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Sex determination and SRY: down to a wink and a nudge?

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Sex-determining region Y (*Sry*) is the crucial gene that initiates male sex determination in most mammals. Although several components of the pathway regulating sexual differentiation have been elucidated, the mechanism of *Sry* action within this was unclear. However, recent discoveries in cellular, genetic and molecular aspects of gonad development are shedding light on the precise role of SRY in the regulation of *Sox9*, a crucial downstream target gene. SRY is thought to act synergistically with SF1, a nuclear receptor, through an enhancer of *Sox9* to promote Sertoli cell differentiation in mice. In this review, we focus on the regulation of these genes and their interaction with other genes involved in promoting testis or ovary development. We also explore the common features between sex determination in mammals and in other vertebrates that lack *Sry*.

What specifies sex? The quest for the Holy Grail

In 1959, the presence of the Y chromosome was identified as the main factor determining maleness in humans and mice, unlike the situation in *Drosophila melanogaster*, in which the decision to be male or female is dependent on X-chromosome dosage (for a review, see Ref. [1]). Over the next three decades, several candidate genes for the elusive testis-determining factor on the Y chromosome (*TDF* in human or *Tdy* in mouse) came and went, often adding confusion to a field that desperately needed a molecular entry point, not just a genetic one, to gain an understanding of how sex is determined. 1990 saw the cloning of the sex-determining region Y genes in human (*SRY*) and mice (*Sry*) (Box 1). Subsequent analyses revealed a conserved 79 amino acid domain within SRY with similarities to a type of DNA-binding domain termed a high mobility group (HMG) box (see Glossary). Work on SRY rapidly led to the discovery of the related SRY-related HMG box protein (SOX) family of transcription factors, with 20 members having been identified in humans and mice to date. These SOX proteins bind the consensus sequence (A/T)ACAA(T/A) and interact with the minor groove, forcing the DNA helix apart to bend the target DNA up to 90° (for reviews, see Refs [1,2]). On the basis of these properties, several proposals were made as to the function of SRY in sex determination: that SRY (i) represses or antagonizes a repressor of male development; (ii) activates transcription of one or more genes that have important roles in male development; (iii) functions as an architectural factor that facilitates or prevents the activity of other chromatin-bound proteins; or (iv) has a role in pre-mRNA splicing [1,2]. However, deciding which of these alternatives is true required knowledge of a direct target for SRY.

Considerable progress has been made over the past 18 years. Several genes involved in various aspects of mammalian sex determination have been identified, including *Sox9*, steroidogenic factor 1 (*Sf1),* GATA-binding protein 4 *(Gata4),* fibroblast growth factor 9 (*Fgf9),* forkhead box L2 *(Foxl2), Wnt4,* a member of the wingless family of genes, and R-spondin 1 *(Rspo1)*, which encodes a secreted factor that is disrupted in a rare recessive syndrome characterized by sex reversal. The origins and the fates of different types of cells within the gonads are also more clearly defined, enabling the temporal and spatial activity of these genes to be investigated. The genes can be divided into

Glossary

Anti-Müllerian hormone (AMH): otherwise known as Müllerian inhibiting substance (MIS). A TGFβ-like growth factor secreted by fetal Sertoli cells, the main role of which in the embryo is to eliminate the Müllerian ducts from male embryos. It is also made by follicle cells in the postnatal ovary, where it is involved in selecting which primordial oocytes will grow from the total pool.
Coelomic epithelium (CE): the epithelium derived from the layer of splanchnic mesoderm of lateral plate origin, which lines the coelomic cavity. In the region of the genital ridge and mesonephros, the CE overlies cells that originate from intermediate mesoderm.
Follicle (granulosa) cells: ovarian cells derived from the bipotential supporting cell lineage of the early genital ridges of XX embryos. These cells support or 'nurse' all stages of female germ cells or oocytes.
Genital ridge: the indifferent gonad, which gives rise to the testis in males and the ovary in females.
HMG box: a DNA-binding motif found in high mobility group proteins.
Leydig cells: The steroidogenic cells of the testis. Leydig cells, which synthesize the male hormone testosterone, first differentiate at ~12.5 dpc in the mouse. The corresponding steroidogenic cells in the ovary are called theca cells, but these do not differentiate until much later.
Mesonephros: a primitive kidney-like organ derived from intermediate mesoderm. This contains transient mesonephric tubules and a portion of the Wolffian duct, which gives rise to much of the male reproductive tract including the vas deferens and seminal vesicles, and the Mullerian duct, which gives rise to the oviduct and uterus in the female.
Peritubular myoid cells (PMC): a cell type unique to testes. These surround and interact with Sertoli cells to form and stabilize the testis cords.
Sertoli cells: testicular cells derived from the bipotential supporting cell lineage of the early genital ridges due to the action of SRY in XY embryos. In the fetal testis, these secrete AMH. In the mature testis, Sertoli cells support or 'nurse' all stages of male germ cells throughout spermatogenesis.

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Box 1. A brief history of SRY

1959

The Y chromosome was identified as the dominant male determinant in humans and mice (for a review, see Ref. [1]). This finding initiated a search for *TDF* or *Tdy* on the Y chromosome. Several candidates were proposed (see below) and later refuted by genetic studies before the isolation of SRY.

1975

H–Y transplantation antigen. Because the Y chromosome was thought to carry few genes, it was proposed that H–Y must be TDF or Tdy.

Y chromosome deletions were eventually found, first in mice (1984) and then in humans (1987), where H–Y was lost, but male development was not.

1982

Bkm (banded krait minor-satellite). A repetitive DNA sequence found on the mouse Y chromosome with similarity to repeats found on a snake heteromorphic (W) chromosome.

Similar repeats could not be readily identified on other mammalian Y chromosomes, including the human Y.

1987

ZFY, a zinc finger nucleic-acid-binding protein, was proposed to be *TDF* owing to its presence within a 280-kb Y-unique sequence in an XX male. It also was deleted from an XY female carrying X,t(Y:22).

All eutherians have a close homologue on the X chromosome (ZFX), which is ubiquitously expressed. ZFY is also ubiquitously expressed in humans. Marsupial homologues are autosomal (1988). Mouse Zfy1 or Zfy2 are normal in XY females (1990).

1990

SRY was identified in a search for conserved sequences within a 35-kb Y unique region found in four XX humans showing some male characteristics. The mouse *Sry* was found to be present in the smallest region of the mouse Y chromosome associated with male development, but was missing in Tdy^{m1} allele (an 11-kb deletion from mouse Y). In line with predictions, *Sry* expression was observed within somatic cells of XY indifferent gonads. Point mutations were discovered within *SRY* in several XY females, indicating that *SRY* is required for male development in humans. Moreover, *SRY* lies within a second deletion in the X,t(Y:22) female.

1991

A 14-kb mouse genomic fragment carrying *Sry* gave XX male development when introduced as a transgene into mice, demonstrating that *Sry* was not only necessary but, rather, sufficient for testis and subsequent male development. The XX male mice were predictably sterile because postnatal spermatogonia cannot tolerate the presence of two copies of the X chromosomes and because other Y-linked genes are required for spermatogenesis.

three categories (not all mutually exclusive) based on their involvement in: (i) pre-sex-determination stages of gonad development; (ii) testicular differentiation; and (iii) ovarian differentiation. Unfortunately, the molecular interactions involving the proteins encoded by the genes are mostly poorly understood, despite the extensive data on their genetic interactions. This was particularly true for SRY.

