CHAPTER EIGHT

The regulation of *Sox9* expression in the gonad

Nitzan Gonen*, Robin Lovell-Badge*
The Francis Crick Institute, London, United Kingdom
*Corresponding authors: e-mail address: nitzan.gonen@crick.ac.uk; robin.lovell-badge@crick.ac.uk

Contents

Abstract

The bipotential nature of cell types in the early developing gonad and the process of sex determination leading to either testis or ovary differentiation makes this an interesting system in which to study transcriptional regulation of gene expression and cell fate decisions. SOX9 is a transcription factor with multiple roles during development, including being a key player in mediating testis differentiation and therefore subsequent male development. Loss of *Sox9* expression in both humans and mice results in XY female development, whereas its inappropriate activation in XX embryonic gonads can give male development. Multiple cases of Disorders of Sex Development in human patients or sex reversal in mice and other vertebrates can be explained by mutations affecting upstream regulators of *Sox9* expression, such as the product of the Y chromosome gene *Sry* that triggers testis differentiation. Other cases are due to mutations in the *Sox9* gene itself, including its own regulatory region. Indeed, rearrangements in and around the *Sox9* genomic locus indicate the presence of multiple critical enhancers and the complex nature of its regulation. Here we summarize what is known about the role of *Sox9* and its regulation during gonad development, including recently discovered critical enhancers. We also discuss higher order chromatin organization and how this might

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be involved. We end with some interesting future directions that have the potential to further enrich our understanding on the complex, multi-layered regulation controlling *Sox9* expression in the gonads.

1. Introduction

The processes of sex determination and gonadal differentiation serve as beautiful systems to understand the role of genes during development and to study gene regulation and cell fate decisions. This is in part because cases of Disorders of Sex Development (DSD) in humans and cases of sex reversal in animals have led to the discovery of many of the genes controlling sex determination and differentiation (Wilhelm, Palmer, & Koopman, 2007). But this is also due to the bipotential nature of many cell types within the gonad and the fact that the early gonad (or genital ridge) is able to differentiate into either ovary or testis. Thus, the supporting cell precursors give rise to Sertoli cells or granulosa cells, the steroid cell precursors give rise to Leydig cells or theca cells, and the primordial germ cells (PGCs) give rise to spermatogonia or oogonia, in the testis and ovary respectively (Lin & Capel, 2015). It is now clear that in addition to the determination of gonadal sex that occurs in the embryo, these cell fate decisions need to be actively maintained in the adult, otherwise Sertoli cells transdifferentiate to granulosa cells or *vice versa*, and this can be followed by more general gonadal sex reversal (Huang, Ye, & Chen, 2017; Matson et al., 2011; Uhlenhaut et al., 2009).

The determination of gonadal sex in mammals, but also in many other vertebrates, relies on a delicate balance of the expression levels of several key pro-male and pro-female transcription factors. Of course, these both interact with and direct signaling pathways, many of which, such as Wnt signaling and steroid hormones, can play critical roles, but it seems that it is the balance of transcriptional controls that both initiates and maintains gonadal fate. Any shift from this delicate balance can result in sex reversal. Because the type of gonad that develops, and the hormones it produces, can affect other sex-specific characteristics, this can lead to many types of DSD (Carre & Greenfield, 2016; Lin & Capel, 2015). While some types of DSD result from the development of gonads of mixed sex, so-called ovotestes, there are also amplification or canalisation processes that usually work to ensure that only one type of gonad develops, even though the whole process is finely

balanced at the beginning. In order to achieve this, multiple layers of regulation are expected to play a role, such as histone modifications, the activity of both enhancers and silencers, as well as higher order of chromatin organization, and these are all likely to operate in a system involving both positive and negative feedback mechanisms (Garcia-Moreno, Plebanek, & Capel, 2018).

Genes located at the top of regulatory cascades involved in sex determination have been proposed to have relatively unique roles, perhaps reflecting recent evolutionary origins (Wilkins, 1995), with some support for this in both invertebrates (Hediger et al., 2004) and vertebrates, including *Sry* (sex determining region Y), the Y chromosome linked testis-determining gene in mammals. While *Dmrt1* (doublesex and mab-3 related transcription factor 1) may have a more complex role, being involved in germ cells as well as playing a sometimes critical role in sex determination *via* its action in somatic cells, notably in birds where it maps to the Z chromosome, derivative homologs, such as *Dmy*, the male-determining gene located on the Y in some species of *Medaka*, and *Dmw*, which acts as a dominant female-determining gene mapping to the W in *Xenopus laevis*. Other genes lower down the pathway, leading to the development of ovaries or testes, have been co-opted for multiple roles during development (Capel, 2017). *Sox9*, which plays a pivotal role in mammalian sex determination, and is probably involved in testis development in all vertebrates, is one such gene.

1.1 The *Sox9* gene

SOX9 is a member of the SRY-related HMG box (SOX) family of transcription factors, which have roles in many tissues. Several types of DNA-binding proteins contain HMG boxes, including the "high mobility group" proteins HMG1 and HMG2, after which the domain is named, UBF, and the LEF/TCF family of transcription factors (Koopman, Sinclair, & Lovell-Badge, 2016; Lovell-Badge, 2010). The class of HMG box found in SOX9 was first identified in SRY (Gubbay et al., 1990; Sinclair et al., 1990). There are 20 members of the SOX family found in mammals, with a well-conserved HMG box of 79 amino acids, which can be divided into eight subgroups, A-H, based on the structural homology outside the box (Schepers, Teasdale, & Koopman, 2002; Wegner, 2010). SOX proteins usually form dimers while regulating target genes. SOX proteins can dimerize with other transcription factors or with homologous or heterologous SOX proteins. It is

only when they bind DNA as a dimer that their gene regulation activity commences (Kamachi, Uchikawa, & Kondoh, 2000).

SOX9, along with SOX8 and SOX10, belong to the SOXE subfamily that shares high homology outside the HMG domain as well as two additional functional domains: a self-dimerization domain located at the N-terminal side and a transactivation domain located at the C-terminus (Gubbay et al., 1990; Jo et al., 2014; Kamachi & Kondoh, 2013; She & Yang, 2017).

SOX9 has been shown to act as both a transcriptional activator and a transcriptional repressor, depending on its binding partner, the target gene and the subsequent recruitment of either co-activators or co-repressors (Ikeda et al., 2004; Leung et al., 2011; Sekido & Lovell-Badge, 2008; Symon & Harley, 2017).

Sox9 is expressed in multiple tissues and cell types during embryonic development and through to adulthood, including cartilage, brain, pituitary, lung, heart, pancreas, testes, hair follicles, retina and several tissue-specific stem cell types (Jo et al., 2014). Genetic and molecular evidence suggests that the regulatory region of *Sox9* is spread over at least a 2 Mb gene desert 5′ to the coding sequence. This is predicted and partially known to contain multiple tissue-specific enhancers (Symon & Harley, 2017), as will be elaborated below.

