct and indirectly regulated targets covered in this review is provided (shown in Fig. 4).

**Fig. 4.** Action of CBX2.1 and CBX2.2 on their downstream targets. In blue male genes and in pink female genes. Targets are grouped according to developmental phases in relation to time. Green lines with arrows show positive regulation, and red lines with bars show negative regulation. Dotted lines indicate an indirect action (regulated but not physically bound by).

**Conclusion**

The discovery of novel genes involved in sex development is indispensable to gain insights into the pathogenesis of human DSD. Advent of advanced technologies has put a spotlight on the relevance of biological interaction between epigenetic and transcriptional factors in development, and also uncovered mechanisms and networks dysregulated in human DSD. The importance of CBX2 was underlined by the report of CBX2 deficiency in 46,XY patients. Studies over the past decade have identified not only different functions of CBX2 during gonadal development as chromatin modifier and transcription factor, respectively, but also a spatiotemporal expression of the CBX2 isoforms during sex development and distinct gonadal phenotype between loss-of-function of CBX2 isoforms, with subsequent evidence that CBX2 is critical for the formation of the reproductive organs. Recent progress in the field has elegantly put this in context by expanding the CBX2-dependent transcriptional landscape and refined the placement of CBX2 within the human sex development cascade. The transcriptional control of CBX2 is still unclear and could further unravel molecular complexities that would enhance the comprehension of this process, ultimately enabling improved diagnosis and clinical management of DSDs.

**Conflict of Interest Statement**

The authors declare no conflict of interest.

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