In this review, we discuss how *Sry* and its protein product (SRY) function in the context of other genes required for testis or ovary development in the mouse. The emerging picture is one in which the early gonad is poised to go in either direction (i.e. male or female), but SRY tips the balance in favor of the male pathway. It seems that SRY acts on a single gene, *Sox9*, the expression of which is then rapidly reinforced by positive regulatory loops. SOX9 then drives Sertoli cell formation and, therefore, testis differentiation. If SRY is absent or fails to act in time, *Sox9* is silenced and development of the follicle cell and ovary ensues, with β-catenin (also called *Catnb*) being one of the crucial components driving this process. Overall, sex determination is a story of opposing forces and crucial alliances but, although the winning team takes all, its rule can be surprisingly tenuous.

Sry and its protein product

To understand the precise role of SRY in sex determination, it is necessary to know where and when it functions. Research using XX–XY chimeric mice predicted that genes on the Y chromosome and thus, SRY, functions in the supporting cell lineage of the developing gonad, driving cells to a Sertoli (i.e. male) rather than a follicle (or granulosa; i.e. female) fate. *Sry* transcripts are expressed for a brief period during early gonad development, which is consistent with the gene initiating rather than maintaining testis differentiation. However, a lack of specific antibodies stalled characterization of mouse SRY (mSRY) until it was first observed *in vivo* as a MYC epitope-tagged protein encoded by a transgene under the control of *Sry* regulatory sequences [3]. mSRY is present for only a few hours in each Sertoli cell precursor at the time they are specified between 10.5 and 12.5 days postcoitum (dpc). The protein is localized within the nucleus, with a distribution that does not seem to support a role in splicing. Although these findings have been confirmed using anti-mSRY antibodies [4,5], the precise timing of expression varies with genetic background [6].

Apart from the HMG box, the SRY protein sequence is very divergent between species. For example, mSRY contains a glutamine-rich domain in the C-terminal half of the protein (derived from CAG repeats within the DNA sequence), whereas human SRY (hSRY) does not. However, hSRY expressed from mouse regulatory sequences drives male development in XX transgenic mice (see Ref. [7]), as does expression of a goat *SRY* BAC clone [8], indicating that the function of SRY is conserved even if much of its sequence is not. Perhaps only an HMG-box domain of the right type (i.e. those found in SOX proteins but probably not those of the related lymphoid enhancer factor/T-cell factor [LEF/TCF] or HMG1/2 proteins), expressed at the right moment, is sufficient to trigger Sertoli cell differentiation.

In addition to its ability to bind to and bend DNA, the HMG box contains several functional motifs, including nuclear localization signals and a calmodulin-binding site (for a review, see Ref. [9]). Nevertheless, other parts of SRY are considered to be functional domains. Studies of human sex reversal patients have identified many point mutations that result in SRY amino acid substitutions. Most of these are within the HMG box but a few affect the N- and C-terminal domains [1]. Moreover, both mouse and human SRY proteins interact with SRY-interacting protein-1 (SIP-1; also called Na^+/H^+ exchanger regulatory factor 2 [NHERF2]) and Krüppel-associated box only (KRAB-O), although their roles in sex determination are unknown. SIP-1 interacts with a motif containing a Thr-Lys-Leu (TKL) amino acid sequence located at the C-terminal

end of hSRY and the bridge domain of mSRY [10]. KRAB-O also interacts with the bridge domains of mSRY and a domain adjacent to the hSRY HMG box C terminus that lacks an identifiable motif [11]. However, to understand SRY function fully, it will be essential to identify binding partners that have a crucial role in sex determination. Studies of SOX proteins indicate that they can have many different types of partner and that these interactions with other transcription factors direct their specificity and activity.

Sry transcriptional regulation

It is still not clear how *Sry* expression is regulated. As with much of the coding sequence, the untranslated regions of the transcript and flanking DNA show a distinct lack of sequence conservation, making the identification of any common enhancer elements a serious challenge. For example, the *Sry* transcribed region is flanked by a large inverted repeat in mouse, but not in other species. Several potential activators of *Sry* have been identified, including the polycomb group protein M33, a splice variant of Wilms' tumor 1 with the presence of Lys-Thr-Ser (KTS) amino acids [WT1(+KTS)] and the GATA4–friend of gata2 (FOG2) complex [1,2]. Receptor tyrosine kinase activity from the insulin receptor (IR), the insulin-related receptor (IRR) and the insulin-like growth factor 1 receptor (IGF1R) might also be involved because combined loss-of-function (LOF) mutations of these genes reduced *Sry* expression [12]. However, this reduction could have resulted from lower levels of *Sry* expression per cell or fewer *Sry*-expressing cells. Moreover, insufficient Sertoli cell proliferation can inhibit testis cord formation or phenotypic maintenance via a *Sox9–Fgf9* regulatory loop (see later and Figure 1).

SF1 (also called Nr5a1) is also a good candidate for an *Sry* activator. SRY-positive Sertoli cell precursors are derived from SF1-positive coelomic epithelium (CE) in mice (for reviews, see Refs [13,14]) (Figure 1) and, in humans, heterozygous mutations in *SF1* can produce XY female sex reversal [15–17], indicating that it is important for regulating genes essential for the male pathway. *In vitro* transfection studies showed that SF1 binds and activates both the human and pig *SRY* promoter [18]. Similarly, WT1 also activates the promoter of human *SRY*, but this ability is limited to the −KTS splice variant [WT1(−KTS)]. In contrast to the *in vitro* data, however, the targeted deletion of WT(−KTS) in mice was associated with apoptotic cell death, whereas reduced *Sry* expression was observed with the deletion of WT1(+KTS), which is thought to bind and stabilize RNA [19]. Moreover, recent *in vitro* studies have demonstrated that both WT1 variants can physically interact with GATA4 and synergistically activate the mouse, pig and human *SRY* promoters [20].