1.2 The role of *Sox9* in gonadal development

In the embryonic gonad, *Sox9* is a direct downstream target gene, and possibly the only critical target, of SRY (Sekido, Bar, Narvaez, Penny, & Lovell-Badge, 2004; Sekido & Lovell-Badge, 2008). The gonads, which are colonized by PGCs, originate from the genital ridges, structures that develop from intermediate mesoderm associated with the mesonephros (a primitive kidney). The genital ridges can follow either a testicular or an ovarian path, depending on the expression (and repression) of key transcription factors (Jakob & Lovell-Badge, 2011; Lin & Capel, 2015; Maatouk & Capel, 2008). The supporting cell precursors that give rise to Sertoli cells in testes and granulosa cells in ovaries originate from a population of rapidly dividing cells in the coelomic epithelium that express several transcription factors, such as WT1 (Wilms tumor 1) and GATA4 (GATA binding protein 4) that are both quite widely expressed in the intermediate mesoderm, and notably, more specifically, the orphan nuclear receptor SF1 (steroidogenic factor-1), although this also has roles in the adrenal and pituitary (Karl & Capel, 1998; Kohler & Achermann, 2010; Schmahl, Eicher, Washburn, & Capel, 2000). After these

cells leave the coelomic epithelium, they begin to express *Sox9* at a low, basal level irrespective of chromosomal sex (Morais da Silva et al., 1996; Zhao et al., 2018). It is SRY activity in the supporting cell precursors in XY gonads that dramatically increases *Sox9* expression and leads to the differentiation of Sertoli cells and the development of a testis (Jakob & Lovell-Badge, 2011; Kent, Wheatley, Andrews, Sinclair, & Koopman, 1996; Morais da Silva et al., 1996; Sekido et al., 2004). Unlike in humans, where SRY appears to be expressed in Sertoli cells throughout life, in the mouse SRY is only transiently expressed starting from embryonic day (E) 10.75, peaks at E11.5, and then disappears by E12.5. However, its expression can persist in XY gonads that have failed to initiate Sertoli cell and testis differentiation (Lee & Taketo, 1994; Sekido et al., 2004). SRY acts with SF1 to upregulate the expression of *Sox9* (Sekido, 2010). Once SOX9 reaches a certain threshold, it is involved in the repression of *Sry* expression (Sekido et al., 2004) as well as the activation of its own expression by interacting with SF1 (Sekido & Lovell-Badge, 2008). SOX9 is also able to further reinforce its expression by activating the FGF9 (Fibroblast Growth Factor 9) and PGD2 (Prostaglandin D2 Synthase) signaling pathways, directly or indirectly repressing ovary determining/anti-testis genes, and probably activating the expression of the related gene *Sox8* (Chaboissier et al., 2004; Schepers, Wilson, Wilhelm, & Koopman, 2003). SOX8 and SOX9 are then maintained in Sertoli cells throughout life, acting redundantly (probably always with SF1) to help maintain the expression of a range of genes required for the differentiation and function of Sertoli cells, including the production of signals instructing other cell lineages in the gonad to follow the testis pathway (Bagheri-Fam et al., 2008; Barrionuevo et al., 2009; Barrionuevo & Scherer, 2010; Chaboissier et al., 2004; Colvin, Green, Schmahl, Capel, & Ornitz, 2001; Kim et al., 2007; Moniot et al., 2009) (Fig. 1).

The key role of *Sox9* in testis determination has been shown by both gain and loss of function studies in mouse and human (Barrionuevo et al., 2006; Chaboissier et al., 2004; Foster et al., 1994; Huang, Wang, Ning, Lamb, & Bartley, 1999; Vidal, Chaboissier, de Rooij, & Schedl, 2001; Wagner et al., 1994). Notably, in humans, heterozygous loss-of-function mutations of *SOX9* lead to campomelic dysplasia (CD, OMIM 114290) (Houston et al., 1983), a severe skeletal malformation syndrome where 70% of XY patients show female development (Foster et al., 1994; Wagner et al., 1994). Duplication of *SOX9* has also been reported in a 46, XX mosaic male patient, suggesting that extra dose of *SOX9* is sufficient to initiate the male pathway in the absence of SRY (Huang et al., 1999).

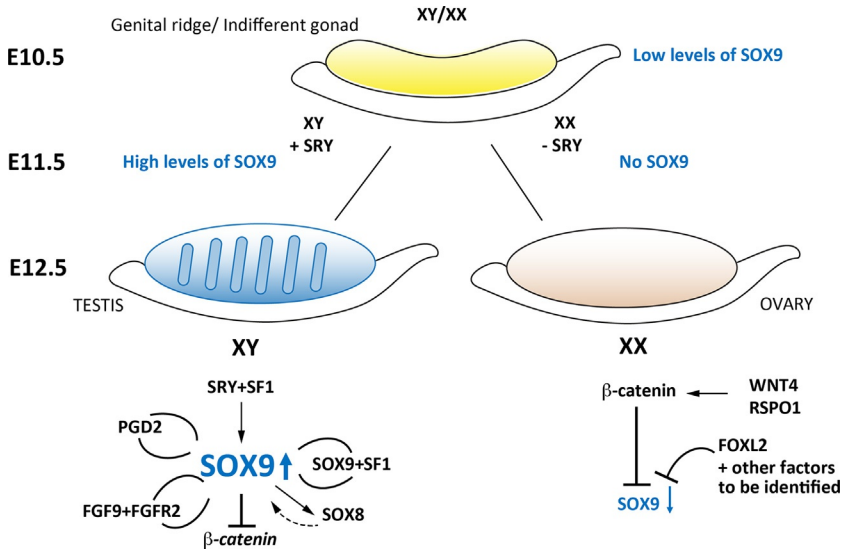

Fig. 1 The expression pattern of *Sox9* during mouse sex determination. SOX9 is expressed at low levels in the supporting cell precursors of both sexes in the bipotential gonad/genital ridge at E10.5. In XY gonads, SRY expression begins in the supporting cell precursors at E10.75, reaches a peak at E11.5 and disappears by E12.5. Once SRY is expressed, it acts along with SF1 to upregulate SOX9 expression, which initiates the differentiation of the supporting cell precursors to Sertoli cells. Once above a critical threshold, SOX9 binds to SF1 and acts in a feed-forward loop to activate its own expression. SOX9 also activates the expression of FGF9 and FGF2R, as well as PGD2 that in turn further increase SOX9 expression levels. *Sox8* expression begins at E12.5, as a direct or indirect target of SOX9, and acts to further enhance and reinforce SOX9 expression, as well as on the same targets as SOX9 in a redundant fashion (Barrionuevo et al., 2009). SOX9 is expressed in Sertoli cells throughout life. In addition to activating pro-males genes, SOX9 represses the expression of β-catenin, a pro-female gene. In XX gonads, SRY is not present and hence other genes and signaling pathways, including WNT4/RSPO1/β-catenin and *Foxl2* are expressed and act to repress the expression of *Sox9* in the ovary.

In the mouse, unlike in humans, heterozygous *Sox9* mutations do not cause XY sex reversal (Bi et al., 2001). Mice carrying a homozygous mutation in *Sox9* die at around E11.5, just at the onset of testis differentiation; however, *ex vivo* organ cultures showed no signs of testis cord formation after 3 days in culture (Chaboissier et al., 2004). To better understand the role of *Sox9* before and after the stage of sex determination, mice with a conditional null mutation of *Sox9* ($Sox9^{fl/fl}$) were used. Early, gonad-specific, loss of *Sox9,* triggered using an *Sf1-Cre* driver allele, demonstrated complete XY male-to-female sex reversal (Chaboissier et al., 2004; Lavery et al., 2011). In contrast, when *Sox9* was deleted using *Amh-Cre*, which resulted

in complete elimination of *Sox9* expression in Sertoli cells, but not until E14, i.e., following sex determination and the onset of *Sox8* expression, testis development was normal and the mice were initially fertile. However, like *Sox8* homozygous mutants (O'Bryan et al., 2008; Sock, Schmidt, Hermanns-Borgmeyer, Bosl, & Wegner, 2001), they became sterile at about 5 months (Barrionuevo et al., 2009). Loss of both *Sox9* and *Sox8* does lead to a transdifferentiation of Sertoli cells into granulosa-like cells, proving their functional redundancy (Barrionuevo et al., 2016). Furthermore, using Wt1 regulatory sequences to drive overexpression of SOX9 in XX gonads leads to XX male development (Vidal et al., 2001) as does the chance insertion of a transgene about 1 Mb upstream of the *Sox9* coding region in "Odd Sex" mice, which also leads to XX male development (Bishop et al., 2000; Qin et al., 2004). When the latter is bred into XY mice lacking *Sry*, this leads to fully functional male testis and normal fertility, indicating that SOX9 is sufficient to trigger all the downstream events needed for the development of a fully fertile male (Qin & Bishop, 2005). All of the above clearly demonstrate that, in human as well as mouse, *Sox9* is both necessary and sufficient to induce testis differentiation and all subsequent aspects of male development.

Proper development of an ovary, on the other hand, requires the repression of *Sox9*, which involves activation of the WNT4 (wingless-type MMTV integration site family, member 4)/RSPO1 (R-spondin homolog 1) /β-catenin signaling pathway (Chassot et al., 2008; Kim et al., 2006; Maatouk et al., 2008; Vainio, Heikkila, Kispert, Chin, & McMahon, 1999) and expression of the transcription factor FOXL2 (forkhead box L2) (Ottolenghi et al., 2005; Schmidt et al., 2004; Uda et al., 2004). Other, as yet unknown factors are probably also involved, including factors that actively promote granulosa cell differentiation. In order to maintain ovarian cell fate, *Sox9* must be actively repressed throughout life in granulosa cells, otherwise they transdifferentiate into Sertoli cells, which then induce the rest of the gonad to become testis-like (Lindeman et al., 2015; Uhlenhaut et al., 2009; Zhao, Svingen, Ng, & Koopman, 2015) (Fig. 1).

In this review, we summarize and discuss the regulatory landscape controlling the gonadal expression of *Sox9*, in both humans and mice. We describe the gene-desert surrounding the *Sox9* locus, both upstream and downstream and how alterations in these non-coding regions such as deletions, duplications and inversions have been implicated in patients with DSD. We describe the previously known as well as newly discovered gonadal enhancers of *Sox9* and discuss the higher order of chromatin

organization as an additional layer of regulation of gonadal *Sox9* expression. We conclude with some exciting future directions that can further accelerate our understanding on the multi-layered, complex, regulation of *Sox9* expression within the gonads.

2. The regulatory genomic locus of *Sox9*

The *Sox9* gene is located on the long arm (q) of chromosome 17 in humans and chromosome 11 in mice. It is embedded within a large gene desert with the neighboring genes located 2 MB upstream and 0.5 MB downstream in humans and 1.7 MB upstream and 0.5 MB downstream in mice (Fig. 2). Several lines of evidence support this region as being highly involved in the regulation of *Sox9* in the various tissues in which it is normally expressed. In this section, we summarize the literature regarding the regulatory, non-coding, regions implicated in the control of the gonadal expression of *Sox9*.

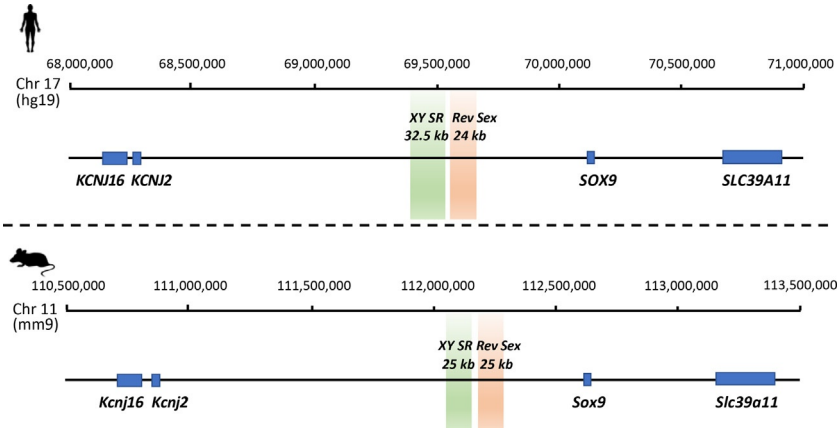

Fig. 2 Genomic view of the *SOX9*/*Sox9* locus in human and mouse. The *SOX9*/*Sox9* gene is located on chromosome 17 in human and chromosome 11 in the mouse. It is surrounded by a gene desert, with the neighboring genes located 2 MB upstream in human and 1.7 MB upstream in mouse, while in both species the next downstream gene is located 0.5 MB away. Based on several DSD human patients, two regions have been associated with sex reversal. The first is called *RevSex* (peach box) and is a minimal region of 24 kb located 559–583 kb upstream of *SOX9,* duplications of which result in XX male development. The second is further upstream and termed XY SR (green box). This is a minimal 32.5 kb region, located 607–639 kb upstream of *SOX9* and when deleted in XY patients leads to female development. The regions in the mouse that show conserved synteny with the human *RevSex* and XY SR are both 25 kb in size and are indicated by peach and green boxes, respectively.