Sox9: an SRY-target gene

Several lines of evidence indicate that *Sox9* is the best candidate for a direct SRY target gene [3,4,21]. First, *Sox9* expression is strongly upregulated soon after the expression of SRY begins, whereas it is downregulated in the ovary. Second, cell-fate mapping experiments show that SRY-positive cells exclusively become SOX9-positive Sertoli cells. Third, heterozygous mutations in *SOX9* are

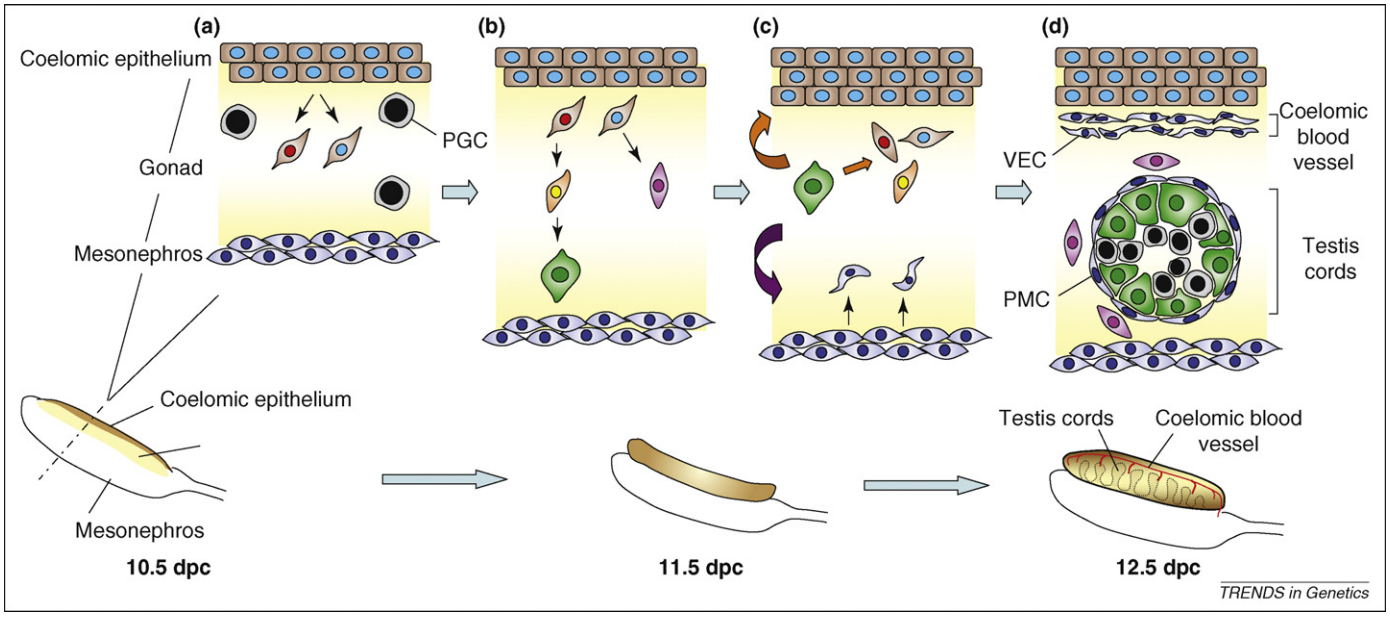

Figure 1. Cellular organization during testis development. The adult testis is composed of germ cells and somatic cells. The latter are divided into three main types: (i) Sertoli cells; (ii) Leydig cells; and (iii) connective tissue cells, notably peritubular myoid cells (PMCs) and vascular endothelial cells (VECs). **(a)** The testis arises from the genital ridge, which is first recognized as a thickening underlying the coelomic epithelium (CE) adjacent to the mesonephros at ~10.5 dpc. **(b)** Primordial germ cells (PGC; black) migrate into the gonad from this stage until ~11.5 dpc. The CE cells, which are SF1-positive (light blue), proliferate and some daughter cells migrate into the ridge. **(c)** A proportion of these daughter cells express SRY to become Sertoli cell precursors (red), with the remainder giving rise to interstitial cells, perhaps including Leydig cells (pink). Soon after SRY is expressed, *Sox9* is upregulated in the Sertoli cell precursors (yellow). SOX9 represses *Sry* expression once it has reached a critical threshold, which generates SOX9 single-positive cells (green). The SOX9-positive cells then release signaling molecules such as FGF9, which reinforce their *Sox9* expression and induce neighboring cells, including the CE cells, to proliferate (orange arrows). This action increases the number of supporting cell precursors that are generated and, therefore, able to express SRY. This indirect regulatory loop is necessary to obtain sufficient Sertoli cells to form a testis (for a review, see Ref. [90]). The SOX9-positive cells also induce cell migration from the mesonephros (blue arrow) and these cells (blue) mainly comprise VECs, which re-aggregate to form the coelomic blood vessel characteristic of the testis. **(d)** The testis cords develop by 12.5 dpc, in which the SOX9-positive cells become Sertoli cells surrounding the germ cells.

responsible for the human skeletal malformation syndrome, campomelic dysplasia (CD; Online Mendelian Inheritance in Man® [OMIM®] #114290), in which most XY patients have male to female sex reversal. Similarly, targeted *Sox9* ablation in mice also leads to ovary development in XY embryos [22,23]. Lastly, gain-of-function (GOF) mutations, such as duplication of the *SOX9* locus in humans and transgene-driven expression in mice, cause male development in XX embryos [1,2]. These LOF and GOF mutations of *Sox9* mimic those of *SRY* and *Sry*, but not of other genes, implying that *Sox9* is not only necessary for testis differentiation, but that it might be the only gene required downstream of *Sry* to activate the rest of the male pathway. This will include the expression of other essential