2.1 Lessons from human patients

Much of what we know about the regulatory region of *SOX9,* and the fact that many tissue-specific enhancers lie in the upstream and downstream regions of *SOX9,* comes from human patients with deletions, duplications, translocations and inversions in and around the human *SOX9* gene. Altogether, data from patients indicate that the regulatory region of *SOX9* might span a 3 MB region located both upstream and downstream of the gene itself (Gordon et al., 2009). Heterozygous mutations in *SOX9* lead to campomelic dysplasia (CD, OMIM 114290), characterized by a severe skeletal malformation syndrome and male-to-female sex reversal in about 70% of the XY patients (Foster et al., 1994; Wagner et al., 1994). It is a severe syndrome and children with CD usually die within a year, often from breathing difficulties due to weakened cartilage in the upper respiratory tract (Foster et al., 1994; Gordon et al., 2009). The majority of mutations causing CD have been identified within the *SOX9* coding sequence and are predicted to result from haploinsufficiency, although dominant negative effects have not been ruled out in cases where an altered protein product is made. Additionally, many other patients have been characterized over the years with an unaffected *SOX9* coding region, but instead carry rearrangements upstream or downstream (Gordon et al., 2009; Symon & Harley, 2017). In CD patients, the severity of the campomelia (the bowing of the long bones) can vary depending on the distance of the translocation breakpoint from the *SOX9* gene. In the more distant lesions, the campomelic feature is missing and the disorder is described as acampomelic campomelic dysplasia (ACD) (Gordon et al., 2009). It is speculated that in these patients, a substantial portion of the *SOX9* regulatory region is present and hence some aspects of skeletal development can occur normally. Another disorder that has been implicated with long-distance alterations of the regulatory regions of *SOX9* is Pierre Robin Sequence (PRS; OMIM 261800), a disorder affecting the craniofacial skeleton (Benko et al., 2009; Jakobsen et al., 2007). Patients with isolated PRS have been identified with translocations and micro-deletions further than 1 Mb upstream of *SOX9* as well as 1.3–1.5 Mb downstream of *SOX9* (Benko et al., 2009; Velagaleti et al., 2005). PRS syndrome appears in most of the CD patients, but can also appear isolated without the campomelia syndrome. As mentioned above, 70% of XY patients with CD present with sex reversal and a female phenotype (Baetens, Mendonca, Verdin, Cools, & De Baere, 2017; Croft, Ohnesorg, & Sinclair, 2018; Symon & Harley, 2017). It is believed that the reason behind the finding that only 70% of XY patients are sex reversed

is that the *SOX9* expression levels required in the gonads to direct male development are around 50% of normal levels. Hence, genetic variability between individuals that either alter the initial expression levels of *SOX9*, modulate how it acts, or affect how ovotestes develop and resolve into an ovary or a testis, can allow some to develop as XY males.

Looking specifically at the gonadal phenotypes, two regions have been identified in humans that affect *SOX9* expression in the testis (Fig. 2). One is a (minimal overlap) 24 kb region located 559–583 kb upstream of *SOX9* (hg19 chr17: 69,534,526–69,558,832), termed RevSex (or XX SR) that was found to be duplicated in XX male patients. The minimal overlap duplication in all known patients has been recently refined from 178 kb (Cox, Willatt, Homfray, & Woods, 2011) to 98 kb (Vetro et al., 2011), 78 kb (Benko et al., 2011), 67 kb (Xiao, Ji, Xing, Chen, & Tao, 2013), 41 kb (Hyon et al., 2015) and most recently to a 24 kb minimal region (Croft, Ohnesorg, & Sinclair, 2018; Ohnesorg et al., 2017). The other region has been defined by overlapping deletions associated with XY female development as a 32.5 kb region (termed XY SR) located 607–639 kb upstream of *SOX9* (hg19 chr17: 69,477,571–69,510,055) (Hyon et al., 2015; Kim et al., 2015) (Fig. 2). Most XY female patients that were originally discovered with deletions also covered the RevSex region, but it was only in Kim et al. that a patient was identified (termed DSD2 patient) with a deletion located 672–607 kb upstream of *SOX9* that did not contained the previously identified RevSex. This led to the separation of the patients into two groups, those with duplications in RevSex, resulting in XX males and those with deletions in XY SR, resulting in XY females (Baetens et al., 2017; Croft, Ohnesorg, & Sinclair, 2018; Kim et al., 2015; Symon & Harley, 2017). For a comprehensive review, which summarizes all identified patients, please see Baetens et al. (2017).

2.2 Lessons from mouse models

Several studies in mice have also strongly supported the existence of a highly complex regulatory landscape involved in *Sox9* gene regulation and pinpointed the location of multiple enhancers mediating tissue-specific expression of *Sox9* for the various tissues in which it is normally expressed, including chondrocytes, pancreas, liver, gut, neural crest and more (Barrionuevo et al., 2006; Mead et al., 2013; Symon & Harley, 2017; Wunderle, Critcher, Hastie, Goodfellow, & Schedl, 1998; Yao et al., 2015). Very recently, Mochizuki and colleagues used CRISPR/Cas9 based

approaches to look for distant upstream enhancers governing cartilage-specific *Sox9* expression (Mochizuki et al., 2018). They used hemagglutinin (HA)-tagged catalytically dead Cas9 (dCas9) and a sgRNA directed against the *Sox9* promoter and using anti-HA ChIP, performed in primary chondrocytes, they found binding of the *Sox9* promoter to an enhancer located ~1 MB upstream of the *Sox9* TSS (transcription start site) which they termed as a rib cage-specific enhancer (RCSE). Deletion of this enhancer *in vivo* resulted in mice with shorter and narrower rib cages compared to wild-type mice. *Sox9* and *Col2a1* (Collagen Type II Alpha 1 Chain) expression levels were downregulated by ~50% in the costal cartilage of RCSE null mice. Since the reduction in *Sox9* expression levels as well as the phenotype observed in the RCSE null mice are weaker than those observed with *Sox9* KO mice, it is likely that other enhancers also participate in regulating *Sox9* expression in cartilage (Mochizuki et al., 2018).

In relation to *Sox9* regulation of expression in the gonads, two mouse models were described. The first is *Odd Sex* (*Ods*), which involves a 134 kb deletion located around 1 MB upstream of the *Sox9* gene associated with the insertion of a tyrosinase minigene under the control of the dopachrome tautomerase (*Dct*) promoter (Bishop et al., 2000). Heterozygous *Ods* mice exhibit XX male development due to constitutive expression of SOX9 in the gonads in the absence of SRY. It was initially hypothesized that the reason for the sex reversal was the loss of a critical gonad-specific repressor of *Sox9* located within the deleted 134 kb region (Bishop et al., 2000). However, a later study recapitulated the 134 kb deletion without the insertion of the *Dct* promoter and demonstrated that the deletion, in itself, was not sufficient to cause the sex reversal. This suggests that the gonad-specific expression of *Sox9* in *Ods* mice was due to the *Dct* promoter element interacting with gonad-specific enhancer elements located upstream of *Sox9*, and strongly inducing *Sox9* expression in XX gonads despite the absence of *Sry* (Qin et al., 2004).

The second model studied is provided by C57BL/6J-YPOS (B6-YPOS) mice. These mice carry autosomes from the B6 strain and a Y chromosome from the *Mus domesticus poschiavinus* (YPOS) wild-derived strain. They show a significant delay in *Sry* expression, and often present with complete XY female sex reversal (Bullejos & Koopman, 2005). The presence of a 55 MB congenic region on chromosome 11 was shown to protect B6-YPOS from sex reversal in a dose-dependent manner. This region, derived from a "semi-inbred" strain POSA, was further refined into a 1.62 MB genomic region that, when homozygous, confers full protection

from XY B6-YPOS female sex reversal (Arboleda et al., 2014). This 1.62 MB region, which had been maintained despite 30 years of backcrossing onto C57BL/6J-YPOS, is located upstream of the *Sox9* gene, covering most of the 1.7 MB upstream mouse gene-desert, and is predicted to promote sufficient expression of *Sox9* to drive testis development despite the delayed *Sry* expression associated with the B6-YPOS background (Arboleda et al., 2014).

Taken altogether, these studies in human patients and mouse models strongly support the presence of several potential gonad-specific enhancers of *Sox9*. In the next section, we describe in detail the discovery of the first known gonad-specific enhancer of *Sox9*, termed TES (Testis-specific Enhancer of *Sox9*), and the very recent finding that several others, more upstream enhancers are able to drive gonadal *Sox9* expression, with one of them, called Enh13, having a critical role specifically at the stage of sex determination.

3. Gonadal enhancers of *Sox9*

An early attempt to explore the regulatory region of human *SOX9* involved making transgenic mice with a yeast artificial chromosome (YAC) vector containing up to 350 kb 5′ and 250 kb 3′ to the human SOX9 coding region, with a *lacZ* reporter gene engineered into the first exon (Wunderle et al., 1998). This gave reporter expression in several mouse tissues known to express *Sox9*, but not in testes. It was suspected that this might be due to lack of gonadal regulatory sequences or to differences in these between human and mouse. However, the analysis was rather minimal in terms of numbers of lines examined, and the latest stage examined was E11.5, which may have been too early. Bagheri-Fam et al. (2006) compared *Sox9* genomic sequences between human and pufferfish (Fugu) and identified eight highly conserved sequence elements between 290 kb 5′ and 450 kb 3′ to human *SOX9*, and then tested these for enhancer activity in transgenic mice, again using a *lacZ* reporter gene. They also failed to detect any gonadal expression, although several elements did give *lacZ* activity in other relevant tissues.

3.1 The TES enhancer

In order to avoid problems associated with potential lack of evolutionary conservation and the use of human genomic sequences in mice, another

early attempt to look for a gonad-specific enhancer controlling *Sox9* expression in the testis was carried out by Sekido and Lovell-Badge (2008). They used a mouse *Sox9* bacterial artificial chromosome (BAC) clone (−70 to +50 kb relative to the *Sox9* transcriptional start site) carrying a minimal *heat-shock protein 68* (*hsp68)* promoter and a *lacZ* reporter gene. Mice carrying this 120 kb construct mimicked several of the characteristics of the endogenous *Sox9* expression pattern, notably including expression in the fetal testis. LacZ gonadal expression started at E10.5 and increased by E11.5 in both sexes and then became restricted to the testis only by E12.5. Serial dissection of the initial 120 kb fragment led to the discovery of a 3.2 kb element termed TES (for Testis-specific Enhancer of *Sox9*) located −13 to −10 kb upstream of the *Sox9* TSS, which was able to fully recapitulate the gonadal expression pattern of *Sox9* (Fig. 3). A core element within TES of 1.4 kb was further defined and termed TESCO (for TES Core) (Sekido & Lovell-Badge, 2008). This enhancer can drive Sertoli cell-specific expression; moreover, by chromatin immunoprecipitation (ChIP) assays, both SRY and SF1 are found bound to TES *in vivo* at E11.5 in XY gonads. SOX9 also interacts with TESCO *in vivo*, suggesting a feed-forward regulatory loop by which SOX9 helps to maintain its own transcription once it reaches a critical threshold level (Sekido & Lovell-Badge, 2008). There has been much support for this

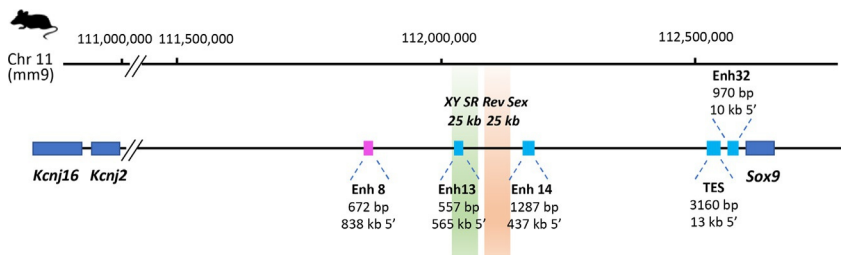

Fig. 3 Enhancers involved in mediating *Sox9* gonadal expression in the mouse. Several gonadal enhancers of *Sox9* have been identified. TES is a 3.2 kb enhancer with a core element of 1.4 kb, located 13 kb upstream of *Sox9*. Three other enhancers giving expression in the testis have been characterized and are labeled in turquoise and called Enh13, Enh14 and Enh32. The sizes and locations are indicated above or below each enhancer. Enh13 is located toward the 5′ side of the mouse homolog of the human XY SR region. Mice carrying homozygous deletions of Enh13 exhibit full XY female development indicating that Enh13 is the sole critical enhancer controlling *Sox9* expression at the stage of sex determination. No enhancer within the *RevSex* region was found in the mouse. Enh8 shows expression in the ovary and may serve either as a repressor, or as an enhancer that under normal conditions in inactive, but under cases of transdifferentiation of granulosa cells to Sertoli cells becomes active.

model and the discovery of TES and TESCO has allowed direct tests of the ability of both mutant SRY and SF1 proteins to act on *Sox9* transcription (Racca et al., 2014; Sreenivasan et al., 2018). For example, Sreenivasan and colleagues showed that the underlying mechanism explaining the variability of phenotypes observed in DSD patients carrying various mutations in SF1 is whether or not they can activate the TESCO enhancer.

In addition to TES being involved in the positive regulation of *Sox9* expression in the testis, it has also been implicated to be involved in the repression of *Sox9* expression in the ovary. It was shown that the WNT4 signaling pathway can inhibit *Sox9* expression at the transcriptional level by reducing the ability of SF1 to activate the TESCO enhancer (Bernard et al., 2012). Similarly, *Dax1* overexpression was shown to repress *Sox9* expression in a TES-dependent manner *via* competition with SF1 over the binding to TES. This study provides an explanation for 46, XY female DSD patients carrying a duplication of the DAX1 gene (Ludbrook et al., 2012). Furthermore, using ChIP techniques on adult ovaries, both FOXL2 and ESR1 (Estrogen Receptor 1) were found bound to TESCO *in vivo*. Transgenic mouse assays using TES sequences mutated for the FOXL2 sites suggested that the latter were also important. This explains the mechanism behind the ability of FOXL2 to repress *Sox9* expression in adult ovaries and hence to maintain granulosa cell fate. Conditional knock out of *Foxl2* in adult ovaries leads to transdifferentiation of the latter to testis, with onset of *Sox9* expression occurring within 2 days of the loss of FOXL2 protein (Jakob & Lovell-Badge, 2011; Uhlenhaut et al., 2009).