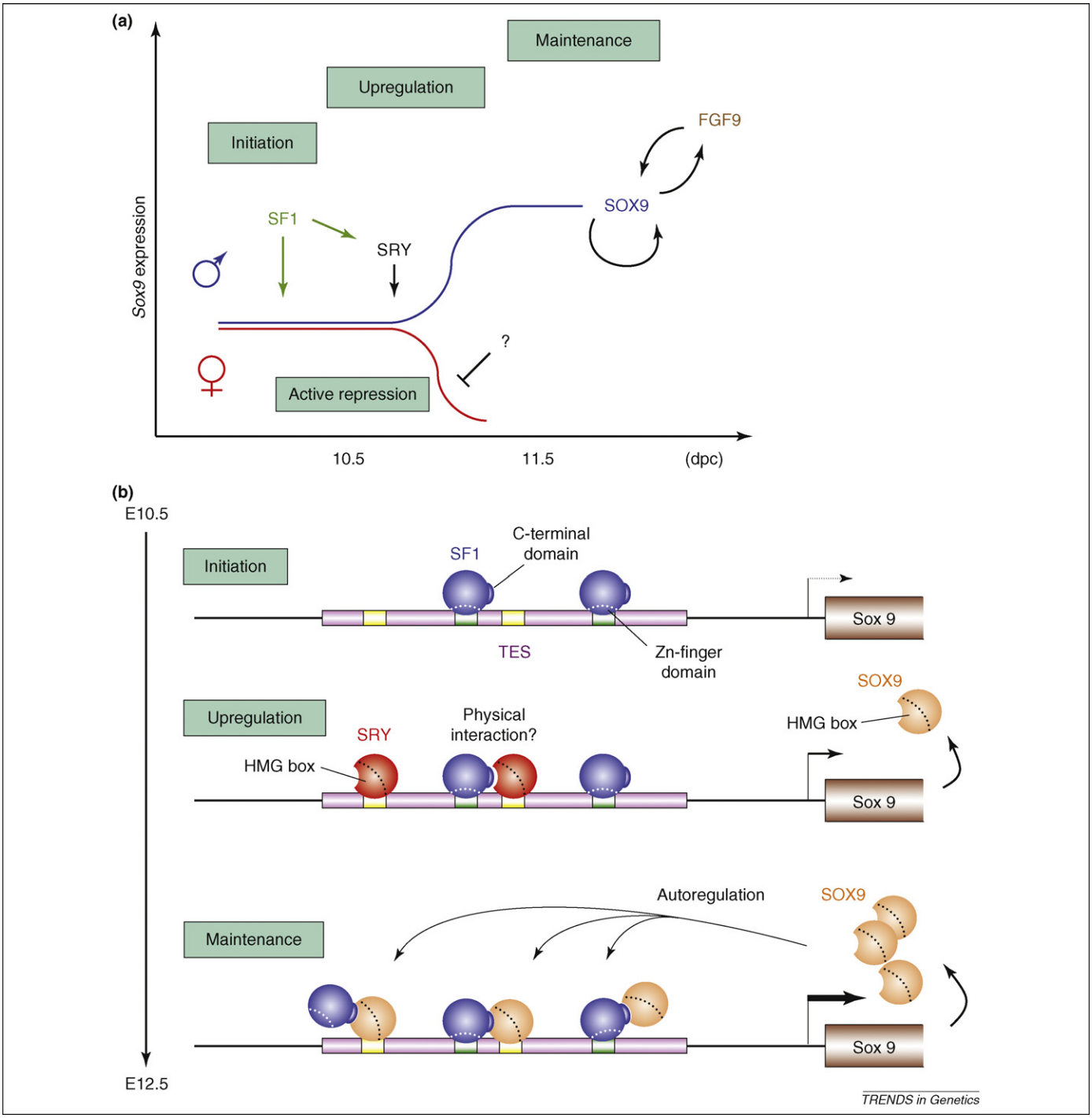

Figure 2. A model for *Sox9* transcriptional regulation in the gonad. Gonadal *Sox9* transcriptional regulation consists of three phases: initiation, upregulation and maintenance. **(a)** SF1 sensitizes *Sox9*, initiating a low level of expression in the genital ridge of both sexes at 10.5 dpc. In the male, SF1 (probably with other factors such as $WT1^{-KTS}$) also activates *Sry* expression. *Sox9* expression is upregulated by the action of SRY together with SF1, whereas it is downregulated in the female. This downregulation is unlikely to be passive, implying the presence of one or more currently unknown repressors. After the transient expression of *Sry* has ceased, high levels of SOX9 are maintained by its direct autoregulation and via FGF9 signaling. **(b)** SF1 binds to SF1-BSs (green) in TES via its Zn-finger domain (white dotted line) at 10.5 dpc. When SRY is expressed, it binds to SRY-BSs (yellow) via its HMG-box (black dotted line) and cooperates with SF1 to upregulate *Sox9* expression. In turn, SOX9, also together with SF1, maintains its own expression. SOX9 binds directly to the enhancer, replacing SRY at some sites, but because the SOX9 HMG box can physically interact with the SF1 C-terminal domain, the two proteins also recruit each other to additional binding sites.

genes for testis differentiation, such as *Fgf9*, and repression of 'anti-testis' or ovary-determining genes, such as *Wnt4* and *Dax1* (also known as NR0B1– a nuclear receptor).

However, the expression of *Sox9* in the genital ridge is not entirely dependent on SRY. Transcripts and protein are detected at very low levels in the genital ridge of both sexes at 10.5 dpc before SRY expression begins (for example, see Ref. [24]). Moreover, *Sox9* expression is maintained long after that of SRY has ceased; indeed, it is characteristic of Sertoli cells throughout life. Thus, normal gonadal *Sox9* transcriptional regulation consists of at least three phases: (i) SRY-independent initiation; (ii) SRY-dependent upregulation; and (iii) maintenance in the absence of SRY (Figure 2a). SF1 is a good candidate for initiating or sensitizing *Sox9* expression because early sex-independent expression of *Sox9* is abolished in *Sf1* null mutant gonads [3,7]. Continuing SF1 expression might then be required for *Sox9* upregulation. This model is supported by a transgenic experiment in which SRY induced *Sox9* expression only in SF1-positive supporting cell precursors even though the *Sry* transgene was ubiquitously expressed [25]. It is possible that upregulation and maintenance comprise several distinct stages. Furthermore, the cases of male sex reversal in *SRY*-negative XX embryos that can result when *Sox9* experiences a GOF mutation, or when *SOX9* itself is unaffected but secondary mutations have led to its activity [26,27], indicate that the usual requirement for SRY can be bypassed. It should be stressed that the model we describe relates to the mouse for which most data exist. It might not be identical in other mammals; SRY expression is thought to persist much longer in some species, for example, humans, although there is no evidence to indicate whether this is relevant to its function.

To explore how SRY influences *Sox9* expression in the gonad, it is necessary to examine how it affects the regulatory elements of *Sox9*. However, *Sox9* is widely expressed, with a complex regulatory region spread over a very long distance; indeed, some CD patients with sex reversal have translocation breakpoints mapping over 1 Mb 5′ of the open reading frame (ORF). By comparing genomic sequences between human and *Fugu rubripes SOX9*, several highly conserved elements were identified from −290 kb to +450 kb of the gene and their enhancer activities were tested in transgenic mice [28]. These studies revealed several conserved tissue-specific enhancers, such as those involved in the cranial neural crest, notochord and telencephalon, but none for the gonad. However, because the primary sex-determining mechanism is different between human and fish, it is likely that the elements required for gonadal expression are not sufficiently well conserved to have been identified in this way. An added complication is that *SOX9* seems susceptible to 'position effects', illustrated by studies of the *Odsex* mutation in mice in which XX male sex reversal results from constitutive *Sox9* activation in the early gonad. Because the phenotype in *Odsex* mice is associated with the insertion of a mini-tyrosinase transgene and a coincident 134-kb deletion ~1 Mb upstream of *Sox9*, it was originally proposed that the sex reversal was caused by the loss of a gonad-specific regulatory element required to mediate *Sox9* repression in XX genital ridges. However, subsequent work ruled out this possibility. Moreover, an additional phenotype seen in the eyes of the *Odsex* mice was attributed to the transgene enhancer aberrantly driving *Sox9* expression, even at such a distance, indicating that the enhancer might also drive expression in the genital ridge [29]. The XY female sex reversal and other phenotypes associated with translocations in human CD patients could be explained by similarly long-range, but repressive, effects on *SOX9* expression.

Recent transgenic studies were prompted by the absence of a *Sox9* gonadal enhancer [21]. These began with a 120-kb mouse *Sox9* BAC clone, which replicated part of the endogenous gene expression pattern, giving robust reporter gene expression in several tissues including the early gonads. Through iterative pruning, a 3.2-kb testis-specific enhancer of *Sox9* (TES) was revealed between −13 and −10 kb of the gene. Subsequently, TES activity was narrowed down to a well-conserved critical region of 1.4 kb, termed TESCO (for TES core element). Although the early sex-independent expression seems to have been lost, this might simply relate to the absence of many more SF1 sites that are in the flanking sequences. Importantly, expression from TESCO was dependent on the presence of SRY and SF1. Moreover, whereas high levels of enhancer activity required the presence of endogenous SOX9, expression of the reporter was still seen in its absence, indicating that SRY is required for enhancer activity at 11.5 dpc and that SOX9 is subsequently involved in positively regulating its own expression (see later). Chromatin immunoprecipitation (ChIP) assays showed that SRY and SF1 directly bind several sites within TES *in vivo*. Moreover, mutating all these sites abolished the enhancer activity in co-transfection assays *in vitro* and in transgenic mice, indicating that SRY and SF1 synergistically upregulate the enhancer. These findings not only demonstrated that *Sox9* is a direct SRY target gene but, also, revealed that SRY contributes to transcriptional activation rather than simply interfering with the action of a repressor (Figure 2b).