Despite having many of the properties expected for an enhancer with a critical role in sex determination, targeted deletion of either TES or TESCO did not result in XY female development (or in XX male sex reversal) (Gonen, Quinn, O'Neill, Koopman, & Lovell-Badge, 2017). Nevertheless, these studies did reveal that TES/TESCO accounts for about 55% of *Sox9* expression levels in the embryonic and postnatal mouse testis. Unlike in humans, where this is likely to correspond to a threshold below which ovary development occurs, in the mouse, 50% of *Sox9* expression levels are more than enough to form a functional testis, as indicated by mice heterozygous for a null mutation of *Sox9* itself (Barrionuevo et al., 2006; Gonen et al., 2017; Lavery et al., 2011). Using the TES deletion, it was possible to further reduce the expression levels of *Sox9*. Mouse embryos carrying one allele of the TES deletion and a second allele in which a conditional allele of *Sox9* was deleted using *beta-actin*-Cre exhibited 23% residual expression of *Sox9* compared to wild-type XY gonads at E13.5, and presented with

ovotestes. This provided evidence that about 25% of normal *Sox9* levels in the mouse gonad correspond to the 50% threshold level found in humans, at or below which XY-female sex reversal occurs (Gonen et al., 2017). Ovotestes can resolve by birth into either ovaries or testes, the latter tending to be smaller than normal, which can explain complete sex reversal seen in some cases of CD (Swain, Narvaez, Burgoyne, Camerino, & Lovell-Badge, 1998).

All of the above strongly indicated the importance of TES in regulating the gonadal expression of *Sox9*. However, the lack of sex reversal upon TES deletion in mice, combined with a failure to date to identify mutations affecting the equivalent human TES sequence, including a study examining 66 patients with idiopathic 46, XY gonadal dysgenesis (Georg et al., 2010), and data from human DSD patients identified with deletions and duplications far upstream of the *Sox9* gene (Symon & Harley, 2017), all indicated that there must be additional enhancers involved in the regulation of *Sox9* expression in the gonads.

3.2 The Enh13 enhancer and other novel enhancers of *Sox9*

To identify additional enhancers involved in the gonadal regulation of *Sox9* expression during embryonic stages, chromatin accessibility was examined in the 2 MB gene desert upstream of *Sox9*. This initially made use of a DNaseI hypersensitivity dataset performed on sorted Sertoli cells at E13.5 and E15.5 as well as H3K27Ac ChIP-seq that mark active enhancers (Maatouk et al., 2017). ATAC-seq data, which similar to DNaseI-seq, marks chromatin accessible regions in the genome that can serve as regulatory elements, was also performed to compare E13.5, sorted Sertoli and granulosa cells. Thirty-three potential sites were identified and 16 of these were screened experimentally, *in vivo*, using transgenic mice. Four novel gonadal enhancers were identified apart from the previously known TES (Gonen et al., 2018) (Fig. 3). Enh13 and Enh14 showed very strong testicular expression while Enh32 showed a much weaker expression only on the ventral side of the testis. Surprisingly, Enh8 conferred strong β-Gal activity in the ovary, while it was barely present in the testis at E13.5.

Although *Sox9* is not expressed in the ovary at E13.5, it is expressed at low levels in both sexes from E10.5 until E11.5, before it is markedly upregulated in the male and all but lost in the female (Kent et al., 1996; Morais da Silva et al., 1996). However, *Sox9* can be expressed in the adult ovary upon either deleting *Foxl2* (Uhlenhaut et al., 2009) or overexpressing

Dmrt1 (Lindeman et al., 2015; Zhao et al., 2015), both of which encode transcription factors required to maintain ovary and testis fate, respectively. The onset of *Sox9* expression in granulosa cells leads to their transdifferentiation into Sertoli cells. Because repression of *Sox9* in granulosa cells is an active process throughout life, otherwise they transdifferentiate into Sertoli cells, the relevant enhancers also must be accessible (Jakob & Lovell-Badge, 2011; Uhlenhaut et al., 2009). Thus, it is perhaps not surprising to identify an enhancer that, when taken out of its original genomic context, can induce ovary expression, and we speculate that in the endogenous *Sox9* locus in females, Enh8 is usually sequestered away from the *Sox9* promoter by DNA looping. It will be interesting to explore whether the activity of this enhancer is involved in adult gonadal sex reversal as described above. Interestingly, and in support of this idea, ATAC-seq at E13.5 reveals a much stronger peak at Enh8 in granulosa cells compared to Sertoli cells. Additionally, this enhancer was found active and marked with H3K27ac in both male and female gonads at E13.5 (Gonen et al., 2018).

Further study of Enh13 revealed that it is critical for sex determination and upregulation of *Sox9* expression in the testis. Homozygous deletion of Enh13, either on a TES deleted or a C57BL/6J wild type genetic background, led to full XY female sex reversal (Gonen et al., 2018). This lowered *Sox9* mRNA levels in XY gonads at E11.5 to that of control XX gonads, explaining the complete and early sex reversal. SRY was found strongly bound to Enh13 *in vivo* using ChIP at E11.5, while by E13.5 SOX9 itself was bound to Enh13, demonstrating that this enhancer is involved both in the initiation as well as maintenance of *Sox9* expression in the testis (Gonen et al., 2018).

Enh13 would appear to be well conserved amongst many mammals, with consensus binding sites for SRY/SOX9, SF1 and other relevant transcription factors (Gonen et al., 2018). Interestingly, Enh13 is located toward the distal 5′end of a 25.7 kb region in mice showing conserved synteny with the 32.5 kb human XY SR locus (Kim et al., 2015) (Fig. 3). Ohnesorg et al. relying on published DNaseI hypersensitivity data in unsorted cells from human embryonic testis and ovaries, found 13 potential sites upstream of *SOX9* (Ohnesorg, Croft, Tan, & Sinclair, 2016). One of these, termed "SOX9 up 2", is 1773 bp-long, maps to the XY SR locus and would include the human equivalent of Enh13. This, as well as other sites, showed enhancer activity assessed by luciferase-assays, although these were carried out in heterologous cell lines (Ohnesorg et al., 2016). The data in Gonen et al. (2018) therefore suggest that Enh13 is the critical enhancer within the XY SR

region, where it also plays a critical role in *Sox9* expression leading to Sertoli cell and testis differentiation in humans. In strong support of this, a recent study which utilized CRISPR genome-editing to delete different parts of the XY SR region found that removing the entire mouse XY SR region results in XY female sex reversal. By further refining the deleted fragment, they identified a 783 bp element (mm9 chr11: 112,078,499–112,079,281) that is able to induce XY female development when homozygously deleted. Enh13 (557 bp) is fully contained within this 783 bp element (Ogawa et al., 2018).

Remarkably, recent work by Croft and colleagues, which focused on identifying enhancers that regulate human *SOX9* expression in gonads, found a homologous enhancer to Enh13. They describe two new 46, XX testicular/ovo-testicular DSD patients carrying a micro-duplication with overlapping 5.2 kb. They were able to identify a 1514 bp enhancer, which they termed Sex Reversal Enhancer-A (eSR-A). Enh13 sequence is fully contained within the 1514 bp eSR-A enhancer. Interestingly, when they compare the human eSR-A and the mouse Enh13 using luciferase assays they show that whereas Enh13 is activated by both mSRY + hSF1 and mSOX9 + hSF1, the human eSR-A enhancer is only activated by hSOX9 + hSF1 but not hSRY + hSF1 (Croft, Ohnesorg, Hewitt, et al., 2018). This suggests that whereas in the mouse Enh13 is important for both activation and maintenance of *Sox9* expression, in the human context, eSR-A is more important for the maintenance of *SOX9* expression. However, there is a possible caveat in that, unlike mouse SRY that has a C-terminal activation domain, albeit one that appears weak, human SRY does not, and it is assumed to rely on an interacting co-activator (Zhao et al., 2014). It is possible that this is not present in the cell line used for the co-transfection experiments. If so, then it is possible that eSR-A and Enh13 have identical roles in activation as well as maintenance.

This work and the newly identified DSD patients further support the critical role of Enh13/eSR-A and show that both deletion of this enhancer in XY patients and duplication in XX patients, in the absence of SRY, are able to induce sex reversal (Croft, Ohnesorg, Hewitt, et al., 2018; Kim et al., 2015). This would also suggest that it is an early acting enhancer.

Croft et al. further identified two novel human enhancers. One of them is located within the XX SR region, termed Sex Reversal Enhancer-B (eSR-B) and the other is located slightly upstream to the human TES enhancer, termed enhancer Alternate Long-Distance Initiator (eALDI). The former seem to be activated by SF1 + SOX9, whereas the latter is

SRY-dependent. Unlike Enh13/eSR-A which seems to be highly conserved between human and mouse, the two other enhancers seem to be human-specific (Croft, Ohnesorg, Hewitt, et al., 2018).

It will be interesting to explore if some DSDs involve specific mutations, including Single Nucleotide Polymorphisms (SNPs), affecting Enh13/eSR-A or the other novel identified enhancers.

The notion of redundancy or "shadow enhancers" within a regulatory region is well established (Lagha, Bothma, & Levine, 2012; Visel, Rubin, & Pennacchio, 2009). Recently, Dickel et al. studied ultraconserved enhancers, showing perfect conservation across mammalian genomes, and found that deletion of single enhancers does not impact viability. They focused on eight ultraconserved enhancers of the *Arx* (*Aristaless related homeobox*) gene and showed that while single deletions do not affect *Arx* expression, deletion of pairs of enhancers can lead to a reduction in *Arx* expression levels. In accordance with this, phenotypes in the mouse brain are more severe upon deletion of pairs of enhancers compared to a deletion of single enhancer (Dickel et al., 2018). Similarly, another study created 23 mouse deletion lines including single and combinatorial deletions of 7 established enhancers required for limb development. Surprisingly, none of the individual deletions caused changes to limb morphology. It was only when a pair of limb enhancers near the same gene were deleted that a phenotype started to appear, strongly indicating that enhancers function redundantly in establishing normal cell fate and morphology (Osterwalder et al., 2018).

As discussed above, the expression of *Sox9* in cartilage also appears to rely on several redundantly acting enhancers (Mochizuki et al., 2018). Hence, knowing the major contribution of TES to maintaining *Sox9* expression levels, as well as the discovery of other novel gonadal enhancers, such as Enh14, it is remarkable to see that deleting a single enhancer phenocopies the loss of *Sox9* itself within the supporting cell lineage (Gonen et al., 2018; Lavery et al., 2011). Substantial evidence points to the time-dependent action of SRY on *Sox9* as the explanation. If *Sox9* fails to reach a critical threshold within a few hours then ovary-determining/anti-testis factors, such as Wnt signaling, accumulate to a sufficient level to repress *Sox9* and make it refractory to male promoting factors, including SRY, even though expression of the latter persists in XY gonads when Sertoli cells fail to differentiate (Hiramatsu et al., 2009). The most likely explanation for the critical role of Enh13, and one that is consistent with all current data, is that it is an early acting enhancer, such that without it *Sox9* transcription

fails to increase to a level where the other enhancers can act before the gene is silenced.

Unlike the critical role of Enh13 during sex determination, deletion of Enh14 did not exhibit any phenotype and did not alter *Sox9* expression levels at the embryonic stages. It is likely that Enh14 functions in a redundant manner along with TES and Enh13 to ensure that high levels of *Sox9* are maintained (Gonen et al., 2018). Enh32 may also correspond to an enhancer used later. While the DNaseI-seq and ATAC-seq data showed it was open at E13.5 and E15.5 in Sertoli cells, transgene expression was low at these stages, but higher at postnatal stages (Gonen et al., 2018) and (unpublished data). Moreover, it should be stressed that because the ATAC-seq data was carried out in early gonads there could well be other enhancers involved at later stages and in the adult testis.

Interestingly, high-throughput methods, including chromosome capture confirmation assays (3C and derivatives 4C, 5C and HiC), indicate that a single gene promoter will interact on average with five different enhancers (Jin et al., 2013; Spitz, 2016). Our data agrees with this, with at least four different enhancers likely to act on *Sox9* expression during embryonic testis development (Gonen et al., 2018). Assuming it will be possible to perform 4C analysis on small number of cells isolated from the embryonic gonad, it will be interesting to verify these long-distance interactions between the remote enhancers and the *Sox9* promoter during sex determination, and to explore if there is a distal to proximal use of enhancers, showing either consecutive or additive roles in *Sox9* transcription in the testis. Finally, given the relevance for SOX9 in sex determination, cartilage development, *etc.*, throughout vertebrate evolution, it will be of interest to explore how well the enhancers and their arrangement are functionally conserved. Despite its obvious importance, sex determination mechanisms show rapid evolution. SRY itself is poorly conserved outside the HMG box, and it is the sex-determining gene only in eutherian and metatherian mammals, indeed it has even been lost in some species, such as mole voles (*Ellobius lutescens*) and spiny rat (*Tokudaia osimensis*) (Capel, 2017; Just et al., 2007; Kuroiwa, Ishiguchi, Yamada, Shintaro, & Matsuda, 2010; Soullier, Hanni, Catzeflis, Berta, & Laudet, 1998). While Enh13 shows good conservation across a range of mammals tested, it was not obvious in the chick (Gonen et al., 2018). Perhaps this reflects the very different mechanism of sex determination present in birds, including the absence of *Sry*, and the more critical involvement of DMRT1 and of estrogen signaling (Capel, 2017; Lambeth et al., 2014; Smith et al., 2009).