How is *Sox9* expression maintained?

Because SRY acts on *Sox9* for only a brief period, other factors must help maintain its expression during Sertoli-cell differentiation and, subsequently, in the adult testis. One possibility, because SRY and SOX9 share the same DNA-binding motif, is that SOX9 regulates its own expression, acting via the same enhancer as SRY after expression of the latter has ceased. Indeed, by using an antibody against SOX9, ChIP analyses revealed that at least some of the SRY-binding sites (BSs) in TES were occupied by SOX9 after 13.5 dpc. Similarly to SRY, SOX9 also synergizes with SF1 to activate the TES sequence and it is far more potent than SRY, presumably because it possesses a strong activation domain [21]. By itself, however, SOX9 had no activity in co-transfection assays. In the ChIP assays, SOX9 was associated with some DNA fragments that lacked SRY-BSs, but these were fragments to which SF1 was bound, presumably via its own consensus sites. This finding indicates that, in addition to interacting with TES via its own sites, SOX9 can be recruited to the enhancer via protein–protein interactions with SF1.

Similar results were obtained with SRY, implying that it might also physically interact with SF1. A direct interaction between the SF1 C-terminal domain and the SOX9 HMG box was previously shown to occur on regulatory sequences upstream of the anti-Müllerian hormone gene (*Amh*) and to be required for transcription (see later) [1]. A similar scenario probably applies for maximal activity of SF1 and SOX9 on TES (Figure 1b). The identification of *bona fide* enhancer sequences will facilitate the detailed study of these interactions and the contributions that DNA bending and binding make to *Sox9* activation need to be addressed.

Although SOX9 contributes directly to the maintenance of its own expression, other feedback loops are also involved. Co-transfection assays indicate that SOX9 is required for maximal SF1 expression [30]. This would provide an additional positive feed-forward mechanism to help maintain SOX9 expression, involving activation of its own partner. Studies in *Fgf9* mutant mouse embryos indicate, however, that these intracellular feedback loops are insufficient; although SRY action is normal and SOX9 levels mirror that of wild-type embryos until 11.5 dpc, with initiation and upregulation occurring as usual, *Sox9* expression is then lost, which results in ovary development and XY female sex reversal [31]. Because *Fgf9* expression is itself lost in the absence of *Sox9*, this finding links the two genes in a positive feed-forward loop, whereby SOX9 is required to upregulate *Fgf9* expression and FGF9 helps to maintain *Sox9* expression. Four FGF receptors (FGFRs) are expressed in the gonad [32], but FGFR2 is the most crucial for transducing the FGF9 signal because Cre recombinase-mediated *Fgfr2* loss in the early gonads leads to XY ovary development [33,34]. FGF9–FGFR2 signaling might antagonize Wnt4 activity, which participates in promoting ovary differentiation (see later) [31], but this action could be secondary to a more direct effect on *Sox9* transcription. Indeed, FGFR2 can signal through the MAPK cascade, a pathway which might activate *Sox9* expression [35].

Two other genes have been identified as direct targets of SOX9 in Sertoli cells: *Amh*, a member of the TGFβ superfamily, and prostaglandin D synthase (*Ptgds*), which is required to synthesize prostaglandin D2 (PGD_2). SOX9, together with SF1, binds to a crucial enhancer close to the promoter of *Amh*. AMH is mainly required in male embryos for regression of the Müllerian ducts, but it has additional minor roles within the mammalian testis (e.g. in germ cells and in Leydig cell proliferation), which might reflect more robust ancestral roles for the protein in species lacking these ducts, such as in the teleost fish Medaka [36]. SOX9 binds to and activates the promoter of *Ptgds* as a homodimer, although the role of other transcription factors in *Ptgds* activation has not been addressed [37]. Autocrine or paracrine signaling via PGD_2 drives the import of cytoplasmic SOX9 into the nucleus, thereby helping to promote Sertoli cell differentiation [4,38]. Because *Ptgds* mutant mice do not show sex reversal, it is widely considered that PGD_2 signaling is a back-up mechanism to ensure that all supporting cell precursors become Sertoli cells, particularly when *Sry* expression is weak or in XY–XX chimeric or XY–XO mosaic gonads.

What happens downstream of SOX9?

SOX9 is probably the pivotal factor in regulating the gene activity that defines Sertoli cells. It must directly or indirectly repress gene activity characteristic of bipotential supporting cell precursors. For example, once SOX9 reaches a critical threshold, *Sry* is repressed via a SOX9-dependent negative-feedback loop [3,22]. SOX9 activity must also ensure that genes, such as *Wnt4*, *Dax1* and *Foxl2*, which are typically expressed in follicle cells, are repressed (see later). SOX9 must also influence the expression of genes that define Sertoli cell phenotype and function. A variety of approaches have identified approximately 1000 genes that become active in the testis after ~10.5 dpc [39]. Many of these are specific to Sertoli cells within the gonad; some, such as cytochrome P450 family 26b (*Cyp26b)* that encodes an enzyme responsible for eliminating retinoic acid (RA), have known functions in testis differentiation. RA drives germ cells to enter meiosis early, which is typical of the fetal ovary, so it must be removed from the testis [40–42]. The roles of many others are unknown, including *Vanin-1 (Vnn1)*, which encodes a membrane-associated pantetheinase involved in generating cysteamine, although there is some evidence that it has a role in tissue remodelling. Here again, SOX9 and SF1 synergize to activate transcription [43]. Although it seems likely that these two factors cooperate to regulate many genes expressed in Sertoli cells, SF1 is also present in follicle cells, albeit at lower levels. SOX9 is clearly the distinguishing factor and it will be interesting to see how much of the burden of maintaining Sertoli cell phenotype and function remains with it throughout life.

The genetic context in which SRY and SOX9 must function

Several genes, including LIM homeobox 9 (*Lhx9*), *Sf1*, *Wt1*, *Gata4* and *Fog2*, the expression of which begins from 9.5 to 10.5 dpc, are required within the early genital ridge. Targeted null mutations of these genes lead to failure of gonadal development (gonadal agenesis) in both female and male embryos, with many somatic cells dying by apoptosis between 11.5 and 12.0 dpc. Some of these genes and their products interact. For example, WT1(−KTS) and LHX9 together activate *Sf1* expression [44] then, in turn, WT1(−KTS) and SF1 activate the AMH receptor gene (*Amhr*), consistent with its lack of expression at 11.0 dpc in *Wt1* null mutant embryos [45]. *Sf1*, *Wt1*, *Gata4* and *Fog2* also have later roles within the supporting cell lineage, particularly in males. Indeed, heterozygous mutations in *SF1* can lead to XY female sex reversal. *Sox9* expression is lost in either $Gata4^{ki}$ (a mutation that disrupts the interaction of GATA4 with FOG2) or *Fog2* null homozygous mutant XY gonads [46], indicating that the GATA4–FOG2 complex is required directly for *Sox9* transcription or within one or more of the regulatory loops that maintains its expression. Similarly, the XX male sex reversal seen in *Wt1:Sox9* (*Wt1* promoter driving *Sox9*) transgenic and *Odsex* mutant mice is suppressed by mutations in both $Gata4^{ki}$ and *Fog2* [47]; both genes are required for *Amh* expression and might also be required later in development, for example, ablation of *Wt1* mediated by *Amh:Cre* leads to loss of *Sox9* expression and disruption of testis cords at 14.5 dpc [48].

Dax1 (also called *Nr0B1*) is an X-linked gene encoding a member of the nuclear receptor superfamily, which lacks a zinc finger DNA-binding domain. It can act as an anti-testis gene because *DAX1* duplication or overexpression in humans causes the XY sex reversal syndrome termed dosage sensitive sex reversal (DSS). *Dax1* overexpression in transgenic mice can also lead to XY female sex reversal. However, the latter only occurs in the presence of either a weak *Sry* allele, such as that provided by the *Mus musculus poschiavinus* Y chromosome (Y^{POS}), or when *Sry* expression is delayed by using a transgene driven by *Dax1* regulatory sequences (for a review, see Ref. [49]). By contrast, no ovarian defect except for multiple oocytes in the follicles was observed in XX mice carrying a targeted deletion of *Dax1* ($X^{Dax1-}X^{Dax1-}$), although this deletion might not generate a *Dax1* null allele [50]. Instead, testis cord organization and spermatogenesis were affected in hemizygous males ($X^{Dax1-}Y$) [51]. Thus, *Dax1* is thought to be required for testis development in mice, but LOF mutations in humans do not lead to XY female development [49,52]. Although *Dax1* expression depends on SF1 in the early genital ridge [53,54], DAX1 antagonizes SF1 function via a direct protein–protein interaction and recruitment of a nuclear co-repressor NcoR (for a review, see Ref. [55]). Perhaps in the mouse, DAX1 has distinct functions in concert with SF1 that are dependent on the developmental stage and the cell type.

Sex determination is sensitive to gene dosage and to genetic background effects, although distinct requirements can vary between species. Haploinsufficiency for *SF1*, *WT1* and *SOX9* can cause XY sex reversal in humans [56–58], but not usually in mice. XY mice heterozygous for *Fog2* or $Gata4^{ki}$ mutations develop normal testes on a C57BL/6J (B6) background, whereas they show sex reversal when the B6 Y chromosome is replaced with that from the AKR strain [59]. Moreover, targeted hemizygous mutations in *Dax1* cause complete sex reversal either in the presence of Y^{POS} or on a B6 background, whereas testes develop on 129Sv/J and DBA/2J backgrounds [60,61]. The levels or timing of *Sry* expression are also important, as shown by the XY sex reversal seen when Y^{POS} is on a B6 background, or with position effects associated with Y chromosome deletions or transgene insertions.

Each of these male-to-female sex reversal cases can ultimately be explained by the failure to generate sufficient levels of SOX9 to promote the positive-feedback loops that maintain its own expression. The effects can be indirect however, involving upstream genes and their regulatory networks and/or a failure to generate sufficient numbers of supporting cell precursors.

The balance between testicular and ovarian genes

Ovarian development has been considered as the 'default' pathway in mammals since Alfred Jost's classical experiments, first carried out using rabbits in the 1940 s, which showed that testes are required for male development throughout the rest of the embryo [1]. This notion was reinforced by the discovery in 1959 that the Y chromosome must carry a dominant male determinant, now known to be *Sry*, which is required to 'overcome' the female pathway. However, this 'default pathway' idea must be reconciled with the occurrence of complete or partial XX male sex reversal seen in humans and mice with LOF mutations of genes such as *Wnt4*, *Rspo1* and *Foxl2* or GOF mutations affecting genes such as *Dax1*. These findings contributed to the hypothesis, now supported by experimental evidence, that ovarian development is established by active repression of one or more genes in the testicular pathway rather than it depending entirely on a passive 'default' pathway.

In XX $Wnt4^{-/-}$ mutant embryos, the gonads are partially masculinized, with transient *Sox9* activation, the specification of Leydig-like cells, which make testosterone, and endothelial cell migration from the mesonephros resulting in a testis-specific vasculature [31,62]. *Rspo1* mutations give an almost identical XX male phenotype in mice, but a more complete sex reversal in some human cases [63–65]. In the presence of Wnt, Rspo1 binds to low density lipoprotein receptor-related protein 6 (LRP6), a co-receptor of the Wnt receptor frizzled, to help stabilize β-catenin. Rspo1 therefore amplifies Wnt signaling, indicating that *β-catenin* is an important ovary determinant or 'anti-testis' gene. In support of this idea, a conditional GOF mutation of *β-catenin* that produces a stable version of the protein in XY gonads results in ovary development [66,67]. This does not affect either SRY expression or the initial upregulation of *Sox9*, but it interferes with the maintenance mechanisms such that *Sox9* expression is rapidly lost. Conversely, the conditional loss of *β-catenin* mediated by *Amh:Cre* does not affect testis development [67]. It is possible that SRY could work, in part, by inhibiting β-catenin and promoting *Sox9* transcription. However, because neither *Wnt4* nor *Rspo1* mutant mice show complete XX male sex reversal, the absence of β-catenin seems insufficient to give sufficiently stable expression of SOX9 to promote its own maintenance and Sertoli cell differentiation.

FOXL2 is another good candidate for a repressor of the male pathway. It was identified in goats as a candidate gene for XX male sex reversal in *polled-intersex* syndrome (PIS). In humans, heterozygous LOF mutations are responsible for the autosomal dominant blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; see OMIM #110100), characterized by eyelid malformations and premature ovarian failure (for a review, see Ref. [68]). In the mouse, *Foxl2* is expressed exclusively in the ovary from 12.5 dpc onward and homozygous null mutations in XX animals cause a dramatic change in ovarian phenotype shortly after birth. Oocytes are lost and follicles transdifferentiate into structures that look more like seminiferous tubules with Sertoli-like cells, whereas Leydig cells producing testosterone are found in the interstitium [69,70]. *Sox9* expression is upregulated in XX $Foxl2^{-/-}$ gonads, but not until approximately one week after birth. Therefore, β-catenin and FOXL2 are both required to maintain *Sox9* in a repressed state during normal ovary development, but β-catenin is the crucial molecule during embryonic stages, with FOXL2 taking over postnatally.