4. Higher order of chromatin organization at the *Sox9* locus

It is now well established that the genome is organized non-randomly into higher order chromatin domains (Andrey & Mundlos, 2017; de Laat & Duboule, 2013; Spitz, 2016). Chromosome conformation capture techniques (3C, 4C, 5C, HiC and derivatives) have revealed the presence of chromatin domains called topological association domains (TADs) that show high internal interactions while being separated by low interacting regions called boundary elements (Dixon et al., 2012; Nora et al., 2012). It is believed that TADs divide the genome into separate regulatory units and hence allow the binding of enhancers only to their target genes (Rao et al., 2014; Symmons et al., 2014). TADs are relatively conserved across different cell types and even between different species (Dixon et al., 2015, 2012).

Recently, Franke et al. (2016) studied the *Sox9* TAD in human and mice and explored how genomic duplications spanning this region affect the TAD formation and mediate disease. They performed 4C experiments on human fibroblasts carrying three different types of duplications. The first was a 0.5 Mb duplication covering the RevSex region but fully contained within the *SOX9* TAD (termed an intra-TAD duplication) in which the patient presented with XX male sex reversal. The second was a larger duplication that included the RevSex region, but extended further toward the neighboring TAD and contained the upstream *KCNJ2* and *KCNJ16* genes (termed an inter-TAD duplication) (Fig. 2). This duplication had no effect on sexual development, but resulted in Cooks syndrome, a congenital limb malformation (Kurth et al., 2009). The third duplication included the RevSex and the entire gene desert upstream of *SOX9* but not the *KCNJ2* and *KCNJ16* genes. These patients were phenotypically normal. Using 4C and capture HiC the authors demonstrate that the intra-TAD duplications within the *SOX9* TAD result in increased contacts of the duplicated regions within the TAD, without change in the overall TAD structure. In contrast, the inter-TAD duplications that did not include the *KCNJ2* and *KCNJ16* genes created a new TAD (neo-TAD) that was isolated from the rest of the genome and hence had no phenotypic effect. Conversely, the duplication that did contain the *KCNJ2* adjacent gene also showed the formation of a neo-TAD, but in this instance, the interactions within the neo-TAD resulted in the ectopic contacts of *KCNJ2* with the duplicated part of the

SOX9 regulatory region and led to the misexpression of *KCNJ2* and to a limb malformation phenotype (Andrey & Mundlos, 2017; Franke et al., 2016). In support of TADs being insulated chromatin environments, another study explored the *Shh* gene and its ZRS limb enhancer and demonstrated that as long as both the enhancer and the gene are located within the same TAD, the relative distance of the enhancer from the gene has no influence on its transcriptional activity (Symmons et al., 2016).

Interestingly, it is notable that in the mouse, we did not discover any putative enhancer in the RevSex region (Gonen et al., 2018). On the contrary, a study of gonadal *SOX9* enhancers in humans did find a putative 744 bp-long enhancer termed "SOX9 up 3" located within the RevSex region, however, luciferase assays did not show strong enhancer activity (Ohnesorg et al., 2016). It will be interesting to understand the underlying reason for the sex reversal observed in XX patients carrying duplications containing the RevSex region (Baetens et al., 2017). It is possible that the RevSex region contains a human-specific enhancer that is not conserved in mouse. This idea is strongly supported by the recent finding of a novel human enhancer located in that region termed eSR-B (Croft, Ohnesorg, Hewitt, et al., 2018). Alternatively, we hypothesize that the RevSex duplication within the *SOX9* TAD causes increased interactions and hence elevated expression of *SOX9,* leading to male development in XX individuals. This could involve an effect on Enh13, which is close by. Perhaps the strategy of looking in the shared minimal region that is missing in all patients is not the way forward, but instead the phenotype arises from changes in higher chromatin organization and amount of interactions occurring within a TAD as a result of the duplication.

5. Perspectives

Advances in recent years have strongly supported the presence of multiple enhancers regulating *Sox9* expression throughout gonadal differentiation. We showed that Enh13 is critical for sex determination at early stages (Gonen et al., 2018). However, apart from an important, albeit not critical, subsequent role for TES, we still do not know which enhancers are involved in controlling *Sox9* expression throughout gonadal development. For example, how is the initial, basal expression of *Sox9* in the genital ridge of both sexes controlled, and what are the enhancers that regulate the expression of *Sox9* in adulthood, which is needed to maintain testis identity

(Matson et al., 2011). Furthermore, it will be interesting to see whether *Sox9* expression at different stages is also controlled by a single, critical enhancer or mediated *via* the activity of several, redundant enhancers. In addition, much focus has been put on enhancers; however, from what we know based on genetic studies, sex determination and sex maintenance heavily rely on gene repression (Carre & Greenfield, 2016). Although more challenging to find, it will be important to understand if the pro-female transcription factors such as β-CATENIN and FOXL2 execute their repressive effect on *Sox9* expression *via* binding to the already known enhancers or to different, novel silencers.

The rapid development and improvements of techniques that allow the identification of potential regulatory elements such as DNaseI-Seq, ATAC-Seq, and ChIP-Seq for TFs and histone marks has allowed much progress and increased our understanding of the mechanism of activity of the critical transcription factors controlling sex determination (Garcia-Moreno, Futtner, et al., 2018; Gonen et al., 2018; Maatouk et al., 2017; Rahmoun et al., 2017). Efforts to map all the potential regulatory elements in a cell- and stage-specific manner in the developing gonads are proving to be very informative, although it is likely that the precise role of each will still need to be assessed in a focused way (Garcia-Moreno, Futtner, et al., 2018). However, it is still challenging to perform chromosome capture confirmation techniques on relatively small numbers of cells sorted from embryonic gonads. In addition, whereas ChIP-Seq is useful at detecting transcription factor binding to promoters at target genes, the ability to detect binding to remote regulatory elements can be very limited due to the small amount of starting material available, particularly in early gonads (Rahmoun et al., 2017). Therefore, further progress in downscaling those methods could allow the unbiased identification of novel regulatory elements that are bound by pro-male and pro-female critical transcription factors such as SRY, SOX9, DMRT1, SF1, FOXL2, β-CATENIN and others that mediate the activation as well as repression of downstream target genes. It will also allow identification, *in vivo*, of enhancer-promoter interactions throughout gonadal development.

Although much effort has been given to discover the genetic causes of Disorders of Sex Development (DSD), this is still not known for ~80% of the patients. Many cases have been analyzed by whole exome sequencing (Baetens et al., 2017; Croft, Ohnesorg, & Sinclair, 2018; Hyon et al., 2015). However, with the understanding that in some patients the mutation occurs in non-coding regions, and include copy number variations (CNV) of critical regulatory elements, whole genome sequencing is now the preferred

method (Croft, Ohnesorg, & Sinclair, 2018). The discovery that Enh13 is responsible for XY female development in DSDs involving deletions of the 32.5 kb genomic region termed XY SR, strongly supports the above (Gonen et al., 2018) and advocates for the search for additional regulatory elements of other genes that may mediate sex reversal phenotypes. Adding Enh13, but also the other novel enhancers to genotyping screens may be informative for other cases of DSD.

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