The late appearance of Sertoli-like cells in the *Foxl2* null mutants should be interpreted with caution, however, because it could reflect indirect consequences of an earlier developmental problem within the supporting cells. For example, it is commonly believed, but without much direct evidence [71], that oocytes are required to maintain follicle

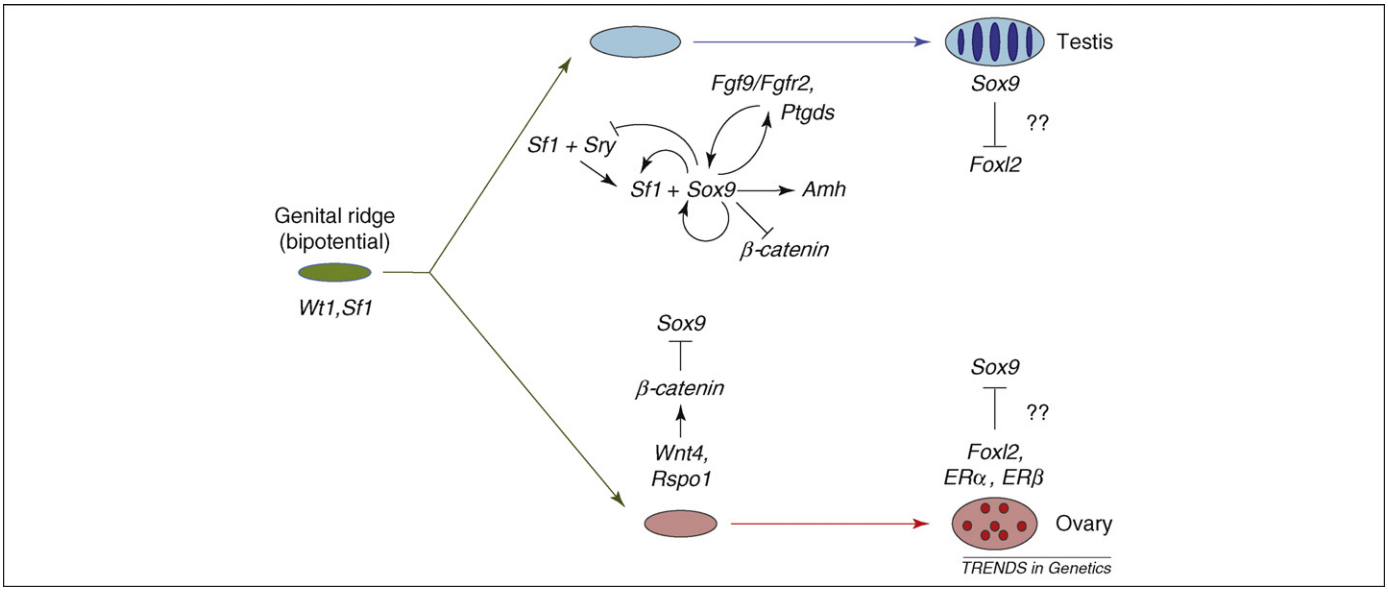

Figure 3. The molecular and genetic events in mammalian sex determination. The bipotential genital ridge is established by genes including *Sf1* and *Wt1*, the early expression of which might also initiate that of *Sox9* in both sexes. *β-catenin* can begin to accumulate as a response to Rspo1–Wnt4 signaling at this stage. In XX supporting cell precursors, *β-catenin* levels could accumulate sufficiently to repress SOX9 activity, either through direct protein interactions leading to mutual destruction, as seen during cartilage development [91], or by a direct effect on *Sox9* transcription. However, in XY supporting cell precursors, increasing levels of SF1 activate *Sry* expression and then SRY, together with SF1, boosts *Sox9* expression. Once SOX9 levels reach a critical threshold, several positive regulatory loops are initiated, including autoregulation of its own expression and formation of feed-forward loops via FGF9 or PGD_2 signaling. If SRY activity is weak, low or late, it fails to boost *Sox9* expression before β-catenin levels accumulate sufficiently to shut it down. At later stages, FOXL2 increases, which might help, perhaps in concert with ERs, to maintain granulosa (follicle) cell differentiation by repressing *Sox9* expression. In the testis, SOX9 promotes the testis pathway, including *Amh* activation, and it also probably represses ovarian genes, including *Wnt4* and *Foxl2*. However, any mechanism that increases *Sox9* expression sufficiently will trigger Sertoli cell development, even in the absence of SRY.

cells (perhaps via the production of signaling molecules such as growth differentiation factor 9 [GDF9]) and when oocytes are lost, the ovaries transdifferentiate into testicular-like structures, similar to those seen in the *Foxl2* mutants [69]. Indeed, this type of secondary sex reversal was suggested to explain the appearance of Sertoli-like cells at late fetal stages in $Wnt4^{-/-}$ mutant XX gonads [62] and also in mice with mutations in the genes encoding the estrogen receptors, ERα and ERβ, or in the *Cyp19* aromatase gene (for example, see Ref. [72]).

Studies in $Foxl2^{-/-}$:$Wnt4^{-/-}$ double mutant XX mice indicate that FOXL2 also represses *Sox9* in embryonic follicle cells, but that its role is normally redundant with respect to Wnt4 activity. [73]. However, the sex reversal in these double knockouts was incomplete; indeed, the phenotype was similar to that seen with either single mutation (although earlier than in $Foxl2^{-/-}$ mice). This finding indicates that R-spondin acting alone or together with other Wnts might still generate sufficient stable *β-catenin* to prevent Sertoli cell differentiation at earlier stages and/or that there are additional, unknown genes that repress *Sox9* during XX gonad development.

Taken together, it seems that both the male and female pathways rely on dominantly acting genes, with SRY actively promoting the male pathway by upregulating *Sox9* expression, and β-catenin and Rspo1 actively promoting the female pathway by repressing *Sox9*. It is a matter of timing (and expression level) that determines which pathway wins (Figure 3).

Sex determination without *Sry*: are there common themes in evolution?

There is a remarkable lack of conservation of sex-determining mechanisms throughout evolution. *Sry* itself is specific to mammals and probably arose from *Sox3* during the evolution of the X and Y chromosomes from a pair of autosomes in an ancestral species. In eutheria, *Sox3* is X-linked and involved in the development of the CNS, the pituitary, pharyngeal region and is perhaps involved in male fertility, but it has no demonstrable role in sex determination [74,75]. The original mutation that led to the origin of *Sry*, therefore, seems to have involved the acquisition of a novel function (neomorph) and it could have been the primary drive for the separation of the two sex chromosomes. *Sry* homologs have been identified on marsupial Y chromosomes, although the functional conservation has not been verified. However, no homologue of *Sry* exists in the prototheria (monotremes) in which *Sox3* is autosomal. Both platypus and echidna have five X and five Y chromosomes and maleness is associated with the presence of the Y chromosomes, but these must have evolved independently from the X and Y of eutheria and metatheria [76].

In birds, sex determination also relies on the inheritance of specific chromosomes, where males are ZZ and females are ZW; however, no crucial sex-determining genes have been identified. Several genes have been proposed as candidates for a dominant female-determining gene on the W chromosome, including those encoding female expressed transcript 1 (*FET-1*) [77] and W-linked PKC inhibitor/interacting protein (*WPKCI*, also known as histidine triad nucleotide-binding protein W-linked [*HINTW*]) [78]. Alternatively, or in addition, avian sex determination could involve Z chromosome dosage. *Dmrt1*, a gene encoding a transcription factor with a DM DNA-binding domain that is expressed in the gonads, is an interesting candidate (for a review, see Ref. [79]). Related DM-containing factors are involved in sex determination in other species, notably in

Caenorhabditis elegans and *Drosophila*, raising the possibility that a double dose of *Dmrt1* is required for testis determination in birds. Some frogs (e.g. genus *Xenopus*) have a ZZ/ZW sex chromosome system. Recently, a W-linked DM domain gene (*DM-W*) was discovered as a paralogue of the autosomal *Dmrt1* in *Xenopus laevis*. Transgenic ZZ tadpoles carrying *DM-W* show ovarian characters including primary oocytes [80]. Species belonging to the Medaka family of teleost fish have an XY/XX system and, in one species, *Oryzais latipes*, another DM domain gene located on the Y chromosome, *DMY*, has been identified as the testis-determining gene [81]. However, the DM genes seen in *X. laevis* and *O. latipes*, which seem to have opposite functions, are not universal sex-determining genes for frogs and fish because they are not found on the relevant sex chromosomes of closely related species such as *Xenopus tropicalis* or *O. luzonensis* [82].

Environmental sex-determining mechanisms, which are found in certain groups of reptiles (notably all alligators, crocodiles and turtles) and fish, are not understood at a molecular level, but must involve differential gene activity rather than the presence or absence of specific genes [83]. In many lower vertebrates, estrogens and androgens can readily influence sex determination and, indeed, the balance between these could be involved in the primary switch between one sex and another. It is even possible to sex reverse XY marsupial gonads with estrogens (for a review, see Ref. [84]). Perhaps, owing to the intrauterine development during the relevant stages, it is only in eutheria in which this seems not to be possible. In birds, male development of ZW embryos can be triggered with aromatase inhibitors, again indicating that sex steroids are close to the genetic switch. In addition, in most birds, although testes form on both sides in ZZ embryos, only the left genital ridge gives rise to an ovary, whereas the other degenerates. Mechanisms driving left-right asymmetry are active in the CE covering the early gonads and these confer an ability on the left only to respond to estrogens made as a consequence of gene activity in the ovarian-determining pathway [85,86]. This interaction with the left-right asymmetry pathway could help to define genes required for sex determination.

Although different genes trigger sex determination among vertebrates and although some of the embryonic origins of the various gonadal cell types can differ, adult testes and ovaries are similar in morphology, cellular organization and physiology [3,83]. Therefore, there is likely to be some conservation of mechanism underlying the different triggers. The effector genes immediately downstream of these might be common, but could use different *cis*-regulatory elements. *Sox9* and *Foxl2* seem to be common effector genes in differentiation of the testis and ovary, respectively, because their expression patterns and secondary structures are highly conserved among vertebrates. The TESCO element identified upstream of the mouse *Sox9* gene is also found in other eutherian mammals, including rat, dog and human, with consensus binding sites for SRY and SF1. Moreover, at least part of TESCO seems to be present upstream of *Sox9* in the opossum. However, the degree of conservation decreases rapidly with evolutionary distance and similar sequences are not apparent in platypus, chick, *Fugu* or zebrafish [21]. TESCO is, therefore, either specific to mammals in which *Sry* functions as the sex-determining gene, or it is too divergent to be easily recognized in the other species. If feedback loops similar to those in the mouse are involved in maintaining *Sox9* expression in males and repressing it in females, then one might predict the existence of at least some conserved elements.

Concluding remarks and future perspectives

Recent discoveries in cellular, genetic and molecular aspects of gonad development have opened up the field of sex determination and differentiation. There is now evidence that *Sox9* is a direct target gene of SRY; moreover, SRY contributes to the transcriptional activation of *Sox9* at a crucial moment during the differentiation of the supporting cell precursor lineage, rather than merely interfering with a repressor [22]. A brief burst, or 'wink', of SRY is sufficient to nudge *Sox9* into action, such that the latter's expression exceeds a threshold level above which it can be maintained. The control of SOX9 activity, which involves several intra- and extra-cellular regulatory loops, is clearly

Box 2. Outstanding questions and future directions

- Detailed mapping of *Sry* regulatory sequences by transgenic assays are required.
- The identification of other genes required for crucial aspects of early gonad, testis and ovary development.
- How is SRY-induced DNA bending crucial to SRY function? This had been suggested by early *in vitro* data comparing mutant and wild-type hSRY proteins found in cases of sex reversal, but on artificial rather than on true target sequences and in the absence of partner proteins. How does SOX9 replace SRY on TES over time?
- What are the consequences of deleting TES from the mouse genome? If XY female sex reversal occurs, then TES is essential for testis development, if not, then it would indicate the presence of one or more additional redundant or 'shadow' enhancers with the same role as TES. These would have to map outside the 120-kb Sox9 BAC used in the initial transgenic experiments.
- What would happen if β-*catenin* and/or *Foxl2* were deleted at the same time as *Sox9*? If ovary development ensues, it would imply that β-*catenin* and/or *Foxl2* represses *Sox9* and that other ovary-determining genes kept silent in XY gonads by SOX9 activity remain to be discovered. By contrast, if ovaries do not form, then β-*catenin* and/or *Foxl2* must actively promote follicle cell and ovary development.
- What mechanisms act intrinsically within germ cells to lead to their epigenetic reprogramming (reactivation of a second X chromosome, erasure of parental genomic imprints) and to either permit their entry into meiosis in response to retinoic acid signaling or to arrest mitosis?
- How is the morphogenesis (and number) of testis cords controlled?
- What leads to the maturation of the ovarian follicle (folliculogenesis)?
- With respect to evolution, how is *Sox9* expression triggered in the non-mammalian testis? Given that the TES sequence has been identified only in eutherians and marsupials, it would be interesting to know which sequences are used in other species. Overall, TES seems to be well-conserved among eutherians, but not all murine SRY- and SF1-BS are conserved. Deletions or rearrangements of these BSs might have occurred during. To test functional conservation, what would happen if mouse TES is replaced with that of other species *in vivo*?
- What is the role of SRY in other tissues, notably in the CNS? Do its non-HMG domains have a greater significance in these other tissues than they seem to have in the gonad?

the crucial issue in the decision of the lineage to differentiate as Sertoli or follicle cells and it might also be important for maintaining this decision throughout life. The importance of SF1 as a partner factor for SRY and SOX9 has also been revealed. There are still many outstanding questions about SRY structure, function and evolution (Box 2). For example, why do SRY non-HMG-box protein sequences seem to diverge with considerable freedom or even with selection for change? Perhaps there are both negative and positive constraints; for example, the sequence could vary widely as long as it is unable to interact with any protein that would render it inactive. β-catenin could be such a protein, although recent reports looking at whether this can interact (in heterologous cell types) with mouse and human SRY gave conflicting results [87,88]. Alternatively, the variation might reflect roles for SRY outside sex determination, for example, in conferring sex-specific differences in the brain and behavior [89].

The identification of enhancer elements regulating *Sox9* expression in the gonads should enable these molecular mechanisms to be dissected in more detail. Indeed, these pathways are likely to be more complex than portrayed so far; for example, TES contains consensus binding sites for several other factors that are likely to have a role in sex determination. Structural details, such as the relative importance of DNA binding, bending and protein interactions can now be addressed using relevant target sequences. It should also be possible to trace the evolution of TES, perhaps helping to reveal sex-determining mechanisms in lower vertebrates. Finally, this knowledge should help us to understand how the protein products of genes involved in specifying and maintaining follicle cells act at a molecular level to repress *Sox9* expression and tip the balance in favor of ovary differentiation.

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